universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Production and Characterization of Cellobiose Dehydrogenase“

verfasst von / submitted by

Elisabeth Tomek BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2016 / Vienna 2016

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 838
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Ernährungswissenschaften
Betreut von / Supervisor:	Univ. Prof. Dipl.-Ing. Dr.techn. Dietmar Haltrich

Content

Acknowledgment

Index

Abstract

1 Introduction 1

1.1 Flavoenzymes 1

1.2 Cellobiose dehydrogenase 1

1.2.1 Occurrence and classification 2

1.2.2 Structure 4

1.3 Function 5

1.4 Expression System 7

1.5 Application 8

1.6 Physical properties of Yoghurt 11

2 Aim of the thesis 12

3 Materials and Methods 13

3.1 Technical equipment 13

3.2 Production and characterization of *A. oryzae* CDH class II and III 15

3.2.1 Cultivation of *Pichia pastoris* on YPD Agar 17

3.2.2 Inoculation of preculture and production in large scale 17

3.2.3 Enzyme purification 19

3.2.4 Hydrophobic interaction chromatography 20

3.2.5 Anion exchange chromatography 21

3.2.6 Absorption spectroscopy 22

3.3 Protein characterization 22

3.3.1 Protein concentration determination 22

3.3.1.1 Bradford assay 22

3.3.2 Enzyme activity determination 23

3.3.2.1 DCIP assay 23
3.3.2.2 Cytochrome *c* assay 24
3.3.3 SDS Polyacrylamid- Gelelectrophorese 25
3.3.4 Further characterization of CDH II 26
3.3.4.1 pH profiles 26
3.3.4.2 Temperature profile 26
3.3.4.3 Determination of the kinetic constants 26
3.4 Characterization of CDH III 28
3.4.1 Substrate screening 28
3.5 Application of *Mt*CDH 33
3.5.1 Production of the enzyme 35
3.5.2 Purification 36
3.5.2.1 Hydrophobic interaction chromatography 36
3.5.2.2 Anion exchange chromatography 37
3.5.3 Kinetic characterization with oxygen as electron acceptor 38
3.5.4 Titration 39
3.5.5 Production of lactobionic acid, gluconic acid, maltobionic acid and cellobionic acid 39
3.5.5.1 FOX assay (= Ferrous oxidation- Xylenol orange) 40
3.5.5.2 Enzymatic assay of catalase 41
3.5.5.3 Analytic method: HPLC 41
3.5.6 Production of enzymatic yoghurt 42
3.5.6.1 Pre experiment for the production of the enzymatic yoghurt 42
3.5.6.2 Production of the enzymatic yoghurt 42
3.5.6.3 Analytic method: HPLC 42
3.5.6.4 Textual properties of yoghurt 43
4 Results and Discussion 44

4.1 Production and characterization of *Aspergillus oryzae* CDH class II and III 44
4.1.1 *A. oryzae* CDH II 44
4.1.1.1 Production of *A. oryzae* CDH II 44
4.1.1.2 Characterisation of *A. oryzae* CDH II 47
4.1.2 *A. oryzae* CDH III 55
4.1.2.1 Production of *A. oryzae* CDH III 55
4.1.2.2 Characterisation of *A. oryzae* CDH III 56
4.2 Application *of Myriococcum thermophilum* CDH 60
4.2.1 Production and purification of *Myriococcum thermophilum* CDH 60
4.2.2 Characterisation of *Mt*CDH 61
4.2.3 Production of lactobionic acid, gluconic acid, maltobionic acid and cellobionic acid 67
4.2.4 Enzymatic production of yoghurt 76
5 Conclusions 80
6 Zusammenfassung 83
7 References 85

Acknowledgment

I would like to thank Univ. Prof. Dipl.-Ing. Dr.techn. Dietmar Haltrich, from BOKU, Department of Food Sciences and Technology for giving me the opportunity to work in the interesting field of enzymes.

My sincere thanks also go to Priv.-Doz. Dr. Roland Ludwig for giving me the chance to be a part of his research team, for his encouragement and insightful comments.

I want sincerely thank my supervisor Dipl.-Ing. Marita Preims for her patience, motivation and enthusiasm. Her guidance helped me through all the time of research and supported me to write my thesis.

I am also very thankful to all co-workers and students of the Institute for their help and the pleasant atmosphere.

Last but not least, I would like to thank my family and friends for motivating and supporting me.

List of abbreviations

AOX	Alcohol oxidase
ABTS	2,2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
CDH	Cellobiose dehydrogenase
cyt c	cytochrome c from bovine heart
DCIP	2,6- Dichloroindophenol sodium salt hydrate
DET	direct electron transfer
FAD	flavin adenine dinucleotide
HQ-water	high quality water
IET	intracellular electron transfer
kDa	kilo Dalton
Km	Michaelis constant
LPMO	lytic Polysaccharide monooxygenaese
µl	microliter
mM	milimolar
*Mt*CDH	*Myriococcum thermophilum* cellobiose dehydrogenase
Min	minuts
nm	nanometer
P. pastoris	*Pichia pastoris*
pH	measure of the acidity or basicity of aqueous solutions
rpm	revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamid gel electrophoresis
U	unit of enzyme activity
YNB	Yeast Nitrogen Base
YPD	Yest Extract Peptone Dextrose

List of figures

Fig. 1: Phylogenetic tree of CDH. Class I CDH is blue, class II in green and class III in brown

Fig. 2: Cellobiose dehydrogenase, 3D molecular structure. Colour coding: (pink) CYT domain, (blue) DH domain, (yellow) FAD, (red) *heme b* [TAN T., 2015]

Fig. 3: Electron transfer in CDH from the substrate to various terminal electron acceptors [LUDWIG R., 2013]

Fig. 4: The figure show the catalytic reaction (CAT), following by the IET and DET of the electrons to the electrode. left: DH_{CDH}, right: CYT_{CDH} [HARREITHER W., 2012]

Fig. 5: Schematic biocatalyst oxidation of lactose to lactobionic acid [GUTIERREZ L., 2012]

Fig. 6: Example of a 96- well plate containing different substrates, *A. orycae* CDH III and DCIP as electron acceptors.

Fig. 7: Protein concentrations measured by Bradford and determination of the biomass during the production process of *A. oryzae* CDH II.

Fig. 8: Determination of the enzymatic activity with cyt *c* and DCIP assays during the production process with *A. oryzae* CDH II.

Fig. 9: SDS-PAGE of the not homogenously purified *A. oryzae* CDH II after Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. [1. Unstained Precision Plus Protein standard; 2. *A. oryzae* CDH II]

Fig. 10: pH profile of *A. oryzae* CDH II with cyt *c* as electron acceptor using McIlvaine buffer solution with pH ranges from 3.0 to pH 8.5.

Fig. 11: pH profile of *A. oryzae* CDH II with DCIP as electron acceptor using McIlvaine buffer solution with pH ranges from 3.0 to pH 8.5.

Fig. 12: Determination of the enzyme activity of *A. oryzae* CDH II at 60 °C and 65 °C using cyt *c* as electron acceptor.

Fig. 13: Determination of the enzyme activity of *A. oryzae* CDH II at 55 °C using cyt *c* as electron acceptor.

Fig. 14: DCIP assay with *A. oryzae* CDH II to determine the specific activity of cellobiose at different substrate concentrations.

Fig. 15: Cyt *c* assay with *A. oryzae* CDH II to determine the specific activity of cellobiose at different substrate concentrations.

Fig. 16: DCIP assay with *A. oryzae* CDH II to determine the specific activity of lactose at different substrate concentrations.

Fig. 17: Cyt *c* assay with *A. oryzae* CDH II to determine the specific activity of lactose at different substrate concentrations.

Fig. 18: DCIP assay with *A. oryzae* CDH II to determine the specific activity of maltose at different substrate concentrations.

Fig. 19: Cyt *c* assay with *A. oryzae* CDH II to determine the specific activity of maltose at different substrate concentrations.

Fig. 20: Protein concentrations measured by Bradford and determination of the biomass during the production process of *A. oryzae* CDH III.

Fig. 21: Determination of the enzymatic activity with cyt *c* and DCIP assays during the production process with *A. oryzae* CDH III.

Fig. 22: SDS-PAGE of fractions of the purified *A. oryzae* CDH III after Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. [1. Unstained Precision Plus Protein standard; 2-9 different fractions]

Fig. 23: Spectral characterization of *A. oryzae* CDH III showing the oxidized (blue) and reduced (red) spectra. Dithionit was used for reduction.

Fig. 24: Spectral characterization of *Neurospora crassa* CDH II B showing the oxidized (black) and reduced (gray) spectra. Lactose was used for reduction. [SYGMUND C., 2012]

Fig. 25: SDS-PAGE of the purified *Mt*CDH after Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. [left: unstained Precision Plus Protein standard; right: FAD domain]

Fig. 26: Oxygen consumption of 30 mM lactose at 25 °C measured with the microsensor oxygen meter, using *Mt*CDH.

Fig. 27: Oxygen consumption of 30 mM lactose at 30 °C measured with the microsensor oxygen meter, using *Mt*CDH.

Fig. 28: Oxygen consumption of 30 mM lactose at 40 °C measured with the microsensor oxygen meter, using *Mt*CDH.

Fig. 29: Oxygen consumption of 30 mM lactose at 50 °C measured with the microsensor oxygen meter, using *Mt*CDH.

Fig. 30: Oxygen consumption of 30 mM lactose at 60 °C measured with the microsensor oxygen meter, using *Mt*CDH.

Fig. 31: Oxygen consumption of 30 mM lactose at 70 °C measured with the microsensor oxygen meter, using *Mt*CDH.

Fig. 32: Titration of 100 ml unskimmed milk with 0.25 M HCL until the milk was clotted.

Fig. 33: HPLC analysis during the conversion process from lactose into lactobionic acid using *Mt*CDH in the reactor with pH regulation.

Fig. 34: HPLC analysis during the conversion process from lactose into lactobionic acid using *Mt*CDH in the reactor without pH regulation

Fig. 35: HPLC analysis during the conversion process from maltose into maltobionic acid using *Mt*CDH in the reactor with pH regulation.

Fig. 36: HPLC analysis during the conversion process from cellobiose into cellobionic acid using *Mt*CDH in the reactor with pH regulation.

Fig. 37: HPLC analysis during the conversion process from glucose into gluconic acid using *Mt*CDH in the reactor with pH regulation.

Fig. 38: Determination of enzyme activity with DCIP assay during the converting process from lactose into lactobionic acid using *Mt*CDH in the reactor with pH regulation.

Fig. 39: Determination of enzyme activity with DCIP assay during the converting process from lactose into lactobionic acid using *Mt*CDH in the reactor without pH regulation.

Fig. 40: Enzymatic activity measured with DCIP during the production process of cellobionic acid, maltobionic acid and gluconic acid

Fig. 41: Measurement of H_2O_2 concentration by FOX assay during the converting process from lactose into lactobionic acid using *Mt*CDH in the reactor without pH regulation.

Fig. 42: Measurement of H_2O_2 concentration by FOX assay during the converting process from lactose into lactobionic acid using *Mt*CDH in the reactor with pH regulation.

Fig. 43: Catalase assay during the converting process from lactose into lactobionic acid using *Mt*CDH in the reactor with pH regulation.

Fig. 44: PH change during the converting process of lactose into lactobionic acid using *Mt*CDH in the reactor without pH regulation.

Fig. 45: PH changes while lactose is converted into lactobionic acid using *Mt*CDH in the reactor without pH regulation.

Fig. 46: HPLC analysis during the conversion process from lactose into lactobionic acid using *Mt*CDH in the reactor without controlling pH.

Fig. 47: *Mt*CDH catalysed oxidation of lactose in milk to yoghurt.

Fig. 48: Determination of the textual properties of a stirred-, set- yoghurt and yoghurt with *Mt*CDH

List of tables

Tab. 1: Pipetting scheme for Bradford assay

Tab. 2: Pipetting scheme for DCIP assay

Tab. 3: Pipetting scheme for cyt *c* assay

Tab. 4: Pipetting scheme for substrate screening with cyt *c* as electron acceptor

Tab. 5: Pipetting scheme for substrate screening with DCIP as electron acceptor

Tab. 6: List of substrates for the substrate screening with *A. orycae* CDH III

Tab. 7: Pipetting scheme for the FOX assay

Tab. 8: Purification scheme of *A. oryzae* CDH II

Tab. 9: Determination of the enzyme activity with cyt *c* and DCIP assays with different concentrations of McIlvaine buffer using *A. oryzae* CDH II as enzyme.

Tab. 10: Determination of the specific activity of lactose, cellobiose, glucose and maltose using *A. oryzae* CDH II. Michaelis-Menten equation was used to determine K_M and V_{max}.

Tab.11: Measurements of the oxygen consumption with different lactose concentrations at 30 °C with the microsensor oxygen meter using *Mt*CDH.

Tab.12: Measurements of the oxygen consumption with 30 mM lactose at different temperatures with the microsensor oxygen meter using *Mt*CDH.

Abstract

The fungal enzyme cellobiose dehydrogenase (CDH) is a monomeric oxidoreductase, consisting of two domains connected by a flexible linker which is found in ascomycetes and basidiomycetes. CDH from different producers shows differences with respect to substrate specificity, pH-, and temperature optima. There is a high scientific interest in CDH because this enzyme has a wide spread field of application in the analytic area as well as in the industry. As CDH can be applied in food science, the production of lactobionic acid is of high importance for industrial applications. The production of CDH by fungi is very complex and expensive. Therefore the yeast *Pichia Pastoris* X33 was selected as expression system. The yeast species *Pichia pastoris* is frequently used as an expression system for recombinant protein production and is suitable for the production in shake flasks or in fermenters.

One class III CDH gene of *Aspergillus oryzae* was selected for recombinant production. This is the first time that a class III CDH was produced successfully. In this project the production process in shaking flasks and the purification of the enzyme is described in detail. Purification was done by Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. After chromatography process a total protein yield of 6.91 mg was measured by Bradford assay. With the purified *A. oryzae* CDH III a substrate screening with cyt *c* and DCIP assay containing different mono-, di-, tri- and polysaccharides, alcohols and amino acids was done. There was no substrate, which showed any activity with the class III CDH. To compare the characteristics of class III CDH with class II, a class II CDH from *A. oryzae* was expressed and characterized. This enzyme was also produced in shaking flasks and purified with Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. 3.20 mg of pure *A. oryzae* CDH II was produced successfully. To compare this enzyme with CDH from other organisms some characteristics were assessed. Kinetic measurements with this enzyme show that the most effective electron donor for *A. oryzae* CDH II is cellobiose. The optimal pH for this enzyme is at pH 5.0 for cyt *c* and for DCIP and the enzyme is best stable at 55 °C.

In general, CDH shows low activity with oxygen as electron acceptors and this hampers the in situ production of H_2O_2. In this work also describes the production process of *Myriococcum*

thermophilum CDH with mutations at positions N700 and N748. This *Mt*CDH variant was chosen because it has increased oxygen reactivity and thereby improves the hydrogen peroxide production. After the production in a fermenter, the purification was done by Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. After purification a total protein yield of 10.7 mg was measured.
Furthermore, an efficient enzymatic bioprocess is described in which lactose converted into lactobionic acid by *Mt*CDH without the formation of any by products in the bioreactor Sixfors. The conversion of lactose to lactobionic acid consists of the oxidation of the free aldehyde group of glucose on the lactose molecule to the carboxyl group. 15.78 g of lactobionic acid were produced successfully.

The oxidation of cellobiose, maltose and glucose by this CDH variant was also performed in the bioreacter system Sixfors. HPLC data show that the converting process of cellobiose into cellobionic acid worked successfully. Further experiments should optimize the production process of gluconic acid and maltobionic acid.

In a further experiment the recombinant *Mt*CDH was used to produce bacteria free yoghurt. Mainly *Streptococcus thermophiles* and *Lactobacillus bulgaricus* are used as starter culture in conventionally yoghurt manufacture. The advantage of enzymatic yoghurt is that patients who are immunosuppressive could benefit from yoghurt which is produced with this *Mt*CDH variant. The *Mt*CDH catalysed oxidation of lactose in milk was performed by using the bioreacter system Sixfors. HPLC analyses have shown that lactose decreased during the production process. In the meantime the concentration of lactobionic acid increased. The product was stored for one week at 4 °C and during that time the texture became firmer. Texture is one of the most essential components of yoghurt quality. Therefore a syneresis test was done to compare the texture of a stirred-, set- and enzymatic yoghurt. Syneresis is an extraction of a liquid from a gel. During the syneresis process whey is collecting on the surface of the yoghurt. The texture from the enzymatic yoghurt is comparable with the stirred and set yoghurt but it is not proven yet whether the yoghurt is mutagenic in humans because a recombinant enzyme was used for the production.

1 Introduction

1.1 Flavoenzymes

Flavoenzymes are colourful oxidoreductases, able to catalyse many different types of reactions. They are involved in many biological processes and have important applications in the pharmaceutical, chemical and food industry. Scientists focus on flavoenzymes because their reactions are selective, controllable and efficient. Some of these enzymes are able to catalyse the oxidative modification of proteins. In the aerobic metabolism flavoenzymes are capable to catalyse both one- and two-electron transfer reactions. As a cofactor flavoenzymes contain either a flavin mononucleotide (=FMN) or a flavin adenine dinucleotide (=FAD). Enzymes use cofactors to enable biological transformations needs in life. In some flavoenzymes the isoalloxazine ring of the flavin is bound covalent to the polypeptide chain. There might be positive effects: saturating the active site, tuning the flavin redox potential and also to prevent flavin modification. Another possibility is that the flavin is bound non- covalently to the apoprotein. [JOOSTEN V., 2007]
CDH has a heme which consists of an iron ion in its centre of the large heterolytic porphyrin ring. The heme cofactor is ligated by methionine at position 65 (Met 65) and histidine at position 163 (His 163) in the CDH of P. chrysosporium. Those positions are uncommon for heme *b* in CYT_{CDH} and show a relatively low redox potential, which is approximately 100- 160 mV vs the normal hydrogen electrode at pH 7.0. [LUDWIG R., 2013]

1.2 Cellobiose dehydrogenase

The fungal enzyme cellobiose dehydrogenase (CDH, EC 1.1.99.18) is secreted by many cultures of wood-degrading, phytopathogenic and saprotrophic fungi within the phyla of *ascomycota* and *basidiomycota.* The widespread occurrence implies an important role of this enzyme in the process of wood degradation. [SYGMUND C., 2013]

CDH was first isolated by Westermark and Eriksson in 1974. The extracellular cellobiose quinone oxidoreductase was isolated from the fungus *Polyporus versicolor* and *Chrysosporium lignorum*. Results of their studies showed that the enzyme catalyses the oxidation of cellobiose to cellobionic acid which is coupled to the reduction of the quinone to the hydroquinone ($FADH_2$). [WESTERMARK U., 1974]

In 1995 the first cDNA sequence coding for CDH was revealed and in the period from 2000 to 2002 the structures of the two domains, which build the holoenzyme, were solved.

The name cellobiose dehydrogenase was changed from the name cellobiose oxidase in the early 90`s. The reason for this change was in response to two findings: cellobiose was not oxygenated, as previously reported and oxygen was a poor substrate/- electrone acceptor. [CAMERON M., 2001]

In the last few years the field of bioelectrocatalysis has shown tremendous developments, mostly recognised for its various applications. The fungal enzyme CDH is a biorecognition element and is able to detect carbohydrates, quinones and catecholamines. It can also be used as an anode biocatalyst for enzymatic biofuel cells to empower miniaturized sensor-transmitter systems. [LUDWIG R., 2013]

CDH has generated recent interest because of its ability to facilitate the development of free radicals. It also constitutes a suitable model to study intermolecular electron transfer. [CAMERON M., 2001]

1.2.1 Occurrence and classification

The enzyme family CDH is a heterogeneous group of proteins. The sequence of those proteins is between 749 and 816 amino acids long and the sequence identities are as low as 35%. Phylogenetic analyses show three well- supported branches. That branches correlate partly with the species classification. The class I CDH is secreted by various fungi: Atheliales, Corticiales and Polyporales corresponding to the phylum Basidiomycete. Class II and III CDH are secreted by ascomycetes. The fungi Sordariales, Xylariales and Hypocreales belong to that phylum. [SYGMUND C., 2013]

It has been shown that CDH is typically secreted during the exponential phase of growth on cellulose as the only substrate source in the extracellular space. Genes coding for CDH were

already identified in 21 organisms. The identification of a new CDH-coding gene in a complete genome is based on sequence similarity along the whole coding sequence. [ZAMOCKY M., 2006]

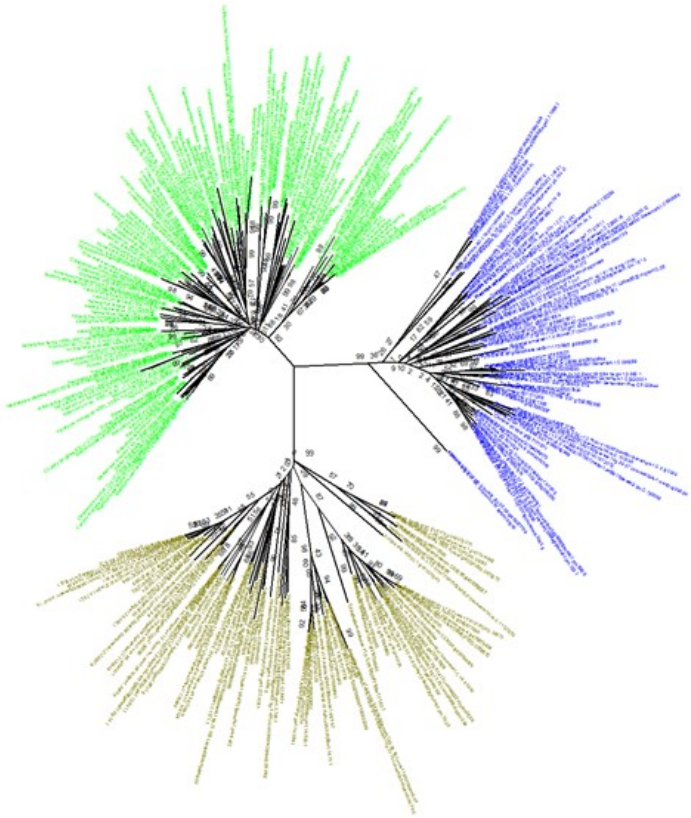


Fig. 1: Phylogenetic tree of CDH. Class I CDH is blue, class II in green and class III in brown.

Phylogenetic studies of these sequences show that the enzyme clusters into three branches. Basidiomycetes secrete CDH sequences and form the branch of CDH class I and they have a carbohydrate binding site at the N-terminal.

Sequences of ascomycete origin form the CDH class II. In contrast to CDH class IIB, CDH class IIA include a carbohydrate binding module. [SYGMUND C., KRACHER D., SCHEIBLBRANDNER S., et.al; 2012], [LUDWIG R., 2013]

While class I CDHs consist of a shorter sequence but have a highly conserved linker region, class II have a less conserved linker region. [LUDWIG R., 2010]

Moreover, class I CDHs do not show a high activity with glucose and the internal electron transfer between the two domains it is only efficient at acidic pH less than 5.5 with pH optimum about 3.5 to 4.0.

Class II CDHs show a broader substrate spectrum and have lower glucose discrimination. Also, depending on their origin, neutral or alkaline intermolecular electron transfer properties have been reported. CDH encoding sequences from members of ascomycetes form a third phylogenetic branch. The secretion and function of these class III CDHs has to be investigated. [ZAMOCKY M., 2006], [LUDWIG R., 2013]

1.2.2 Structure

CDH is a 90 kDa protein existing of about 750 amino acids. 80 kDa belong to the molecular mass of the protein, while the other 10 kDa belong to the glycosylation.

CDH is a monomeric enzyme built of two domains. This extracellular fungal flavocytochrome consist of the N- terminal CYT_{CDH} and the C- terminal DH_{CDH}. The cytochrome domain contains a heme *b* as a cofactor and the dehydrogenase domain FAD as a cofactor. [LUDWIG R., 2013]

Cox et al found some ligands to the heme iron in 1992. The heme type b is hexacoordinated and unusual ligand by histidine and methionine. This is only found in *cytochrome c*. During oxidation or reduction the ligands are not exchanged or lost.

The heme domain shows a higher degree of glycosylation than the flavin domain.

The CYT_{CDH} and the DH_{CDH} are connected by a flexible linker of about 20 amino acids. This linker keeps the two domains in a close contact allowing an intermolecular electron transfer. The flavin domain is about 60 kDa and the cytochrome domain approximately 30 kDa.

The isoelectric point of CDH is 4.2 while the independent flavin domain has an isoelectric point at 5.45 and the heme domain at 3.42. Regarding pH and temperature, this enzyme is very stable and remains active at room temperature for 24 hours in a range of pH from 3.0 to 10.0. If CDH is incubated at pH 2.0, the enzyme becomes inactive within 2 hours due to the release of FAD. At a temperature of 60 °C the enzyme is most stable at pH 4.5. [CAMERON M., 2001]

TAN et al. screened some basidiomycete and ascomycete fungi and achieved successful crystallization and structure determination of two CDHs from the *Myriococcum thermophilum* and *Neurospora crassa.* [TAN T., 2015]

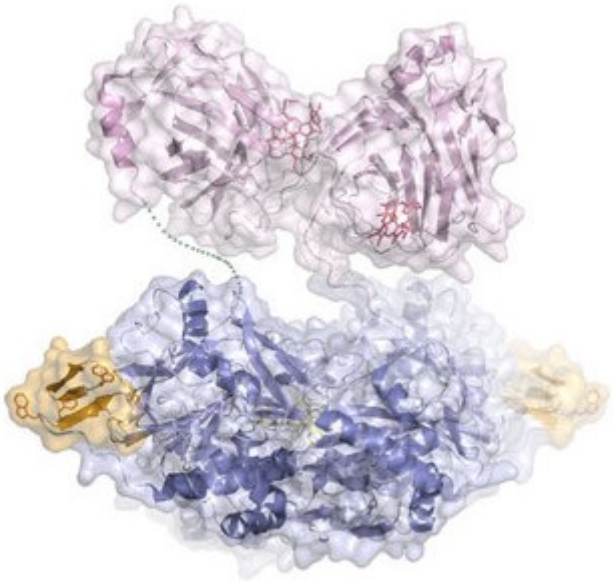


Fig. 2: Cellobiose dehydrogenase, 3D molecular structure. Colour coding: (pink) CYT domain, (blue) DH domain, (yellow) FAD, (red) heme *b* [TAN T., 2015]

1.3 Function

The favoured substrate of all CDHs is the ß- 1,4 linked disaccharide cellobiose which is a breakdown product of cellulose or cellodextrin. Lactose is also converted by CDHs although it is not the natural substrate. As already mentioned, class I CDHs strongly discriminate glucose turnover, whereas some classes of CDH II can also oxidise monosaccharides but with lower catalytic efficiencies. [LUDWIG R., 2013]

There are many speculations about in vivo biological function suggested for CDH. One of the hypothesis is the Fenton reaction with the formula:

$$Fe\ (II) + H_2O_2 \longrightarrow Fe(III) + OH^- + OH^\circ$$

Electrons from cellobiose oxidation are transferred one-by-one to the *heme* domain and subsequently to a one electron-acceptor, which is suggested to be a ferric complex. This type of reaction produces a hydroxyl radical and further it reacts with cellulose or lignin. [ZAMOCKY M., 2006]

The enzyme has a very wide spectrum of electron acceptors including ABTS, ortho- and para-quinones, phenoxazine and phenothiazine dyes, ferricyanide or cyt *c* and iron-, osmium- and ruthenium complexes. Recently a new group of electron acceptors for CDH was identified, the lytic polysaccharide monooxygenase (LPMOs). These copper- dependent carbohydrate active enzymes accept electrons from CDH. Through an oxidative mechanism the LPMO is able to cleave cellobiose. [HARREITHER W., 2012], [LUDWIG R., 2013]

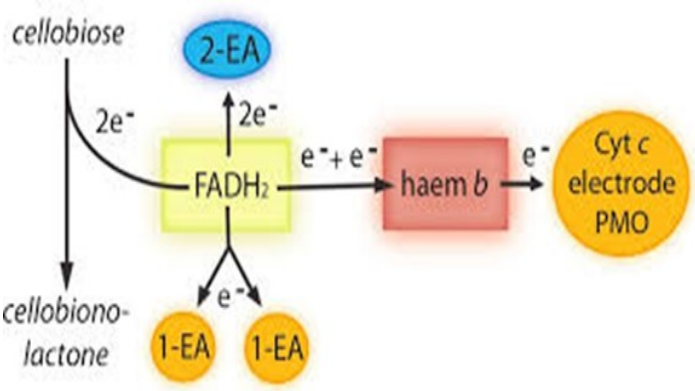


Fig. 3: Electron transfer in CDH from the substrate to various terminal electron acceptors. [LUDWIG R., 2013]

During the reductive half reaction, the dehydrogenase domain oxidizes carbohydrates at the anomeric C1 atom into intermediary lactones. They hydrolyse spontaneously to the corresponding aldonic acid. Re-oxidation of the FAD can be conducted by two electron acceptors or by one electron acceptors. The reduction of electron acceptors takes place at the DH_{CDH} domain. In the literature it is often described as a ping- pong mechanisms.

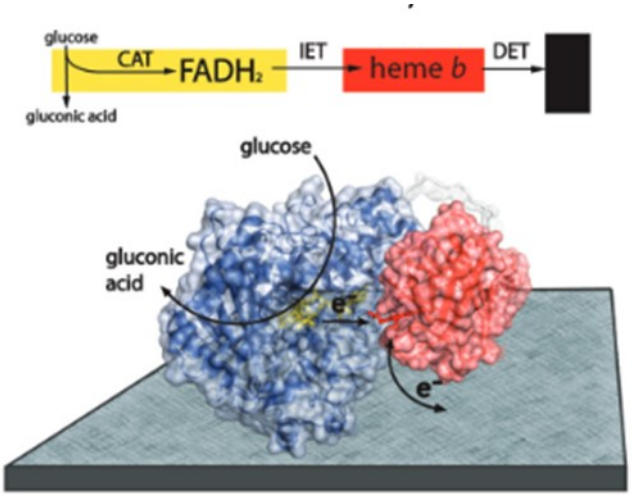


Fig. 4: The figure shows the catalytic reaction (CAT), following by the IET and DET of the electrons to the electrode. left: $DH_{CDH,}$ right: CYT_{CDH} [HARREITHER W., 2012]

Examples of two electron acceptors are DCIP and quinones. Another option for the reoxidation with FAD can also be performed very slowly by oxygen.
Electrons can also be transported by intramolecular electron transfer (=IET) to the heme *b* in the CYT_{CDH}, which works as a relay for the reduction of macromolecular electron acceptors like LPMO, cyt *c* or an electrode. [SYGMUND C., 2013]

1.4 Expression System

Protein expression is a method to produce proteins in living organism. The yeast species *Pichia pastoris* is frequently used as an expression system for recombinant protein production. [ZAMOCKY M., 2006]

P. pastoris is suitable for the production in shake flasks or in fermenters. AOX 1 and AOX 2 are the two alcohol oxidase genes in *P. Pastoris*. Both genes have a strongly inducible promoter. These genes allow *Pichia* to use methanol as a carbon and energy source. The AOX 1 promoters are induced by methanol. These enzymes are only present when the cells are grown on methanol. [DALY R., 2005] To insert a foreign gene into *P. Pastoris*, the desired gene has to be integrated into an expression vector, like pPICZ-α, which then is transformed into a yeast cell. [CREGG J., 1989]

1.5 Application

CDH have a wide spread field of application in the analytical area and in the industry. Depending on the different pH optima, the best pH milieu for IET and the substrate specificity from different CDHs, they constitute a wide range for applications.

In the field of analytics it can be used in **biosensors** in different ways: The enzyme can determine a sugar substrate, or it can be used for the determination of electron acceptors. To detect carbohydrates with biosensors, the principles are based either on DET or MET. CDHs are flavocytochromes belonging to a limited number of oxidoreductases, which show efficient direct electron transfers (=DET) between the electrode surface and the active site in their native wild type form. The position of the active site is in the flavodehydrogenase domain and the electrode is located in the CYT_{CDH}. The flavodehydrogenase domain is larger than the CYT_{CDH} and is catalytically active whereas the smaller domain contains heme *b* as a cofactor. CYT_{CDH} acts as the electron transfer domain between DH_{CDH} and the electrode surface. [LUDWIG R., 2013]

To conclude CDH can directly transfer electrons on various carbon and thiol- modified gold electrodes, which is the main reason why this fungal enzyme can be used as biocatalyst in biosensors and biofuel cell anodes. In general, class I CDH are ideal bioelements for cellobiose and lactose because they have a very high specificity for ß- 1,4-linked substrates. In order to work efficiently they need an acidic milieu. CDH class II show widespread pH optima and are able to oxidise monosaccharides. Therefore they are used for biosensors to detect glucose. [HARREITHER W., 2012], [LUDWIG R., 2013]

Other examples for the usage in the analytical field are **enzymatic assays**. With the help of CDH, it is possible to measure the cellulose saccharification process. CDH can be used to quantify lactose in dairy products. It is based on the oxidation of lactose with electrone acceptor, like ferricyanide and the enzyme CDH. That action describes the context between the activity and the substrate concentration.

There is also a usage of CDH in **enzymatic biofuel cells** as anode catalyst. An enzymatic biofuel cell uses enzymes as a biocatalyst to convert the chemical energy of a fuel into electrical energy. [ZAMOCKY M., 2006]

Another application is to produce **lactobionic acid**, which is used in high price products. During an enzymatic process lactose can be converted into lactobionic acid. CDH can be used as a biocatalyst for the conversion. Lactobionic acid tastes mild, sweet and has good chelating properties. Hence lactobionic acid is applied in the food, pharmaceutical and detergent industry. In the food industry lactobionic acid can be used in dairy products like in the cheese and milk production. Some benefits of using lactobionic acid include the reduction of souring and ripening time in some dairy products. Further it can enhance the flavour and eliminate the bitterness.

[MIKKEL N., 2006]

The conversion of lactose to lactobionic acid consists of the oxidation of the free aldehyde groups of glucose on the lactose molecule to the carboxyl group. In 1889, two researchers produced lactobionic acid by oxidizing lactose with bromine. Since then the chemical oxidation method has been optimized. Scientists found out that some organisms are able to oxidise lactose to lactobionic acid. LUDWIG et al. used cellobiose dehydrogenase for the production of lactobionic acid. The catalytic production of lactobionic acid obtained the oxidation of lactose with enzymes or by using microorgansism as biocatalysts. Cellobiose dehydrogenase oxidizes several b-1,4-linked disaccharides including lactose at position C-1 of the reducing sugar to the corresponding lactones. The used electron acceptor is continually regenerated with laccase, which is a H_2O producing, copper containing oxidoreductase.

The reaction mechanism outgoing from lactose involves the formation of lactobiono-δ-lactone as an intermediate product. That product is hydrolyzed to lactobionic acid.

[GUTIERREZ L., 2012], [MIKKEL N., 2006], [LUDWIG R., 2004]

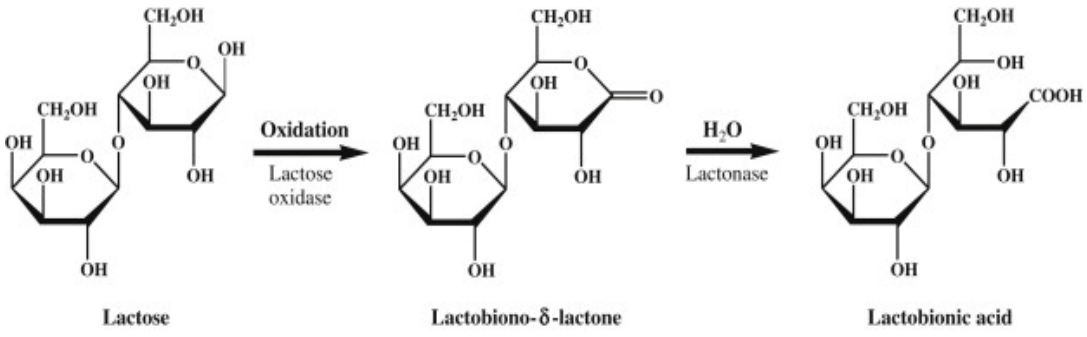


Fig. 5: Schematic biocatalyst oxidation of lactose to lactobionic acid. [GUTIERREZ L., 2012]

In further consequence it is possible to produce **gluconic acid**.

Gluconic acid is produced from glucose through oxidation. This reaction is catalyzed by glucose oxidase. The products of the oxidation of the aldehyde group on the C-1 of β –D-glucose to a carboxyl group are glucono-δ-lactone and hydrogen peroxide. Either spontaneously or by lactone hydrolysing enzyme glucono-δ-lactone is hydrolysed to gluconic acid. Gluconic acid has wide applications in food and pharmaceutical industry. At alkaline pH gluconic acid is a good chelator. The acid is mild and for example it is used in the pickling of foods. It can also be used as a component of leavening agent and as a flavouring agent.

To produce gluconic acid, there are three different approaches available: chemical, electrochemical and bioelectrochemical. The fermentation process, which mostly involves the fungus *Aspergillus niger*, is one of the most efficient techniques for manufacturing gluconic acid. [RAMACHANDRAN S., 2006]

1.6 Physical properties of Yoghurt

Cultured milk products are made as a result of the production of lactic acid, during the bacterial fermentation of lactose by adding a starter culture. Mainly *Streptococcus thermophiles* and *Lactobacillus bulgaricus* are used. The production consists of a lactic fermentation that brings milk to gelification due to destabilization of the protein. Acidification impacts many properties of casein. Casein influences the gelation properties during the formation of cultured products. [LUCEY J., 2004]

There are three types with different physical states of yoghurt: set, stirred and fluid yoghurt. Texture is one of the most essential components of yoghurt quality. To determine the yoghurt texture, there are analytical methods to measure the sensory characteristics, the structure and the rheology. The starter culture, fat content, incubation temperature, homogenization, pH at breaking, cooling conditions are factors which influence the yoghurt texture. It is well established that fermented dairy products have benefits in managing intestinal disorders such as lactose intolerance or acute gastroenteritis. By addition of probiotics, prebiotics and different food processes the rheology and texture properties of yoghurt can be changed. There is still need to optimize these dairy products. [OZCAN T., 2013]

2 Aim of the thesis

CDH clusters phylogenetically into 3 branches. They are secreted by different fungi. Members of class I and class II have already been characterized. There is almost no information published for class III CDH.

The target organism of this master project is CDH of *Aspergillus oryzae*. One class III CDH gene of *Aspergillus oryzae* was selected for recombinant production. To compare the characteristics of class III CDH with class II, a class II from *Aspergillus oryzae* is expressed and characterized. Since cellobiose dehydrogenase can be applied in food science, the production of lactobionic acid and yoghurt with the produced and purified *Myriococcum thermophilum* CDH mutant was another goal of this project.

3 Materials and Methods

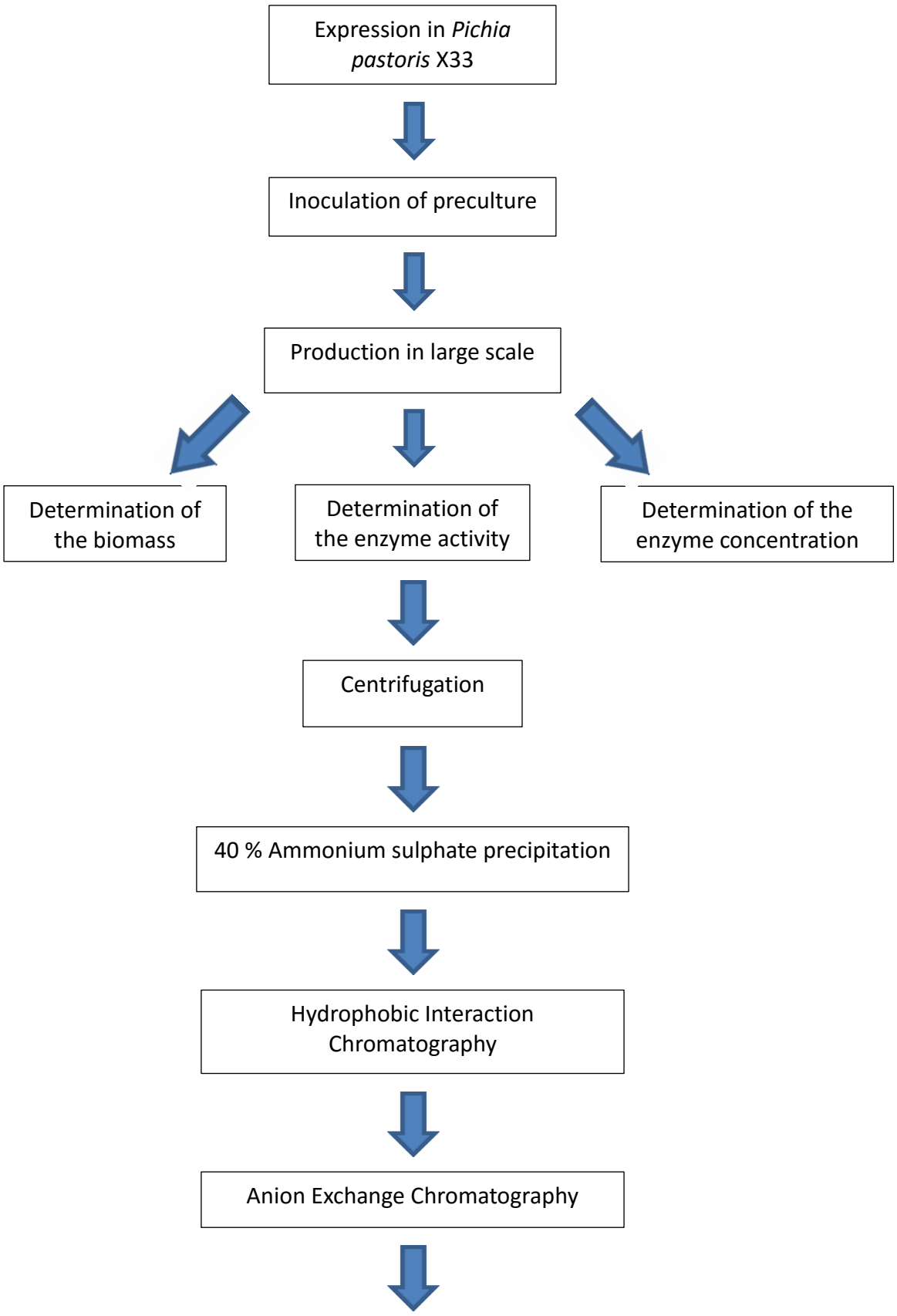
All chemicals and media components used were purchased in the highest grade of purity available from Sigma Aldrich (Steinheim, Germany). Substrates for kinetic measurements such as lactose, maltose, cellobiose, glucose and the electron acceptors 2.6-dichloroindophenol (DCIP) and cytochrome c (cyt c) were obtained from Sigma Aldrich. The Bradford assay was obtained from Bio- Rad (Hercules, CA, USA).

3.1 Technical equipment

Apparatus	Producer
Autoclave	Laboratory autoclave, Certoklav EL, 10L/12L, Kelomat
Balance	Sartorius analytic
Centrifuges	Eppendorf Centrifuge 5415R Amikon 10 K
Fermenter	Multifors, 6fors
Laminar flow bench	Sterile bench HS-P 12/2, Haraeus
Microplate reader	Perkin Elmer, 2300 EnSpire Multimode Plate reader
Microsensor Oxygen Meter	Instrument Manual MICROX TX 3
pH Meter	pH- and conductivity electrodes
Pipettes	Eppendorf
Purification equipment	ÄKTA Explorer, Amersham Bioscience Phenyl-Sepharose Fast Flow gel in XK50 column, GE Healthcare Q- Source HR 20/5 column, GE Healthcare

	Amicon ultra 30mm centrifugal filters Kross-flow
SDS-PAGE equipment	Electrophoresis system, Bio- Rad Bio- Safe Coomassie staining solution Bio-Rad
Sterile bench	Thermo Scientific
Thermoshaker	Eppendorf
UV-VIS spectrophometer	Beckmann DU- 800
Water bath	Julabo TW 12

3.2 Production and characterization of *A. oryzae* CDH class II and III



Characterisation of *A. oryzae* CDH II

- SDS-PAGE
- pH-profile
- Temperature profile
- Kinetic measurements

Characterisation of *A. oryzae* CDH III

- SDS-PAGE
- Substrate screening

3.2.1 Cultivation of *Pichia pastoris* on YPD Agar

Equipment:

Sterile bench: Thermo Scientific

The gene was already cloned into pPICz α vector for expression in *Pichia pastoris* X33. Petri dishes with YPD agar and zeocin were used for the cultivation of *Pichia pastoris*. Zeocin is used as a selection marker to prevent growth of yeast cells without plasmid. Under sterile conditions in the laminar flow bench a single colony was picked with a toothpick and streaked out onto the agar plate. The subculture cells were incubated for 2 days at 30 °C.

3.2.2 Inoculation of preculture and production in large scale

Material:

- YPD liquid medium

compound	concentration [g/L]
Peptone for casein	20.0
Yeast extract	10.0
D- Glucose	4.0

- YNB (=Yeast Nitrogen base) 10X YNB; 13.4 %

compound	concentration [g/L]
YNB	34.0
Ammonium sulfate	100.0
H_2O	1.0 L

- Biotin 500XB (0.02 %)

compound	concentration [g/L]
Biotin	0.2
Dist. water	1.0

- 1 M Potassium phosphate buffer (pH 6.0)

compound	Amount [ml]
Potassium hydrogen phosphate	132.0
Potassium dihydrogen phosphate	868.0

- BMMY (= Buffered Methanol complex Medium)

compound	concentration [g/L]
Yeast extract	10.0
Peptone	20.0
H_2O	0.79
Autoclave for 20 min on liquid cycle, 120 °C	
1M potassium phosphate buffer (pH=6.0)	0.1
YNB	0.15
Methanol	0.0075
Biotin	0.003

Equipment:

Laboratory autoclave, Certoklav EL, 10L/12L, Kelomat

After two days *P. pastoris* was transferred into baffled shaking flasks for liquid precultures under sterile conditions. In each 4 of 1 L flasks was given 150 ml YPD as medium and 150 µl zeocin. The antibiotic zeocin was added in a concentration of 100 µg/ml to prevent contaminations. Cells were picked and dissolved from tooth stick within 1 ml 0.9 % NaCl and added to the flasks. Flasks with *A. oryzae* CDH class II were incubated in the thermoshaker at 25 °C for two days with 110 rpm. *Mt*CDH at 30 °C with 150 rpm.
Afterwards production was started in 5 L baffled shaking flasks. The precultures were transferred each into 1.5 L BMMY and were again incubated in the shaker for five days. Induction of AOX promotor by addition of 0.24 g/ml methanol 4 times per day for *A. oryzae* CDH II and 8 times per day for the production of *A. oryzae* CDH III. The production process was aerated with air.

During the production process, the biomass, enzyme activity and protein concentration were determined every day. To determine the wet biomass the pellet was weighted after centrifugation at 6400 rpm for 5 min. Samples were taken on a daily basis by aseptically pipetting 1 ml sample into Eppendorf tubes.

3.2.3 Enzyme purification

For the protein purification, the ÄKTA Protein Purification system was used. Protein purification was started by centrifugation of the fermentation broth. (CDH II: 4000 rpm for 15 min), (CDH III: 6000 rpm for 20 min)

The purification of CDH was implemented with usage of two chromatography applications: hydrophobic interaction chromatography and anion exchange chromatography. Before and after each purification step the enzyme activity and protein concentration were measured to calculate the purification factor.

3.2.4 Hydrophobic interaction chromatography

Material:

0.2 M disodium hydrogen phosphate (Na_2HPO_4)

0.1 M citric acid

Mcllavine Buffer System:

- to prepare 20 ml of the buffer, mix 0.2 M disodium hydrogen phosphate and 0.1 M citric acid as shown below:

pH= 5.0		pH= 8.0	
Na_2HPO_4	51.5 ml	Na_2HPO_4	19.45 ml
Citric acid	48.5 ml	Citric acid	0.55 ml

Column:

Column: Phenyl Sepharose Fast Flow gel in XK50 column

Size: 750 ml

maximal pressure: 0.3 MPa

maximal flow rate: 10 ml/min

Buffer A: 50 mM sodium acetate + 40 % ammonium sulphate

Buffer B: 50 mM sodium acetate

Equipment:

ÄKTA Explorer

GE Healthcare

Kross-flow

The clear supernatant was loaded onto a 750 ml Phenyl- Sepharose Fast Column equilibrated with 50 mM sodium acetate buffer pH 5.0 containing 40 % ammonium sulphate. The conductivity and the wavelength of 280 nm for total protein, 420 nm for the heme domain and 450 nm for the FAD domain were monitored during the purification.

To remove unbound protein, the column was washed with Buffer A for Hydrophobic Interaction Chromatography. To remove target protein from the column, a linear elution gradient from 0 to 100 % buffer B for Hydrophobic Interaction Chromatography was applied. Fractions of interest were collected as soon as there was an increase in the absorbance at 420 and 280 nm. From these fractions the protein concentrations by Bradford, DCIP activity and cyt *c* were determined. The fractions with the highest specific activities were pooled together and concentrated by kross-flow.

3.2.5 Anion exchange chromatography

Material:

Column: Q- Source

Size: 19 ml

maximal pressure: 1.5 MPa

maximal flow rate: 5 ml/min

Buffer A: 50 mM sodium acetate

Buffer B: 50 mM sodium acetate + 1 M NaCl

Equipment:

HR 20/5 column, GE Healthcare

Amicon ultra 30 mm centrifugal filters

Before the second step of purification, the samples and buffers were degassed and filtrated. To degas bottles with solutions, they were put into an ultrasonic bath for 10 min. For the filtration process cellulose filters were used.

The filtrated CDH pools were applied onto a 19 ml column packed with Q- Source pre-equilibrated with 50 mM sodium acetat buffer pH 5.0. The conductivity and the wavelength of 280 nm for the total protein, 420 nm for the heme domain and 450 nm for the FAD domain were monitored during the purification. Again the column was washed with Buffer A for Mono Q- Source- strong anion exchange to remove unbound protein. Elution of CDH with the gradient from 40 % ammonium sulphate to 0 %. As soon as the absorbance at 280 nm

increased the fractions were collected, pooled and afterwards concentrated by using Amikon Ultra centrifugal filters devices with a cutoff of 30 kDa.
The purified enzymes were stored at -30 °C for further analytical applications and measurements.

3.2.6 Absorption spectroscopy

Every substance has its own specific absorption spectrum. Purified CDH shows a characteristic absorption spectrum in the oxidized as well in the reduced state. The soret band of the oxidized spectrum is at 420 nm due the heme cofactor. The peak of the FAD group shows a broader shoulder between 450 and 500 nm. [ZAMOCKY M., 2006]

3.3 Protein characterization

The purified enzymes were characterized by using cyt *c*, DCIP, Bradford assays and SDS-PAGE. With the *A. oryzae* CDH II pH- and temperature profiles were determined. Steady-state kinetic constants were determined for *A. oryzae* CDH II. A substrate screening was done with *A. oryzae* CDH III using the DCIP and cyt *c* assay.

3.3.1 Protein concentration determination

3.3.1.1 Bradford assay

Equipment:
UV- VIS Spectrophometer, Beckmann DU- 800

Protein concentrations were determined by Bradford method using the Bradford reagent from Bio- Rad Laboratories. 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 a binding to a protein occurs. Coomassie Brilliant Blue G- 250 dye binds especially to arginine and to aromatic amino acids like phenylalanine and tryptophan. [COMPTON S., 1985]
In practice it means that if the dye binds to protein, the colour will change from brown to blue. For the calibration, bovine serum albumin was used at a concentration ranging from 0.1 mg/ml to 1.0 mg/ml.

15 µl of sample were mixed with 600 µl Bradford solution in a cuvette. Before measuring the absorbance, the solutions were incubated for 15 min at room temperature.

Tab. 1: Pipetting scheme for Bradford assay

Reagent	Volume µl/ cuvette
Bradford Reagent	600
Sample	15

3.3.2 Enzyme activity determination

3.3.2.1 DCIP assay

Material:

- DCIP solution: dissolve 78.03 mg 2,6-dichloroindophenol (=DCIP) in 10 ml 96 % ethanol and 100 ml distilled water
- 300 mM lactose solution

Equipment:

Water bath, Julabo TW 12

UV- VIS spectrophometer

This method is used as a standard method to determine CDH activity in purified enzyme samples. To prove the presence of the dehydrogenase domain, DCIP can be used as an

electron acceptor. After oxidation of the substrate, two electrons are transferred to DCIP. [SYGMUND C., 2013]

For the measurement all reagents except the sample were mixed in a 1 ml micro cuvette and were incubated for 20 min at 30 °C. After the cuvette was placed in the photometer, enzyme was added and mixed. The absorbance was measured at 520 nm for 180 seconds.

Tab. 2: Pipetting scheme for DCIP assay

Reagent	Volume µl/ cuvette
DCIP solution	100.0
300 mM lactose	100.0
McIlvaine`s buffer, pH 5.0	780.0
Sample	20.0

3.3.2.2 Cytochrome *c* assay

Material:

- According to your needs dissolve 3.1, 6.2 or 12.4 mg cyt *c* in 0.25, 0.5 or 1.0 ml of distilled water
- 300 mM lactose solution

Equipment:

Water bath, Julabo TW 12

UV- VIS spectrophometer

Compared with the DCIP assay, the cyt *c* assay determines the activity of the holoenzyme CDH, which shows whether the FAD and the heme domain are present. The activity is determined by monitoring the reduction of cyt *c*. This electron acceptor is exclusively reduced at the cytochrome domain. [SYGMUND C., 2013]

For the measurement all solutions except the sample were pipetted into a 1 ml micro cuvette and were incubated in a waterbath at 30 °C for 20 min. Afterwards the sample was added and the reaction was started. The absorption was measured at 550 nm for 180 seconds.

Tab. 3: Pipetting scheme for cyt *c* assay

Reagent	Volume µl/ cuvette
Cyt *c* solution	20.0
300 mM lactose	100.0
McIlvaine`s buffer, pH 5.0	860.0
sample	20.0

3.3.3 SDS Polyacrylamid- Gelelectrophorese

Equipment:

Min-Protean Tetra System from Bio-Rad, 4-20 % SDS- Page gel from Bio-Rad

To analyse the molecular weight of an enzyme and to prove the purity of enzymes a SDS-PAGE was done. Through this method it is possible to separate proteins according to their size in an electrical field.

Dilutions of the samples were prepared and two fold concentrated Laemmli Buffer was added to each sample (1:1).

The mixtures were heated for 5 min at 99 °C. 20 µl of each sample and 10 µl of an unstained protein standard were used for mass determinations. Protein bands were visualized by staining them with Bio- Safe Coomassie. The gel had been run at 120 V for an hour.

3.3.4 Further characterization of CDH II

3.3.4.1 pH profiles

Material:

Glycine sodium hydroxide buffer, McIlvaine buffer

Equipment:

pH meter

PH profiles for DCIP and cyt *c* assays were done to determine the pH optimum. McIlvaine buffer was used for pH 3.0, pH 8.5 and for pH 8.5 to pH 10.0 a glycine sodium hydroxide buffer was used. The measurements were done at the photometer. As a sample a purified enzyme of defined dilution (cyt *c:* 1:100, DCIP: 1:50) was used.

3.3.4.2 Temperature profile

Equipment:

thermometer

Temperature profiles with cyt *c* assay were done. The pipetting scheme for the assay is shown in table 3. The enzyme was diluted 1:100 and was heated in a thermomixer. The McIlvaine buffer solution had a constant pH of 5.0 and instead of lactose, 200 mM cellobiose was used. The enzyme activity from 100 % to 20 % was measured at temperatures 55 °C, 60 °C and 65 °C.

3.3.4.3 Determination of the kinetic constants

Kinetic constants were determined with DCIP and cyt *c* assays for glucose, maltose, lactose and cellobiose. For the DCIP assays the purified enzyme was diluted 1:50 and for the cyt *c*

assay 1:100. Catalytic constants were calculated by fitting the observed data to the Michaelis- Menten equation with the support from Sigma Plot 11. The Michaelis Menten equation was used to calculate from the experimental data the Michaelis Menten constant (K_M) and the maximum reaction velocity ($_{Vmax}$).

$$v_0 = \frac{v_{\max} \cdot [S]}{K_m + [S]}$$

3.4 Characterization of CDH III

3.4.1 Substrate screening

Material:

96 deep well plates

Equipment:

Multichannel Pipette

Microplate reader, Perkin Elmer, 2300 EnSpire Multimode Plate reader

The DCIP and cyt *c* assay were used to determine the activity of *A. oryzae* CDH III with different substrates.

Tab. 4: Pipetting scheme for substrate screening with cyt *c* as electron acceptor

Reagent	Volume µl/ well
Cyt *c* solution	8.0
Undiluted enzyme	20.0
Substrate	222.0

Tab. 5: Pipetting scheme for substrate screening with DCIP as electron acceptor

Reagent	Volume µl/ well
DCIP solution	20.0
Undiluted enzyme	20.0
Substrate	210.0

The required amount of substrate (see table 6) was dissolved in McIlvaine buffer pH 5.0 and pH 8.0 by heating and/or ultrasonic bath. DCIP solution and substrate were transferred into 96 well plates and preincubated at 30 °C for 30 min. The reaction was started by adding

undiluted enzyme and absorption was measured by Microplate reader. End point determinations were carried out after 1 h.

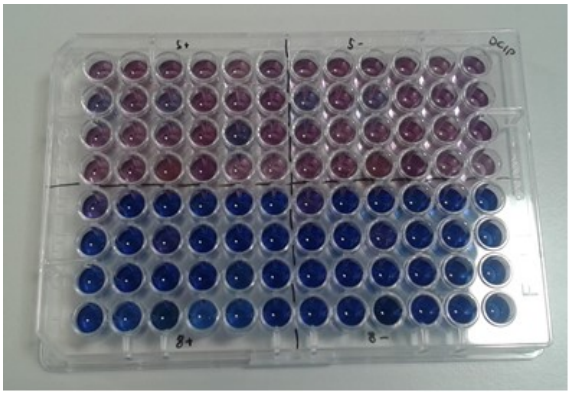


Fig. 6: Example of a 96- well plate containing different substrates, *A. orycae* CDH III and DCIP as electron acceptors.

Tab. 6: List of substrates for the substrate screening with *A. orycae* CDH III

Sugars

monosaccharides		
L-Arabinose	M= 150.13 g/mol	x= 0.0165 g
Arabitol	M=152.1 g/mol	X=0.001521 g
L-Erythrulose hydrate	M=138.121 g/mol	x= 0.01519331 g
Fructose	M= 180.16 g/mol	x= 0.019817 g
D- Fucose	M=164.16 g/mol	x= 0.01804 g
L- Fucose	M=150.13 g/mol	x= 0.01804 g
Galactose	M=180.16 g/mol	x=0.0198 g
Glucose Monohydrate	M= 198.17 g/mol	x= 0.0217987 g
α -D- glucose-1-Phosphat	M=372.36 g/mol	x=0.0037236 g
Lyxose	M= - g/mol	x= small tip of spatula
D-Mannose	M=180 g/mol	x= 0.0018016 g
Methyl α-D- glucopyranoside	M=194.19 g/mol	x=0.0019419 g
Methyl β -D- glucopyranoside	M=203.19 g/mol	x=0.0020319 g

Rhamnose	M= 342.3 g/mol	x= 0.0376 g
Sedoheptulose	M= 594.51 g/mol	x= 0.0653961 g
Sorbose	M= 182.18 g/mol	x= 0.0200398 g
Talose	M= 738 g/mol	x= 0.08118 g
Xylose	M= 180.16 g/mol	x= 0.0198176 g
disaccharides		
Gentibiose	M=342.30 g/mol	x=0.003423 g
Leucrose	M=342.30 g/mol	x=0.037653 g
Maltulose	M= 150.13 g/mol	x= 0.0165 g
Maltose	M= 342.30 g/mol	x= 0.037653 g
Mellibiose Dihydrate	M=378.33 g/mol	x=0.0037833 g
Palatinose	M=342.296 g/mol	x=0.003784 g
Sucrose	M= 180.16 g/mol	x= 0.01982 g
Trehalose	M= 320.30 g/mol	x= 0.0352 g
trisaccharides		
Raffinose pentahydrate	M= 180.16 g/mol	x= 0.0198 g
Xylotetraose	M= 342.30 g/mol	x= 0.03765 g
Cellotriose	M=504.44 g/mol	x=0.0025222g (5mM)
polysaccharides		
Amylose from potato	M= 162.14 g/mol	x=0.01782 g
Amylopektin from maize	M=162.14 g/mol	x= 0.0178354 g
Arabinohexaose	M= - g/mol	x= small tip of spatula
Carboxymethylcellulose Sodium salt		
α-Cyclodextrin	M=972.86g/mol	x=0,0097286 g
β- Cyclodextrin	M=1134.98g/mol	x=0,011498 g
Dextran T500	M=162.14g/mol	x=0,0016214 g
Dextrin from potato starch		
Ficoll		
Glycogen	M=666.57 g/mol	X=0,0066657 g

Gum arabic from acacia tree		X=0,002 2g
Gum guar		
Gum, Locust Bean		
Inulin		
Maltotetraose	M= 360.32 g/mol	X=0.03963 g
Mellobiose	M=666.58 g/mol	x= 0.0733238 g
Pektin from citrus peel		X= 0.1 g
Stachyose	M= 210.18 g/mol	x= 0.02312 g
Xanthine	M=152.11 g/mol	X=0.0015211 g
Xylan	M=132 g/mol	X= 0.00132 g

Alcohols

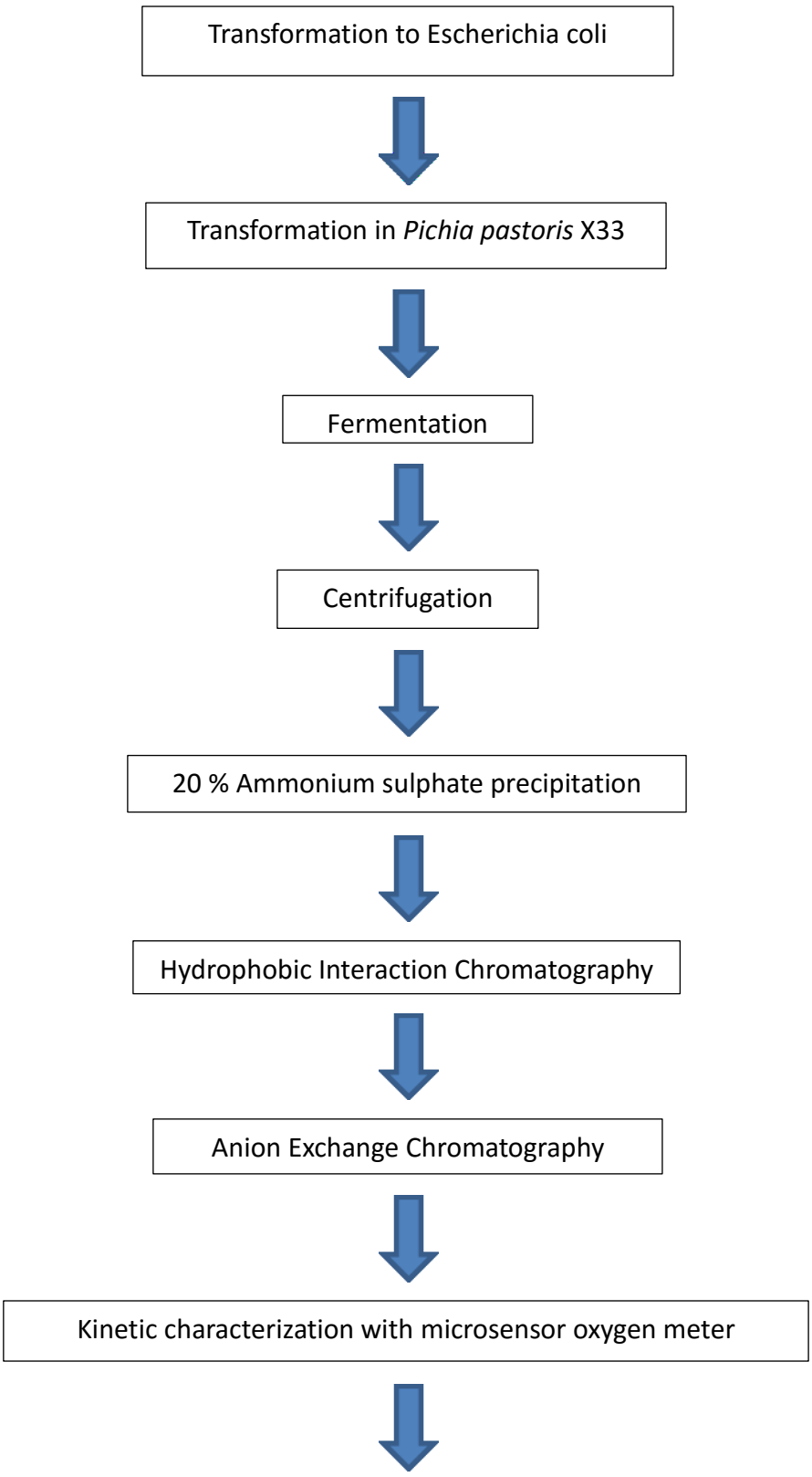
Benzaldehyd	M=106.12g/mol	X=0,00021224g (2mM)
Benzalkohol	M=198.14g/mol	X=0,00010814g (1mM)
1-Butanol	M= 74.12g/mol	X=0,00007412g (1mM)
2,4- Dimethyloxybenzylalc.	M= 168.9g/mol	X=0,00033638g (2mM)
2,6-Dimethoxyphenol	M=154.17g/mol	X=0,00015417g (1mM)
Cholin		
Cinnamylalcohol	M=134.18 g/mol	
Dulcitol	M=182.2 g/mol	X=0.0000911 g (500 µM)
Ethanol	M=46.10 g/mol	X=0.0000461 g (1mM)
Ethylenglycol	M=62.07 g/mol	X=0.00006207 g (1mM)
Guaiacol	M=124.1 g/mol	X=0.0001241 g (1mM)
Hydroxybezylalc.	M=124.14 g/mol	X=0.00024828 g (2mM)
1-Hydroxybenzotriazole	M=135.13 g/mol	X=0.00013513 g (1mM)
Isomylalc.	M=88.15 g/mol	X=0.000176296 g (2mM)
Methanol	M=32.04 g/mol	X=0.00001602 g (1mM)
Methoxybenzylalc.	M=138.16 g/mol	X=0.000138 g (1mM)
Nitrophenol	M=139.1 g/mol	X=0.0001391 g (1mM)
D-Pentolacton	M=94.11 g/mol	X=0.00009411 g (1mM)

Phenol	M=94.11 g/mol	X=0.00009411 g (1mM)
2-Phenylethanol	M=122.2 g/mol	X=0.0001222 g (1mM)
Propylenglycol	M= 76.09 g/mol	X=0.00007609 g (1mM)
Resorcinol	M=110.11 g/mol	X=0.00011011 g (1mM)
Thymol	M=150.22 g/mol	X=0.00015022 g (1mM)
Vanillylalcohol	M=154.16 g/mol	
Veratrylalc.	M=186 g/mol	X=0.000372 g (2mM)
Xylitol	M= 153.1 g/mol	X=0.0001512 g (1mM)

Amino acids

Alanin	M=89.10g/mol	X=0,000891 g (10mM)
Alginic acid		
L- Arginin	M=174.2 g/mol	X=0,001742 g (10mM)
L- Asparagin	M=165.19 g/mol	X=0,0016519 g (10mM)
L-Glutamin	M=146.15 g/mol	X=0,0014615 g (10mM)
Glycin	M=75.07 g/mol	X=0,00075 g (10mM)
L-Histidin	M=155.16g/mol	X=0,0015516 g (10mM)
L-Isoleucin	M=131.18g/mol	X=0,0013118 g (10mM)
L- Leucin	M=131.17 g/mol	X=0,0013117 g (10mM)
L-Lysin monohydrochloride	M=182.65g/mol	X=0,0018265 g (10mM)
L-Methionin	M=149.21g/mol	X=0,0014921 g (10mM)
L-Prolin	M=115.13g/mol	X=0,0011513 g (10mM)
L-Tyrosin	M=181.19g/mol	X=0,0020423 g (10mM)
L-Valin	M=117.15g/mol	X=0,0011715 g (10mM)
Aromatic Amino acids		
L- Phenylalanin	M=165.19 g/mol	X=0.0016519 g (10mM)
L- Tryptophan	M=294.23 g/mol	X=0.0020423 g (10mM)
L- Tyrosin	M=181.19 g/mol	X=0.0018119 g (10mM)

3.5 Application of *Mt*CDH



Production of lactobionic acid, gluconic acid, cellobionic acid and maltobionic acid

- HPLC analysis
- Enzyme activity during the production processes: DCIP assays
- FOX assay during the production process of lactobionic acid

  Enzymatic assay of catalase during the production process of lactobionic acid
- pH change during the production process of lactobionic acid

Enzymatic production of yoghurt

- Pre- experiments in petri dishes
- Enzymatic production of yoghurt in the Sixfors system
- pH change during the production process of yoghurt
- HPLC analysis
- Textual properties of yogurt

3.5.1 Production of the enzyme

Media:

- LB

compound	concentration [g/L]
Peptone for casein	10.0
Yeast extract	5.0
NaCl	10.0

Myriococcum thermophilum CDH was already cloned into pPICz α plasmid.

Amplification of plasmid

- Transformation to *Escherichia coli* (NEB5 α chemical competent cells).
- 4 colonies were picked up for liquid culture in 2 ml LB with zeocin and were incubated at 37 °C over night. Each selected clone was put on LB and zeocin agar plate and incubated at 37 °C for 2 days.
- MiniPrep: Plasmid isolation is a method for isolation of the plasmid DNA

Sequencing: Sequencing was done to determine if the enzyme is correctly cloned into the plasmid.

- Transformation in *Pichia pastoris* X33 (electrocompetent cells)

The plasmid was linearized with SacI restriction enzyme for 2 h in 37 °C.

4 µl of DNA was added to 50 µl of *P. pastoris* electrocompetent cells. The mixture was kept into a chilled cuvette. After the electroporation process, 500 µl cold 1 M sorbitol and YPD pH 7.0 were added. The sample was transferred into a 2 ml eppendorf tube. The cells were incubated in the thermoshaker at 30 °C for 4 hours at 100 rpm and then centrifuged with 1400 rpm. 700 µl of the supernatant were streaked out onto an YPD plate pH 7.0 containing 100 µg/ml zeocin. Colonies were growing after 2 days. The best clone was taken for the production of enzymes by fermentation process.

3.5.2 Purification

For the protein purification the ÄKTA Protein Purification system was used.

Protein purification was started by centrifugation of the fermentation broth. (*Mt*CDH: 6000 rpm for 15 min)

The purification of CDH was performed by a two-step purification: hydrophobic interaction chromatography and anion exchange chromatography.

3.5.2.1 Hydrophobic interaction chromatography

Material:

- 0.2 disodium hydrogen phosphate (Na_2HPO_4)
- 0.1 citric acid

Mcllavine Buffer System:

- to prepare 20 ml or 100 ml of the buffer, mix 0,2 M disodium hydrogen phosphate and 0,1 M citric acid as shown below:

pH=5.0	100 ml	pH=8.0	20 ml
Na_2HPO_4	51.5 ml	Na_2HPO_4	19.45 ml
Citric acid	48.5 ml	Citric acid	0.55 ml

Column:

Column: Phenyl Sepharose Fast Flow gel in XK50 column

Size: 750 ml

maximal pressure: 0.3 MPa

maximal flow rate: 10 ml/min

Buffer A: 50 mM sodium acetate + 20 % ammonium sulphate

Buffer B: 50 mM sodium acetate

Equipment:

ÄKTA Explorer

GE Healthcare

Kross-flow module

The clear supernatant was loaded onto a 750 ml phenyl-Sepharose Fast Column equilibrated with 50 mM sodium acetate buffer pH 5.0 containing 20 % ammonium sulphate. The conductivity and the wavelength of 280 nm for total protein, 420 nm for the heme domain and 450 nm for the FAD domain were monitored during the purification.

To remove unbound protein, the column was washed with Buffer A for Hydrophobic Interaction Chromatography. To remove target protein from the column, a linear elution gradient from 0 % to 100 % buffer B for Hydrophobic Interaction Chromatography was applied. Fractions of interest were collected as soon as there was an increase in the absorbance at 420 and 280 nm. From these fractions the protein concentration by Bradford, DCIP activity were determined. The fractions with the highest specific activities were pooled together and concentrated by kross-flow.

3.5.2.2 Anion exchange chromatography

Equipment:

HR 20/5 column, GE Healthcare

Column:

Q- Source

Size: 200 ml

maximal pressure: 1.5 MPa

maximal flow rate: 6 ml/min

Buffer A: 50 mM sodium acetate

Buffer B: 50 mM sodium acetate+ 1M NaCl

Before the second step of purification, the samples and buffers were degassed and filtrated. To degas bottles with solutions, they were put into an ultrasonic bath for 10 min. For the filtration process cellulose filters were used.

The filtrated CDH pools were applied onto a 200 ml column packed with Q- Source pre-equilibrated with 50 mM sodium acetate buffer pH 5.0. The conductivity and the wavelength of 280 nm for total protein and 450 nm for the FAD domain were monitored during the purification. Again the column was washed with Buffer A for Mono Q- Source- strong anion exchange to remove unbound protein until a stable conductivity. As soon as the absorbance at 280 nm increased the fractions were collected, pooled and afterwards concentrated by using the kross-flow because of the large volume. Then the concentrate enzymes were stored at -30 °C for further analytical applications and measurements.

3.5.3 Kinetic characterization with oxygen as electron acceptor

Equipment:

Microsensor Oxygen Meter, Instrument Manual MICROX TX 3

The fiber- optic oxygen microsensor is based on dynamic fluorescence quenching to measure oxygen concentration. The microsensor is fabricated by immobilizing an oxygen- quenchable fluorophore at the tapered tip of an optical fiber. [KLIMANT I., 1995]

Before the measurements started lactose solutions with concentration of 30 mM, 10 mM, 3 mM, 0.3 mM and 0.1 mM were prepared with McIlvaine buffer pH 7.0. 3 ml of each solution were pipetted into an eprouvette and put into a water bath of 30 °C for 10 min. During this incubating time the solutions were enriched with oxygen. Then 1.85 ml of each solution was pipetted into a vial. Before the microsensor was inserted into the solution 20 µl of the enzyme was added. Measurements ended when the oxygen concentration reached approximately 7 µmol/L. During the measurements the liquid is mixed by a magnetic stirrer and the temperature is held constant at 30 °C by a water bath.

For the temperature optimum 30 mM lactose were prepared with McIlvaine buffer pH 7.0 and measured at 25 °C, 30 °C, 40 °C, 50 °C, 60 °C and 70 °C.

3.5.4 Titration

Material:

0.25 M HCl: 1.04 ml 37 % HCl dissolved with 50 ml dest. water

To determine the pH dependent clotting of casein a titration was carried out with 100 ml unskimmed milk (*"Milfina"*) and 0.25 M HCl. The pH was measured by pH meter and 0.25 M HCl was added until the casein coagulated.

3.5.5 Production of lactobionic acid, gluconic acid, maltobionic acid and cellobionic acid

Material:

1 M sodium carbonate

Equipment:

Sixfors

The *Mt*CDH catalyzed oxidation of lactose, cellobiose, glucose and maltose was performed at 30 °C using the bioreacter system Sixfors with a working volume of 1 liter. The reactor was equipped with standard probes for the measurement and control of the pH- values, temperature and oxygen. The reaction system contained 130 mM lactose, 130 mM cellobiose, 130 mM glucose and 130 mM maltose. Further each reactor contains 0.304 U/ml *Mt*CDH. 1800 U/ml 0.036 % catalase were added to the reactors with cellobiose, maltose and glucose and 1620 U/ml 0.036 % catalase in the reactors which contained lactose. Two initial solutions were prepared for the implementation from lactose into lactobionic acid. In one solution the pH was not controlled but in the other one the pH was held constant at pH 4.0 by addition of 1 M Na_2CO_3. In the reactors, which contained cellobiose, maltose and glucose, the pH was also held constant at pH 4.0 by addition of 1 M Na_2CO_3. Each bioreactor had a starting volume of 312.22 ml. The reaction system was stirred and dissolved oxygen was automatically controlled at 20 % saturation. The reactions ran for 24 hours.

3.5.5.1 FOX assay (= Ferrous oxidation- Xylenol orange)

Material:

FOX reagent: mix 0.2 ml Fox reagent A and 20 ml Fox reagent B

FOX reagent A: 25 mM ammonium ferrous sulfate and 2.5 mol/L H_2SO_4

FOX reagent B: 125 µM xylenol orange

Equipment:

UV- VIS spectrophometer, Beckmann DU- 800

During the production process of lactobionic acid the hydrogen peroxide concentration was measured by using the FOX assay. The samples were immediately heated for 5 min at 95 °C and placed on ice. Then they were centrifuged for 10 min at 14600 rpm and the supernatant was transferred into a new Eppendorf tube.

The assay is based on the oxidation of ferrous iron by H_2O_2 to ferris irons, which binds to xylenol orange to give a coloured complex. The absorbance was measured at 560 nm.

First a calibration curve was done to evaluate the concentrations of H_2O_2 of unknown samples by comparing the unknown to a set of standard samples of known concentrations. The known concentration of H_2O_2 for the calibration curve were: 250 µM, 150 µM, 100 µM, 50 µM and 10 µM

For the measurement, the FOX reagent and methanol were mixed into a cuvette and incubated at room temperature for 30 min. Before the measurement in the photometer started the sample was added.

Tab. 7: Pipetting scheme for the FOX assay

Reagent	Volume µl/ cuvette
sample	90 µl
methanol	10 µl
FOX reagent	900 µl

3.5.5.2 Enzymatic assay of catalase

Material:

- hydrogen peroxide solution [0.036 % (w/w)]

To prepare 20 ml of the H_2O_2 solution mix 240 µl of a 3 % H_2O_2 and 19.76 ml of 50 mM Potassium phosphate buffer pH 7.0.

Equipment:

UV- VIS spectrophometer

During the production process of lactobionic acid, the catalase activity was measured by using the catalase assay. Catalase was added to prevent the accumulation of hydrogen peroxide.

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2\ H_2O + O_2$$

A blank against a quartz cuvette containing 3000 µl phosphate buffer pH 7.0 was done.
Then 2.90 ml of hydrogen peroxide solution [0.036 % w/w] was pipetted into a suitable cuvette. For each cuvette monitor the A 240 until constant and then 100 µl of catalase was added and mixed well. The time required for the A 240 decrease from 0.45 to 0.40 was taken for the calculation of the enzyme activity. To calculate the catalase activity following formula was used:
U/ml= time* 0.1* df (dilution factor)

3.5.5.3 Analytic method: HPLC

Lactose and lactobionic acid, glucose and gluconic acid, maltose and maltobionic acid and cellobiose and cellobionic acid were analyzed by HPLC using an Aminex HPX87-K column from Bio-Rad at 80 °C with HQ- H_2O as eluent (0.5 ml/min) and refractive index detection. The injection volume was 20 µl.

3.5.6 Production of enzymatic yoghurt

3.5.6.1 Pre experiment for the production of the enzymatic yoghurt

Equipment:

Petri dishes, pH- meter

To find out the optimal conditions pre experiments were done in petri dishes. In each petri dish 30 ml unskimmed, pasteurized milk ("milfina") were pipetted and 500 µl *Mt*CDH. They were incubated at different temperatures in duplicates: 4 °C, 25 °C and room temperature; with 722 µl catalase (0.036%) and without catalase. The pH values were measured over a time period of 3 days.

3.5.6.2 Production of the enzymatic yoghurt

Equipment:

Sixfors

The *Mt*CDH catalyzed oxidation of milk was performed at 30 °C using the bioreacoter system Sixfors with a working volume of 1 liter. The reactor was equipped with standard probes for the measurement and controlled in temperature and oxygen. The reaction system contained 300 ml unskimmed, pasteurized milk ("Milfina"), and 1620 U/ml catalase. The specific activity of *Mt*CDH was 2.76 U/mg and the reaction was started with 5 ml *Mt*CDH. The pH value was not controlled. The reaction system was stirred and dissolved oxygen was automatically controlled at 20 % saturation pure oxygen. The reaction was run for 24 h.

3.5.6.3 Analytic method: HPLC

Lactose and lactobionic acid were analyzed by HPLC using an Aminex HPX87-K column at 80 °C with HQ- H_2O as eluent (0.5 ml/min) and refractive index detection.

3.5.6.4 Textual properties of yoghurt

Equipment:

Centrifuge, weighing scale

Syneresis is an extraction of a liquid from a gel. During the syneresis process whey is collecting on the surface of yoghurt.

After the 25 hours production of yoghurt in the Sixfors, the product was stored at 4 °C for one week. In that time the texture became firmer.

Then the texture of the yoghurt, which was produced with *Mt*CDH, was compared with a conventional set and stirred yoghurt from the supermarket.

15 g of sample was weight out into a Greiner Bio- One was centrifuged at 2000 rpm for 10 min at 10 °C. After every centrifugation step the supernatant was weighed out and then removed. The following centrifugation step started at 3000 rpm and this one with 4000 rpm. The temperature and time conditions were the same in all centrifugation steps.

4 Results and Discussion

4.1 Production and characterization of *Aspergillus oryzae* CDH class II and III

4.1.1 *A. oryzae* CDH II

4.1.1.1 Production of *A. oryzae* CDH II

Production of *A. oryzae* CDH class II in *Pichia pastoris* was performed as described in the chapter “materials and methods”. Wet cell weight, protein concentration and enzyme activity were measured during the production process. To determine the cell mass the weight of precipitate and the Eppendorf tube was measured. Then the weight of an empty Eppendorf tube was removed. The protein concentration was measured with the Bradford assay.
The biomass and protein concentrations increased during the production.
After 5 days fermentation broth of all 4 shaking flasks were pooled together and were centrifuged at 6000 rpm for 20 min. In the supernatant a protein concentration of 0.1056 mg/ml was measured.

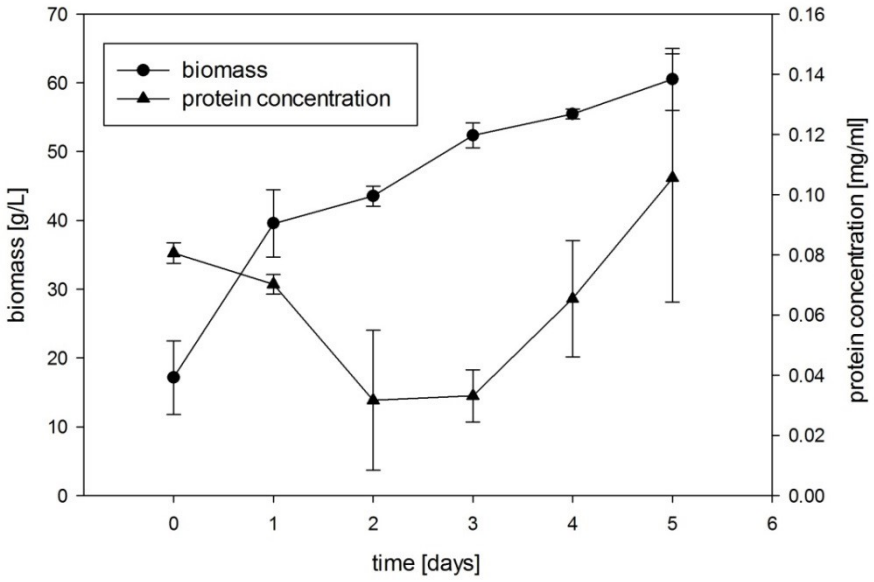


Fig. 7: Protein concentrations measured by Bradford and determination of the biomass during the production process of *A. oryzae* CDH II.

Once a day the CDH activity in the culture was measured with DCIP and cyt *c* assay during the 5 days production in the shaking flasks. After 5 days the fermentation broth of all 4 shaking flasks were pooled together and DCIP and cyt *c* assays were measured again. With the DCIP assay an activity of 0.1843 U/ml was reached and with the cyt *c* assay an enzyme activity of 0.0780 U/ml was determined.

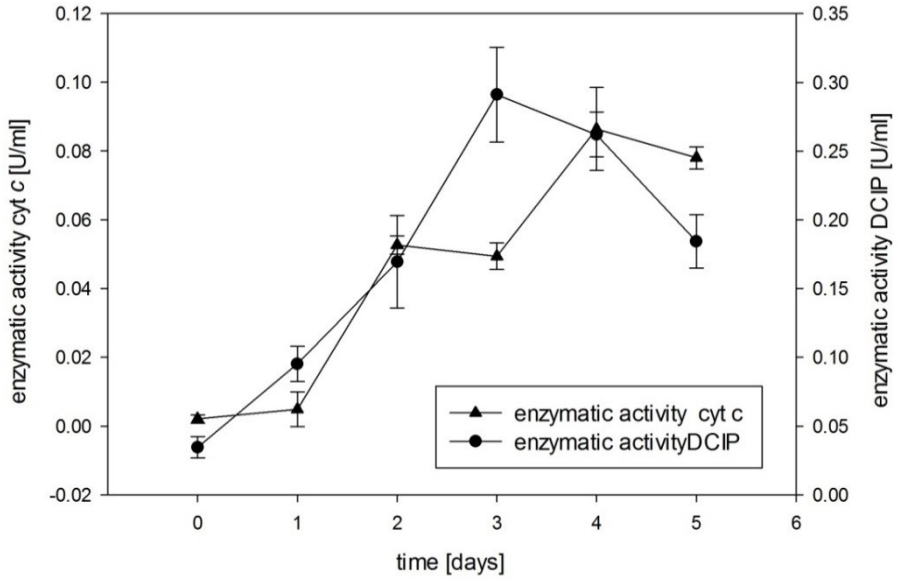


Fig. 8: Determination of the enzymatic activity with cyt *c* and DCIP assays during the production process with *A. oryzae* CDH II.

Purification was carried out by Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. The flow through was collected and measured for activity before rejecting. All purifications were assayed by using DCIP, cyt *c* and Bradford. The fractions with the highest specific activities were pooled together. The purification table is presenting the results of the chromatography.

After the first and second purification step, the specific activity increased from 1.33 U mg^{-1} to 6.79 U mg^{-1}. As a last purification step again a Hydrophobic Interaction Chromatography was performed again to separate the FAD domain from the rest CDH. A total protein yield of 3.20 mg was reached.

Tab. 8: Purification scheme of *A. oryzae* CDH II

Purification step	Total activity [U]	Total protein [mg]	Specific activity [U mg^{-1}]	Yield [%]	Purification (fold)
Culture supernatant	337.05 U/ 4500ml	254.25	1.33	100	1.00
Phenyl-Sepharose	638U/950ml	104.79	6.13	189.3	4.61
Q-Source	87.59U/143ml	12.90	6.79	25.99	5.11
Phenyl-Sepharose	21.98 U/ 1.8 ml	3.20	6.86	6.52	5.16

4.1.1.2 Characterisation of *A. oryzae* CDH II

To determine the state of enzyme purity and the molecular mass, a sample of purified CDH were loaded to a SDS-PAGE. The lane at 150 kDa is characteristic for CDH and the lane at 75 kDa shows most probably a contamination with an isolated dehydrogenase domain.

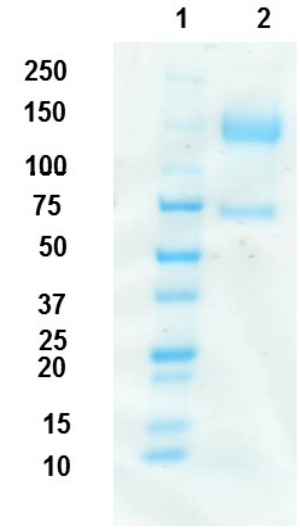


Fig. 9: SDS-PAGE of the not homogenously purified *A. oryzae* CDH II after Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. [1. Unstained Precision Plus Protein standard; 2. *A. oryzae* CDH II]

PH profiles were obtained to determine the pH with the highest enzyme activity for enzymatic reactions. They were determined with cyt *c* and DCIP assays using McIlvaine`s buffer solution with pH ranges from pH 3.0 to pH 8.5.

The graphs show the optimal pH for the enzyme at 5.0 for cyt *c* and for DCIP.

For pH ranges from pH 8.5 to 10.0 a glycin-sodium hydroxide buffer solution was used but *A. oryzae* CDH II shows no activity under this condition.

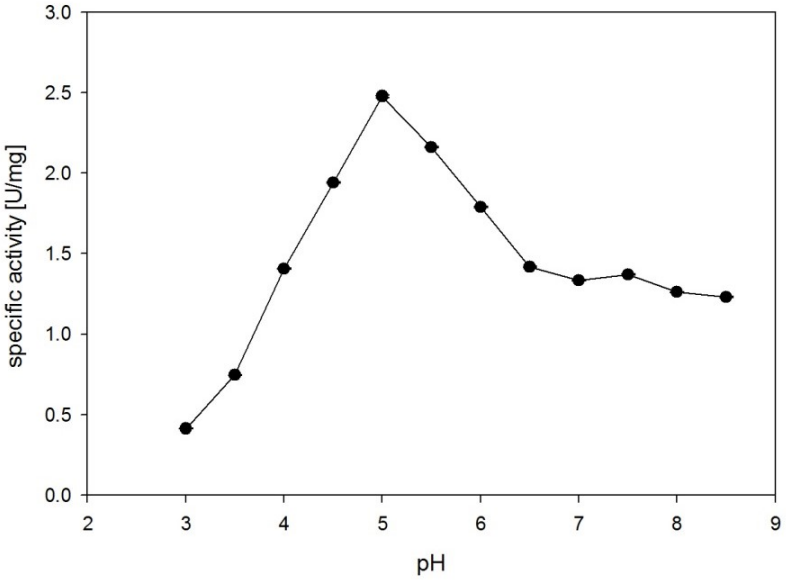


Fig. 10: pH profile of *A. oryzae* CDH II with cyt *c* as electron acceptor using McIlvaine buffer solution with pH ranges from 3.0 to pH 8.5.

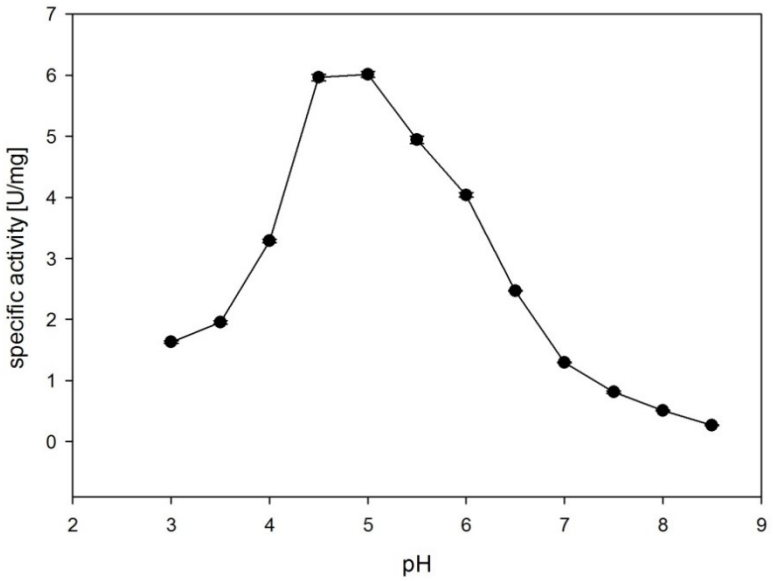


Fig. 11: pH profile of *A. oryzae* CDH II with DCIP as electron acceptor using McIlvaine buffer solution with pH ranges from 3.0 to pH 8.5.

Temperature profiles with cyt *c* assay were done to find out at which temperature the enzyme is most stable. For all measurements 20 mM cellobiose and McIlvaine`s buffer solution at a constant pH of 5.0 were used. With help of the measured outcomes the

biological half-life of *A. oryzae* CDH II and the reduction rate per minute were calculated. The biological half-life of an enzyme is the time it takes to lose half of its activity.

The biological half-life for the enzyme at 65 °C was 2.31 min and the reduction per minute was 0.29 U. At 60 °C the biological half-life amounted 9.30 min and showed a reduction of 0.075 U/ min. The best outcomes were measured at a temperature of 55 °C. The biological half time was 85.3 min and a reduction time of 0.008 U/ min was determined. At higher temperatures the enzymes fully denaturized and no activity remained.

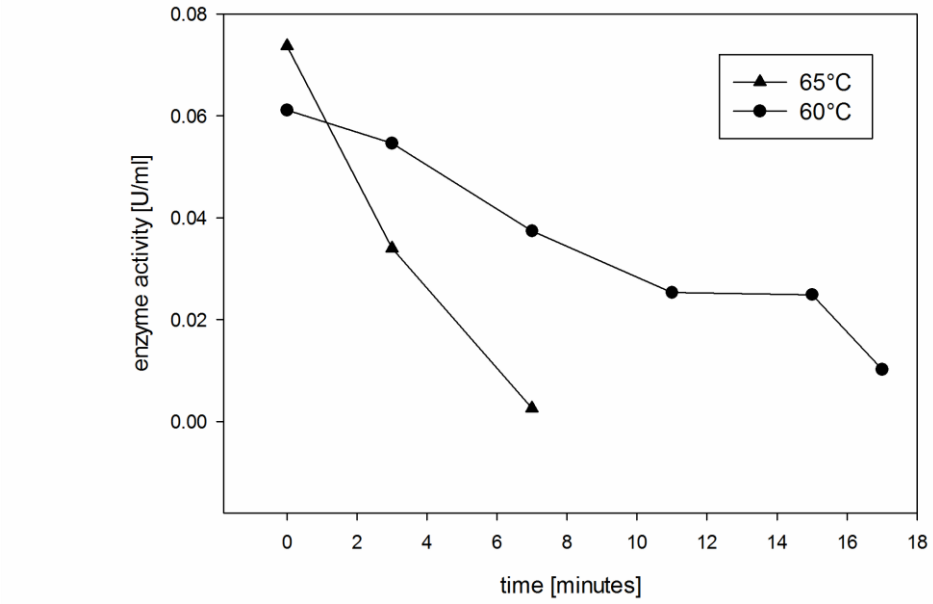


Fig. 12: Determination of the enzyme activity of *A. oryzae* CDH II at 60 °C and 65 °C using cyt *c* as electron acceptor.

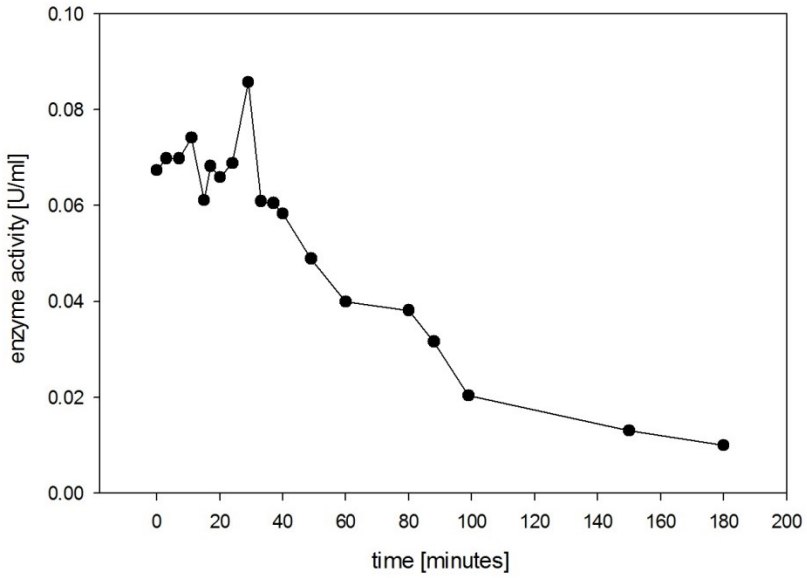


Fig. 13: Determination of the enzyme activity of *A. oryzae* CDH II at 55 °C using cyt *c* as electron acceptor.

Further the question was considered whether the concentration 200 mM of the McIlvaine buffer is the optimum. DCIP and cyt *c* assays were measured and 200 mM cellobiose was used as substrate.

The following list shows that the reaction was more efficient when only 20 mM of McIlvaine buffer was used.

Tab. 9: Determination of the enzyme activity with cyt *c* and DCIP assays with different concentrations of McIlvaine buffer using *A. oryzae* CDH II as enzyme.

McIlvaine buffer [mM]	*cyt c* average [U/ml]	DCIP average [U/ml]
200	0.07	0.35
50	0.10	0.37
20	0.11	0.30
5	0.09	0.34

To describe the steady- state kinetic constants, Michaelis-Menten equation was used to calculate from the measured data the Michaelis-Menten constant (K_M) and the maximum reaction velocity (v_{max}).

Initial rates of substrate turnover were recorded over a substrate range of 1 mM to 960 mM for glucose, 2 mM to 980 mM for maltose, 0.01 mM to 30 mM for lactose and cellobiose by using DCIP and cyt *c* as electron acceptors. Kinetic data are shown in the following figures.

The substrate, the concentration of the substrate, the pH and the temperature influence the activity of an enzyme. The activity of an enzyme is based on how fast it converts the given substrate. The velocity increases with higher substrate concentration until it is saturated with substrate and the maximum velocity is reached ($_{vmax}$).

The most effective electrone donor for *A. oryzae* CDH II is cellobiose. Glucose seems not to be convenient as a substrate for *A. oryzae* CDH II. The unfavourable maltose shows a higher K_M value than cellobiose and glucose has the highest Km value from the tested substrates.

Tab. 10: Determination of the specific activity of lactose, cellobiose, glucose and maltose using *A. oryzae* CDH II. Michaelis-Menten equation was used to determine K_M and V_{max}.

	DCIP				**cyt *c***			
	K_M [mM]	Standard deviation	V_{max} [U/mg]	Standard deviation	K_M [mM]	Standard deviation	V_{max} [U/mg]	Standard deviation
Cellobiose	0.03	0.01	5.61	0.08	0.01	0.01	2.04	0.017
Lactose	0.46	0.26	6.72	0.85	0.04	0.01	2.17	0.06
Maltose	8.10	2.45	0.08	0.01	20.12	3.78	0.11	0.01
Glucose	5129	1143	11.48	2.3	3876	1541	8.89	2.96

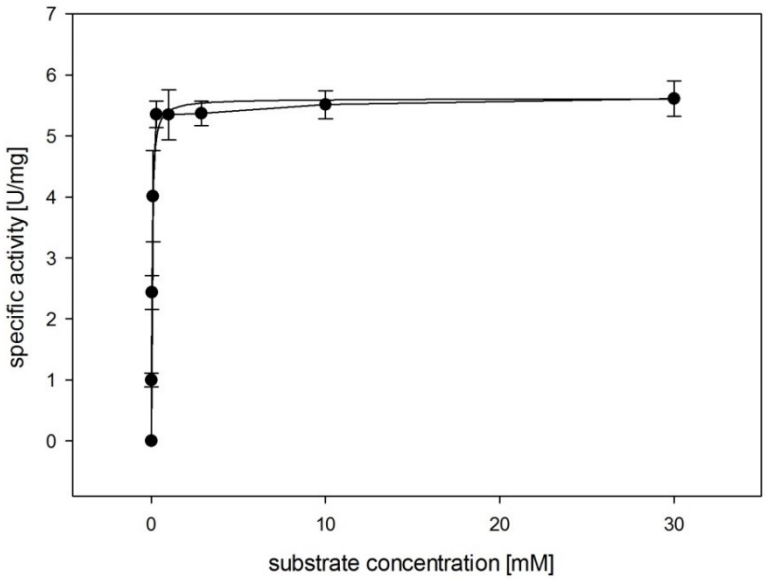


Fig. 14: DCIP assay with *A. oryzae* CDH II to determine the specific activity of cellobiose at different substrate concentrations.

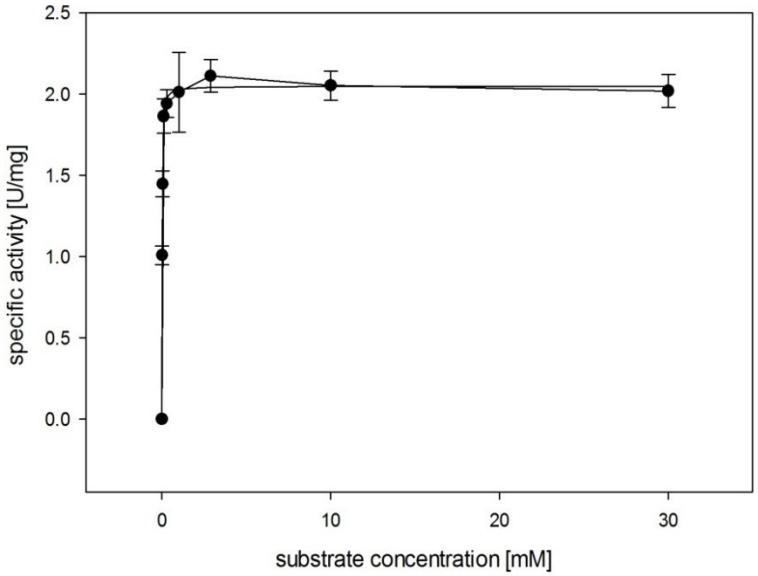


Fig. 15: Cyt *c* assay with *A. oryzae* CDH II to determine the specific activity of cellobiose at different substrate concentrations.

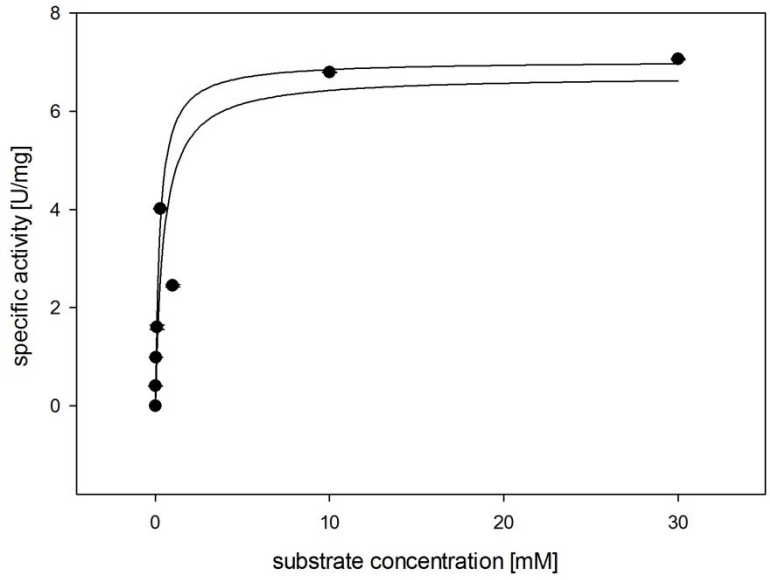


Fig. 16: DCIP assay with *A. oryzae* CDH II to determine the specific activity of lactose at different substrate concentrations.

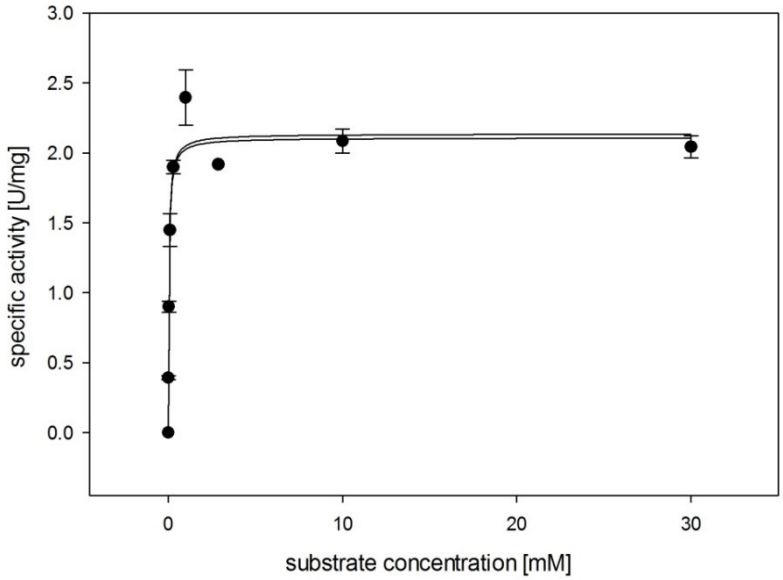


Fig. 17: Cyt *c* assay with *A. oryzae* CDH II to determine the specific activity of lactose at different substrate concentrations.

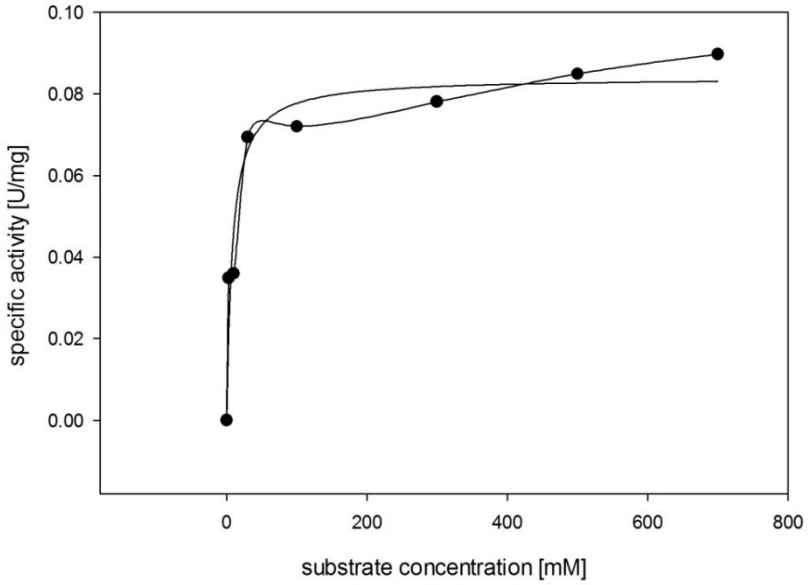


Fig. 18: DCIP assay with *A. oryzae* CDH II to determine the specific activity of maltose at different substrate concentrations.

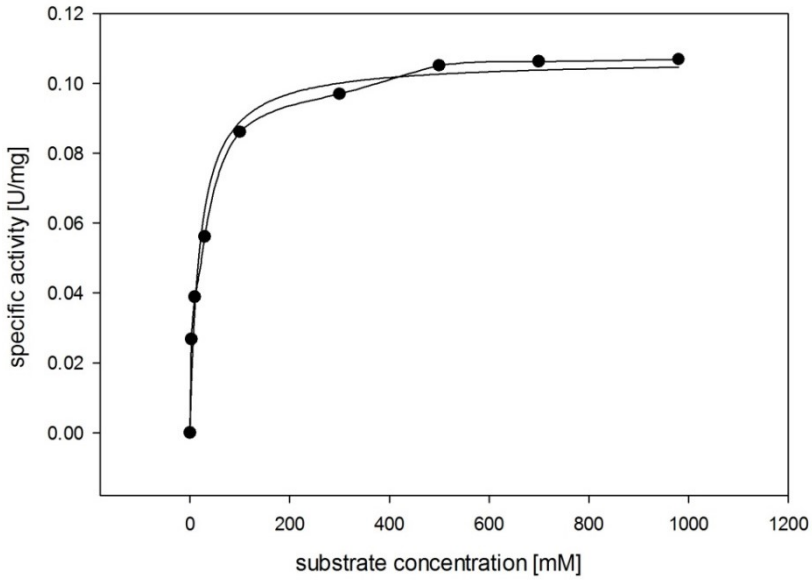


Fig. 19: Cyt *c* assay with *A. oryzae* CDH II to determine the specific activity of maltose at different substrate concentrations.

4.1.2 *A. oryzae* CDH III

4.1.2.1 Production of *A. oryzae* CDH III

Production of *A. oryzae* CDH III in *Pichia pastoris* was performed as described in the chapter "materials and methods". Wet cell weight and protein concentration were measured during the production process.

The biomass and protein concentrations increased during the production. This confirmed that cells were growing. In the end of the production the fermentation broth of all 4 shaking flasks was pooled together and was centrifuged at 6000 rpm for 20 min. In the supernatant a protein concentration of 0.26 mg/ml was measured.

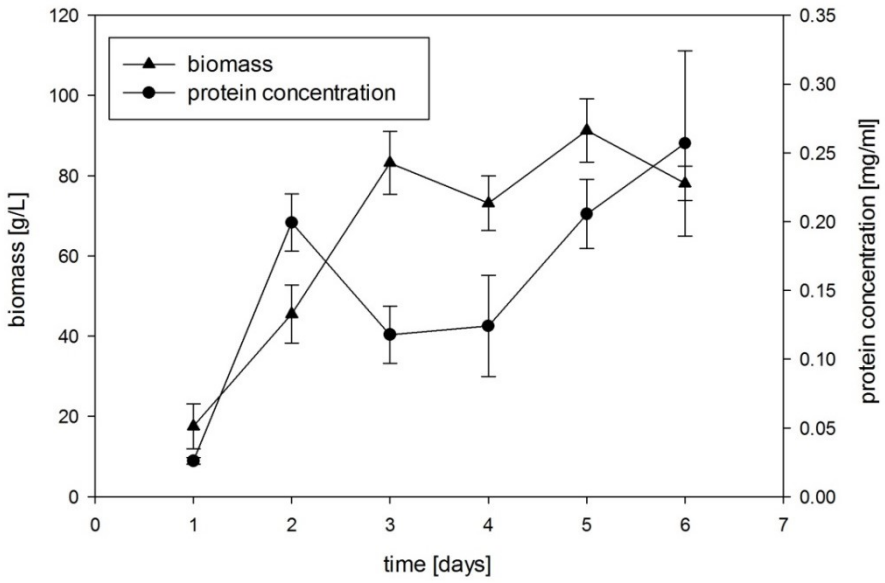


Fig. 20: Protein concentrations measured by Bradford and determination of the biomass during the production process of *A. oryzae* CDH III.

CDH activity in the culture was measured with DCIP and cyt *c* assay daily during the 5 days production in the shaking flasks but there was no activity with lactose. This means that the enzyme is not able to eliminate lactose. Because of the increasing biomass and protein concentration it can be assumed that cells were growing, but it is unknown if the right ones were active.

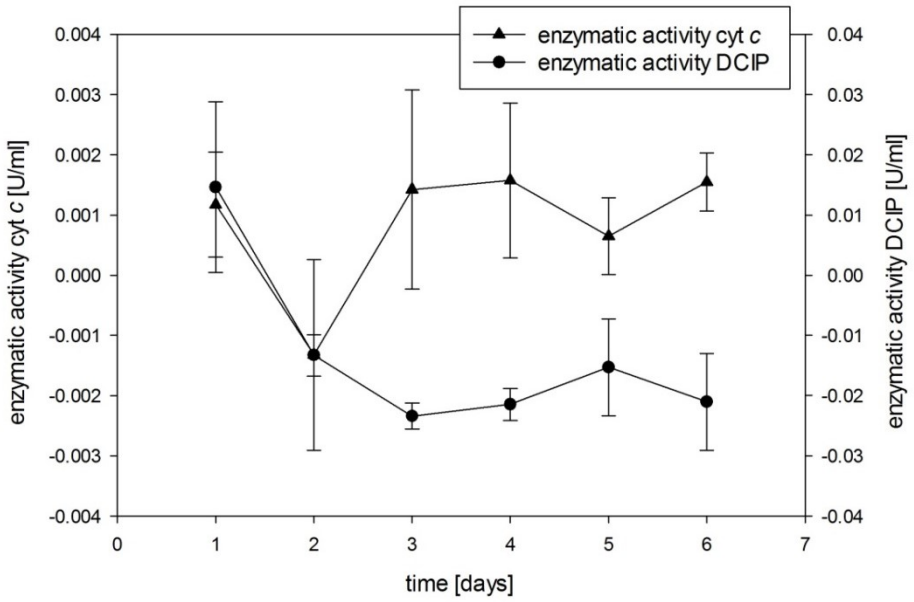


Fig. 21: Determination of the enzymatic activity with cyt *c* and DCIP assays during the production process with *A. oryzae* CDH III.

After 5 days the fermentation broth of all 4 shaking flasks was pooled together and the purification processes was started. Purification was done by Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. The fractions were pooled together, mainly oriented on absorptions spectrum to collect the fractions of interest. It was not possible to measure the enzyme activity with DCIP and cyt *c* assay because it does not show activity with lactose. After chromatography processes were done, a total protein yield of 6.91 mg was measured by Bradford assay.

4.1.2.2 Characterisation of *A. oryzae* CDH III

To determine the state of enzyme purity, samples of purified CDH were loaded to a SDS-PAGE. Fractions of interest which were seen at position 2 to 5, were pooled together. These fractions contained a protein with the size of 100 kDa, which is the characteristic molecular weight of CDH.

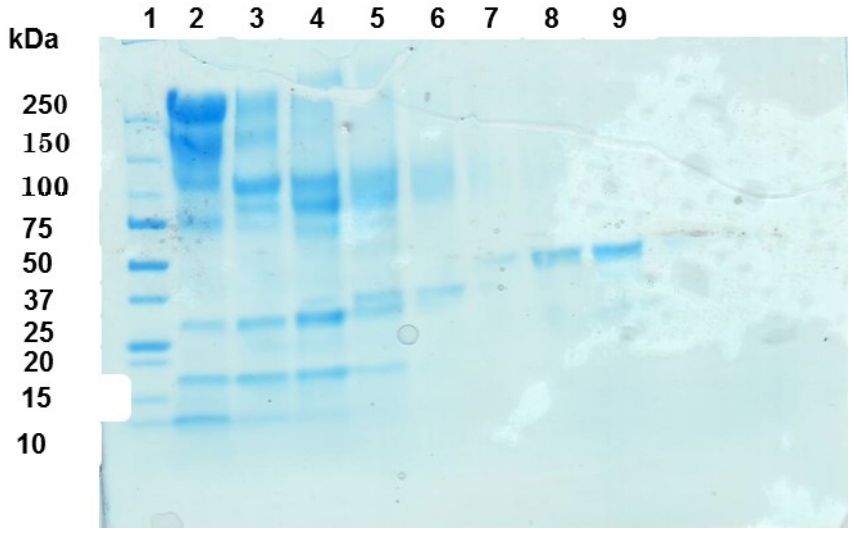


Fig. 22: SDS-PAGE of fractions of the purified *A. oryzae* CDH III after Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. [1. Unstained Precision Plus Protein standard; 2-9 different fractions]

The UV-Vis spectrum with the purified CDH shows a typical CDH spectrum which indicates that the right enzyme was produced. Upon reduction with dithionit, a typical Soret band shift was observed, while two peaks appeared at 534 nm and 564 nm. The absorbance in the region between 430 nm and 450 nm decreased due to a reduction of FAD.

Figure 24 shows a similar UV-Vis spectrum with a purified CDH from Neurospora crassa. Lactose was used for reduction. The α- and β- peaks appeared at 563 nm and 533 nm, while the typical Soret band was monitored. [SYGMUND C., 2012]

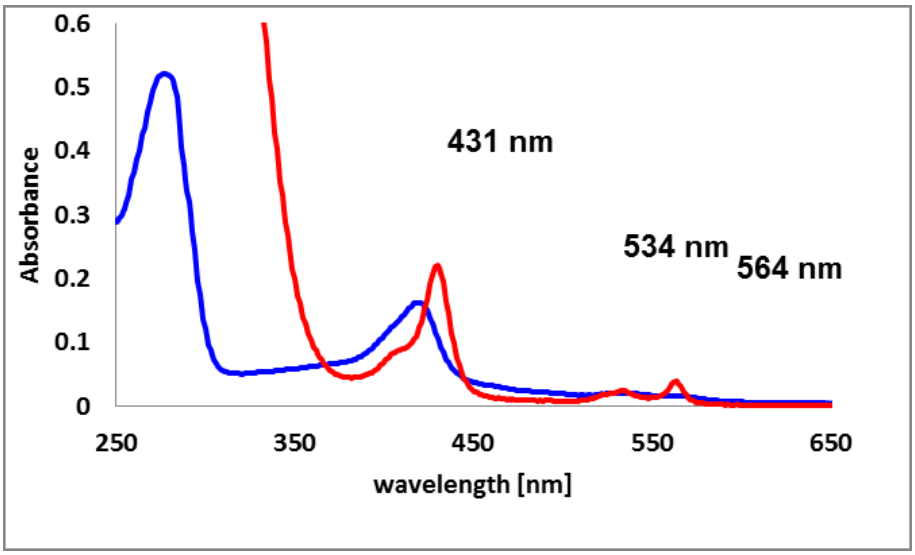


Fig. 23: Spectral characterization of *A. oryzae* CDH III showing the oxidized (blue) and reduced (red) spectra. Dithionit was used for reduction.

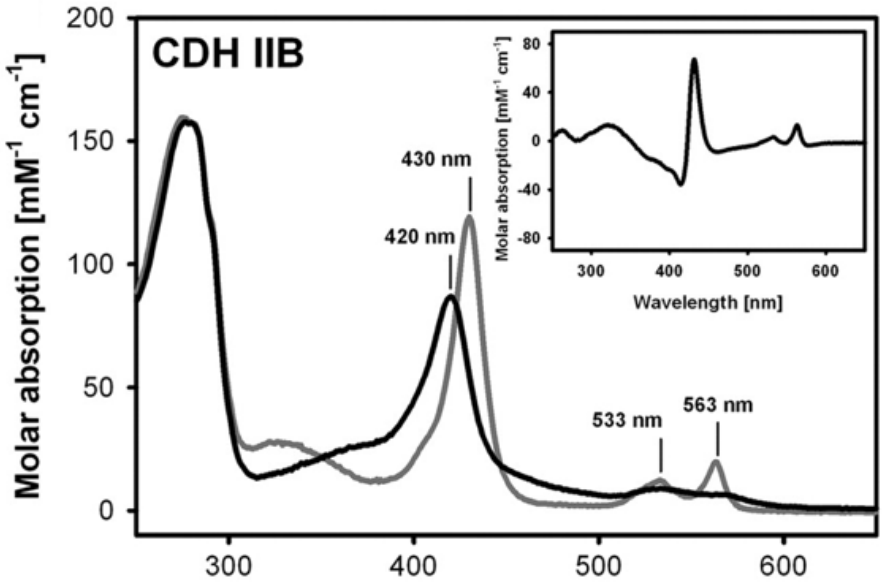


Fig. 24: Spectral characterization of *Neurospora crassa* CDH II B showing the oxidized (black) and reduced (gray) spectra. Lactose was used for reduction. [SYGMUND C., 2012]

A.oryzae CDH III activity was determined with cyt *c* and DCIP assay containing different mono-, di-, tri- and polysaccharides, alcohols and amino acids. With glucose, L- arabinose, D-galactose, mannose and xylose the screening was also performed at pH 3.0; 5.0; 7.0 and 8.5 in order to exclude that measurements were not done at the correct pH range.

During the incubation time erythrulose, sorbose, talose and xanthine had changed their colour but for these substrates the negative control reactions also were positive. The screening for these substrate was repeated by adding the substrate and recording an UV-Vis spectrum, but the spectrum did not change, which means that the enzyme did not react with the substrate.

In the end no substrate was found which showed any activity with *A. oryzae* CDH III. It might be possible that a wrong expression system was chosen. Yeast does not represent the natural expression system for the enzyme and is known to highly glycosylate proteins. Posttranslational modifications in different organisms can alter the enzymatic activity and cause the expression of an inactive enzyme.

4.2 Application *of Myriococcum thermophilum* CDH

4.2.1 Production and purification of *Myriococcum thermophilum* CDH

Ludwig et al. investigated the use of cellobiose dehydrogenase for the production of lactobionic acid. For that the enzyme was coupled to a redox mediator and laccase. Laccase is capable of re-oxidizing the mediator using oxygen as electron acceptor. The disadvantage of this method is that the toxic mediator and laccase is in the end product. [LUDWIG R., 2004] In general CDH shows low activity with oxygen as electron acceptors and this hampers the *in situ* production of H_2O_2.
The dehydrogenase domain of *Mt*CDH with mutations at positions N700 and N748 is a CDH variant with increased oxygen reactivity. This CDH variant was produced to improve the H_2O_2 production without the usage of a mediator and laccase. Therefore it is of high importance for biotechnical applications.

Oxidation of lactose to lactobionic acid by this *Mt*CDH variant without the formation of any by-products was studied. For further experiments this enzyme was also used to produce gluconic acid from glucose, maltobionic acid from maltose and cellobionic acid from cellobiose. In the application in the chapter “Introduction” it is described that lactobionic acid has severe possible applications in food technology.
In a further experiment instead of lactose milk was used to produce yoghurt. In conventional yoghurt manufacture bacteria are used to convert lactose into lactic acid. Patients, who are immunosuppressive, suffer from acute gastroenteritis or have intestinal disorders such as lactose intolerance could benefit from yoghurt, which is produced with that *Mt*CDH.

Myriococcum thermophilum CDH was already cloned into pPICz α plasmid. For amplification, the plasmid was transformed into *Escherichia coli.* In a liquid culture selected colonies were incubated with zeocin in 2 ml LB at 37 °C. Each selected clone was put on LB and zeocin agar plate and incubated at 37 °C for 2 days. A MiniPrep to isolate the plasmid DNA and sequencing was carried out. By sequencing it was proven that the gen was incorporated correctly. Then that the gen was transformed in *Pichia pastoris* X33. After the electroporation

process, which is detailed described in the methods, cells were streaked out onto an YPD plate. After 2 days colonies were growing. The best clone was taken for the production of enzymes.
Production was done with the fermenter by my supervisor with a starting volume of 30 L. During the production samples were taken and DCIP and Bradford assays were done every day. The cell mass was determined as well.
In the end the fermentation broth with a total volume of 30 880 ml was centrifuged at 6000 rpm for 15 min in 1 L bottles. The pellets were removed and the supernatant was pooled together.

Purification was done by Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. The flow through was collected and measured for activity before rejecting. All purifications were assayed by using DCIP and Bradford. Fractions of interest were pooled together and a total protein yield of 10.7 mg was reached.

4.2.2 Characterisation of *Mt*CDH

To determine the state of enzyme purity, the purified CDH was analyzed by SDS-PAGE. The band at 75 kDa showe the characteristic dehydrogenase domain.
With that SDS-PAGE it was proven that the purification was successful and the protein was homogenous.

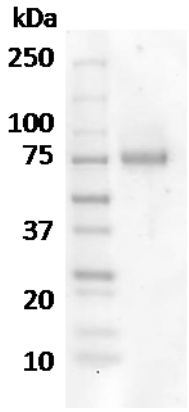


Fig. 25: SDS-PAGE of the purified *Mt*CDH after Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. [left: unstained Precision Plus Protein standard; right: FAD domain]

To determine kinetic constants with oxygen instead of DCIP as electron acceptor, measurements were done with the microsensor oxygen meter. Oxygen microsensors measure the oxygen content in a volume of 1.85 ml.

Initial rates of substrate turnover were recorded over a substrate range of 0.1 mM to 30.0 mM lactose. The different lactose solutions were prepared with McIlvaine buffer pH 7.0.

The following list shows the substrate concentration and the oxygen consumption (rate):

Tab. 11: Measurements of the oxygen consumption with different lactose concentrations at 30 °C with the microsensor oxygen meter using *Mt*CDH.

Substrate concentration [mM]	Rate [µmol/l/min]
0.1	73.8
0.3	110
1.0	202
3.0	225
10.0	308
30	305

This means that with 30 mM lactose, 305 µmol/ml oxygen per minute are consumed by the enzyme. The specific activity with lactose as substrate and oxygen as electron acceptor at pH 7.0 of *Mt*CDH is 2.76 U/mg.

Michaelis-Menten saturation curve for *Mt*CDH reaction shows the relation between the substrate concentration and the reaction rate. V_{max} at 2.75 U/ represent the maximum rate achieved at maximum substrate concentration. The Michaelis constant K_M was determined at 0.52 mM and is the lactose concentration at which the reaction rate is half of V_{max}. When the concentration of lactose is lower, the enzymes consume less oxygen/min and the end point measurement is later than in reactions with higher lactose concentrations. The results show that higher substrate concentrations correlate with a better rate.

Further the oxygen consumption of 30 mM lactose was measured at temperatures of 25 °C, 30 °C, 40 °C, 50 °C, 60 °C and 70 °C.

The following table summarizes the results of the temperature and the oxygen consumption (rate). The representation shows that if the temperatures increases the rate is increasing too. At 60 °C the enzyme shows the best results. At that milieu *Mt*CDH had a rate of 2256 µmol/l/min.

Tab. 12: Measurements of the oxygen consumption with 30 mM lactose at different temperatures with the microsensor oxygen meter using *Mt*CDH.

Temperature [°C]	Rate [µmol/l/min]
25	327
30	433
40	455
50	937
60	2256
70	2000

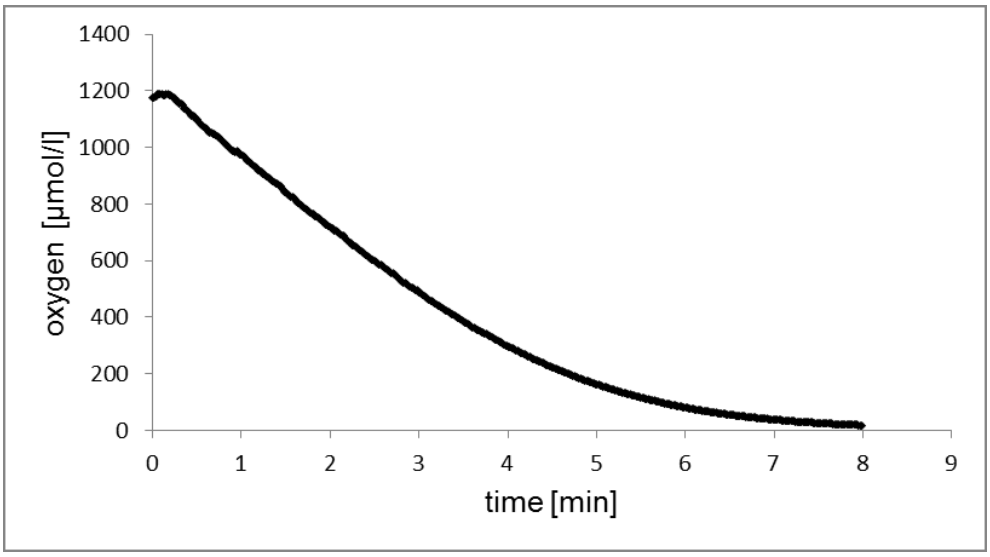


Fig. 26: Oxygen consumption of 30 mM lactose at 25 °C measured with the microsensor oxygen meter, using *Mt*CDH.

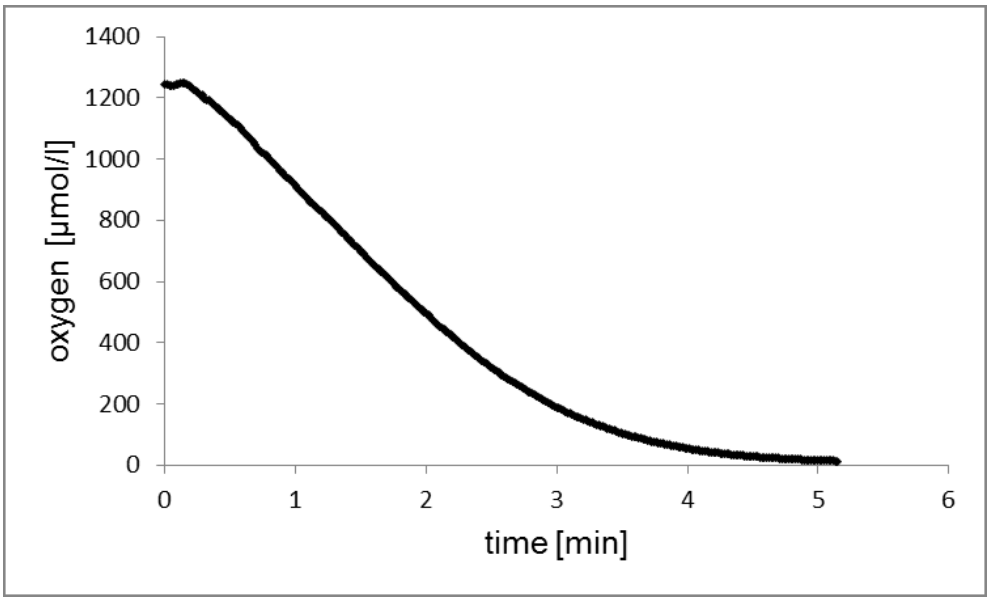


Fig. 27: Oxygen consumption of 30 mM lactose at 30 °C measured with the microsensor oxygen meter, using *Mt*CDH

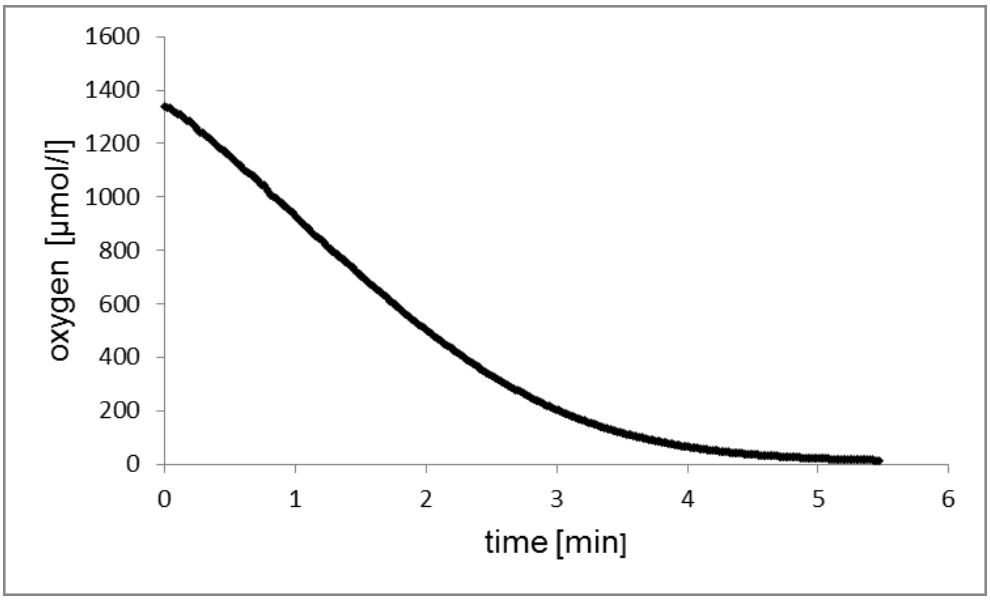


Fig. 28: Oxygen consumption of 30 mM lactose at 40 °C measured with the microsensor oxygen meter, using *Mt*CDH.

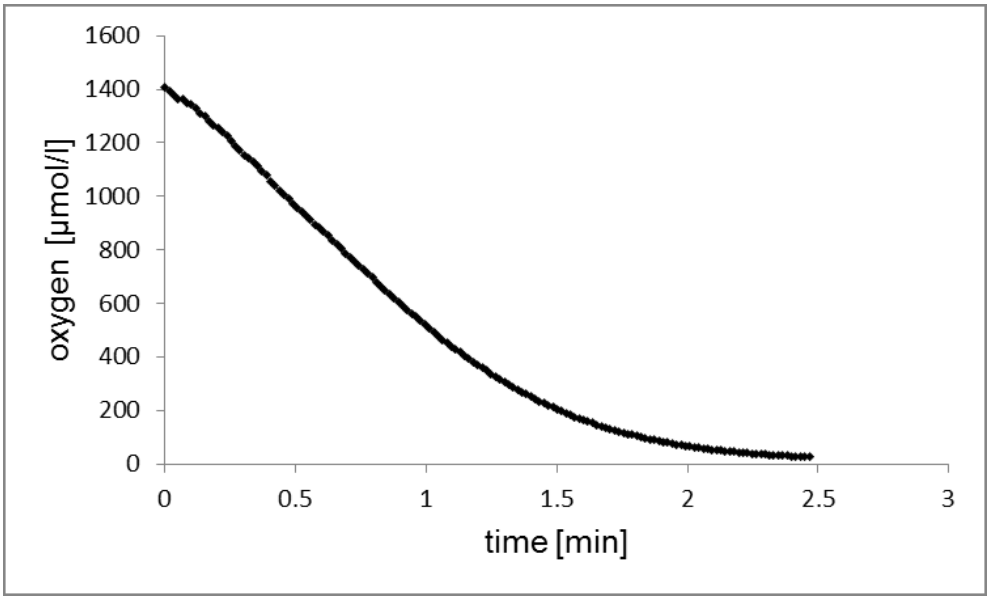


Fig. 29: Oxygen consumption of 30 mM lactose at 50 °C measured with the microsensor oxygen meter, using *Mt*CDH.

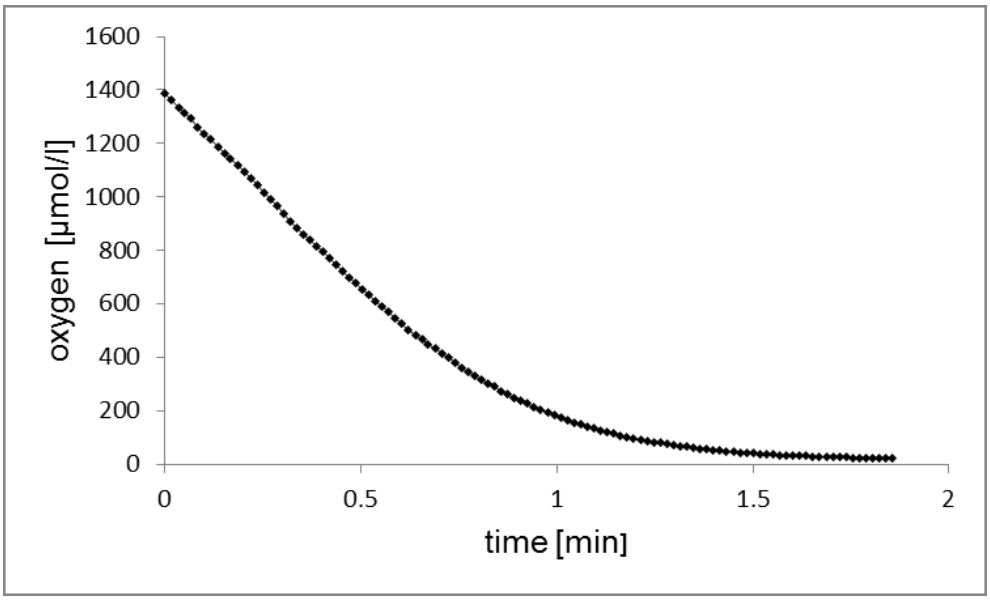


Fig. 30: Oxygen consumption of 30 mM lactose at 60 °C measured with the microsensor oxygen meter, using *Mt*CDH.

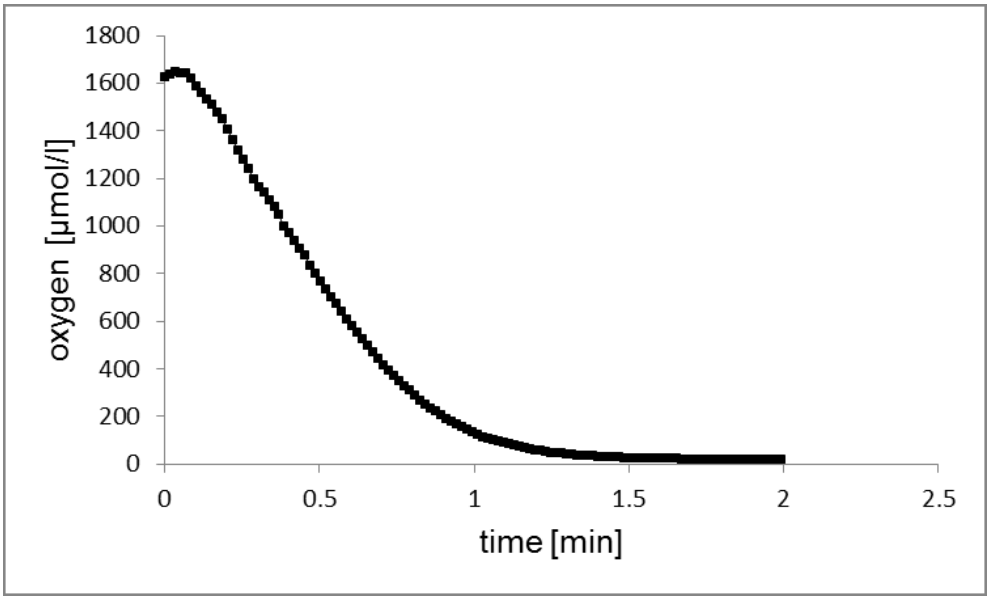


Fig. 31: Oxygen consumption of 30 mM lactose at 70 °C measured with the microsensor oxygen meter, using *Mt*CDH.

In the next experiment milk was titrated with 0.25 M HCl in order to determine the buffer capacity. Casein clotting occurs between pH 4.0 and pH 5.0, depending on the type of bacteria used in the production of yoghurt. [LUCEY J., 2004]. 18 ml of 0.25 M HCl were slowly

added to 100 ml unskimmed milk until the milk clotted. At pH 4.7 the milk started to coagulate which is within the reference level.

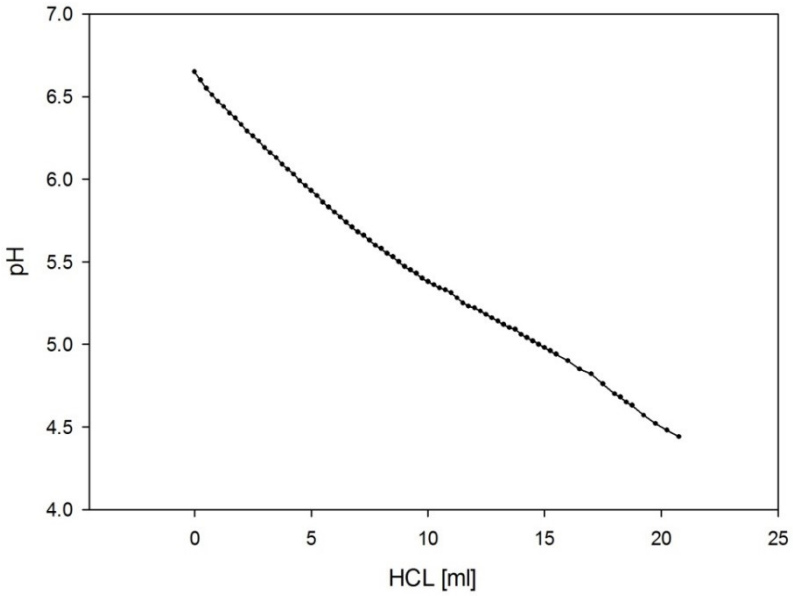


Fig. 32: Titration of 100 ml unskimmed milk with 0.25 M HCL until the milk was clotted.

4.2.3 Production of lactobionic acid, gluconic acid, maltobionic acid and cellobionic acid

For the enzymatic production of lactobionic acid, gluconic acid, maltobionic acid and cellobionic acid, a *Mt*CDH with mutations at positions N700 and N748 with increased oxygen reactivity was used. That recombinant CDH variant was used because it improves the H_2O_2 production.

The *Mt*CDH catalysed oxidations of these sugars were performed at 30 °C using the bioreacter system Sixfors. The reactor was equipped with standard probes for the measurement and a controller of the temperature and oxygen. The reaction system was stirred and dissolved oxygen was automatically held constant at 20 % saturation with pure oxygen. The pH of the reactors was held constant at pH 7.0 with 1 M sodium carbonate. A second attempt with lactose in which the pH was not regulated was also performed.

Samples were taken regularly within 24 hours and lactose and lactobionic acid were analyzed by HPLC. The conversion of lactose was complete within 24 hours in the reactor with pH regulation. At the beginning a concentration of 104.7 mM lactose was measured. After 24

hours the concentration decreased to 0 mM. In the meantime the concentration of lactobionic acid increased from 2.21 mM to 129.8 mM. Without pH regulation the conversation of lactose into lactobionic acid was not complete. *Mt*CDH is not active when the pH is lower than 3. No other product than lactobionic acid could be detected by HPLC.

After the production process the fermentation broth with the pH regulator was concentrated with a rotavapor. A weight of 15.8 g of lactobionic acid was determined.

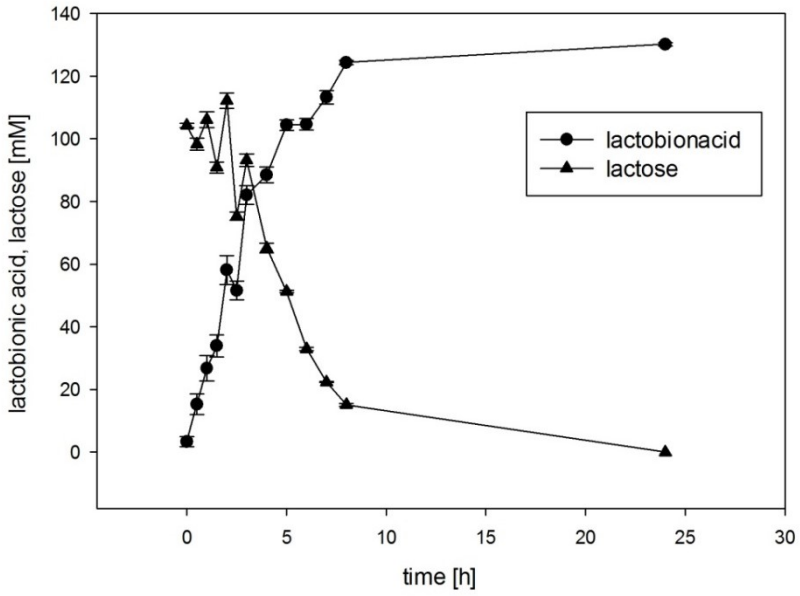


Fig. 33: HPLC analysis during the conversion process from lactose into lactobionic acid using *Mt*CDH in the reactor with pH regulation.

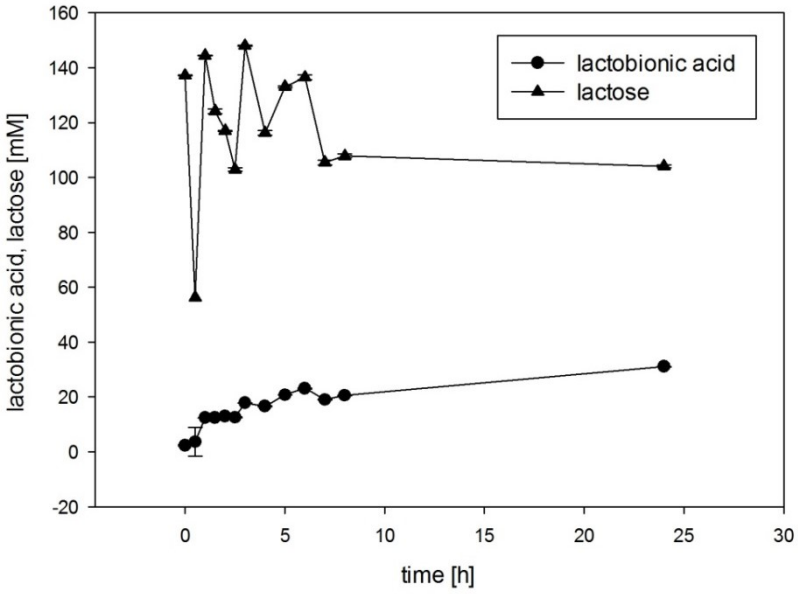


Fig. 34: HPLC analysis during the conversion process from lactose into lactobionic acid using *Mt*CDH in the reactor without pH regulation.

Figure 35 represents the analysed HPLC data of the implementation from maltose into maltobionic acid. Data from maltobionic acid were not available. At the beginning the concentration of 113.8 mM maltose was measured. After 24 hours the concentration decreased to 110.0 mM. The *Mt*CDH catalysed oxidation of maltose did not worked well, since otherwise the concentration would have decreased more.

In figure 36 the measured HPLC data of conversion from cellobiose into cellobionic acid are shown. At the beginning a substrate concentration of 107.8 mM was measured. The substrate was fully implemented within 24 hours. In the meantime, the concentration from cellobionic acid increased from 22.2 mM to 101.4 mM. No other product exept cellobionic acid could be detected by HPLC. For the first time the *Mt*CDH catalysed oxidation of cellobiose into cellobionic acid had worked.

The conversion of the monosaccharide glucose into gluconic acid was not possible as the graph in figure 37 represents. Neither the concentration of glucose nor the concentration of gluconic acid changed within the reaction time.

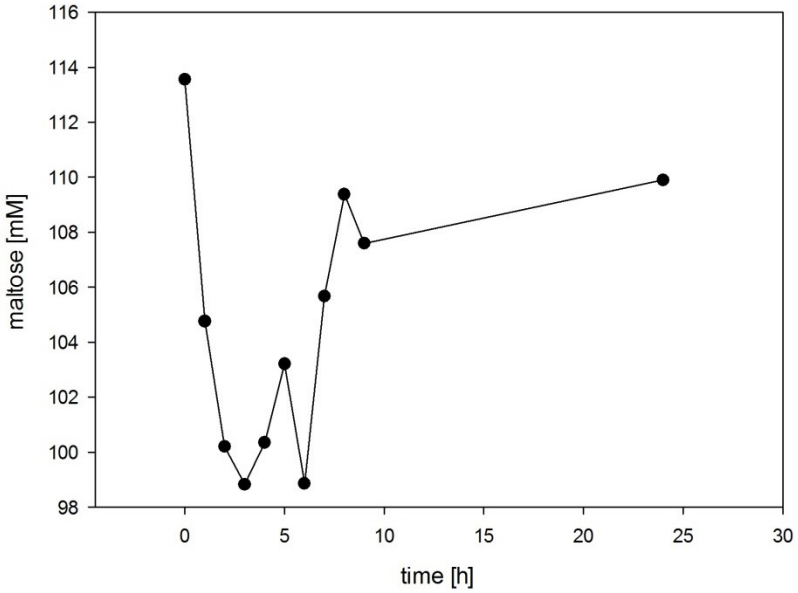


Fig. 35: HPLC analysis during the conversion process from maltose into maltobionic acid using *Mt*CDH in the reactor with pH regulation.

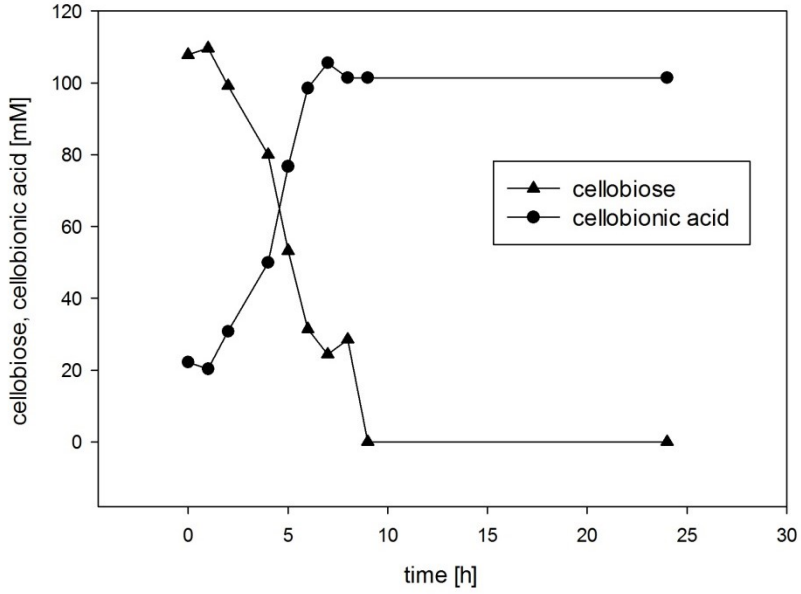


Fig. 36: HPLC analysis during the conversion process from cellobiose into cellobionic acid using *Mt*CDH in the reactor with pH regulation.

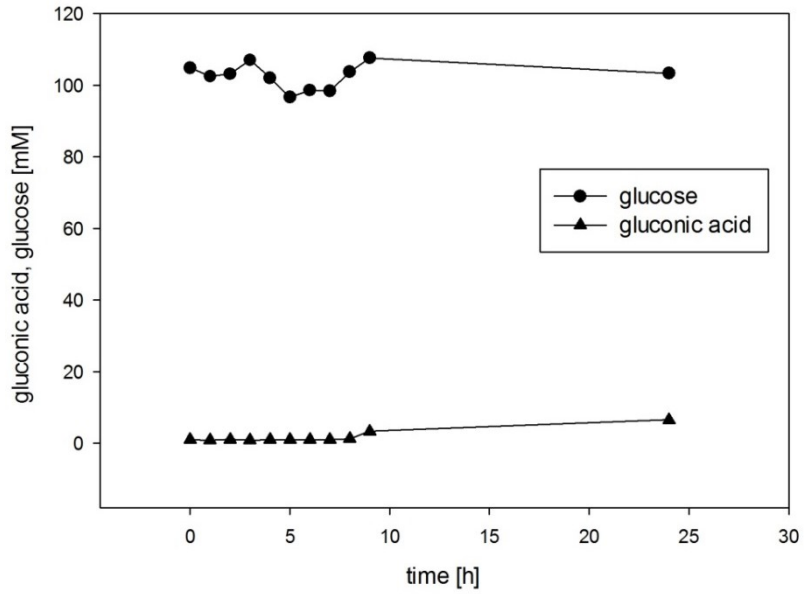


Fig. 37: HPLC analysis during the conversion process from glucose into gluconic acid using *Mt*CDH in the reactor with pH regulation.

DCIP assays were done to prove if the enzyme is active during the production process. In the reactor with pH regulation the enzyme activity decreased from 0.22 U/ml to 0.09 U/ml during the converting process from lactose into lactobionic acid within 24 hours. In the other reactor where the pH was not controlled the activity of *Mt*CDH decreased from 0.21 U/ml to 0.10 U/ml within 24 hours.

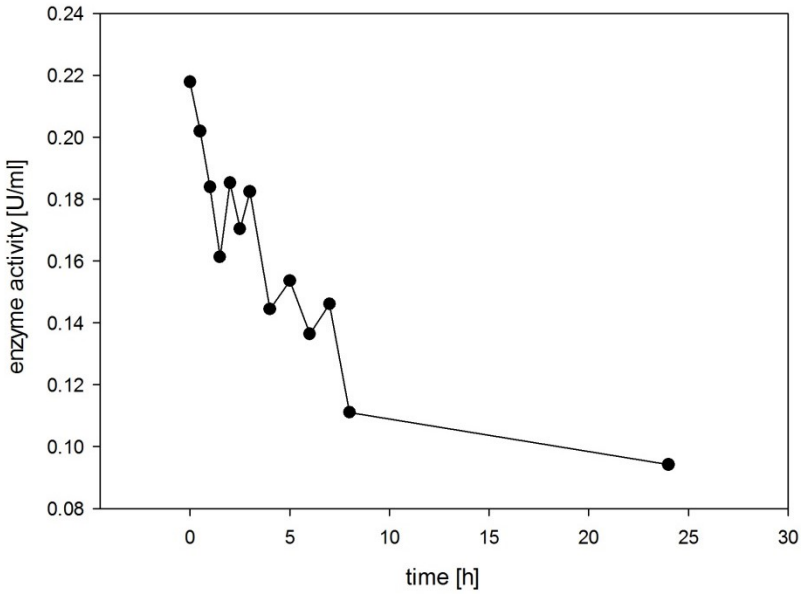


Fig. 38: Determination of enzyme activity with DCIP assay during the converting process from lactose into lactobionic acid using *Mt*CDH in the reactor with pH regulation.

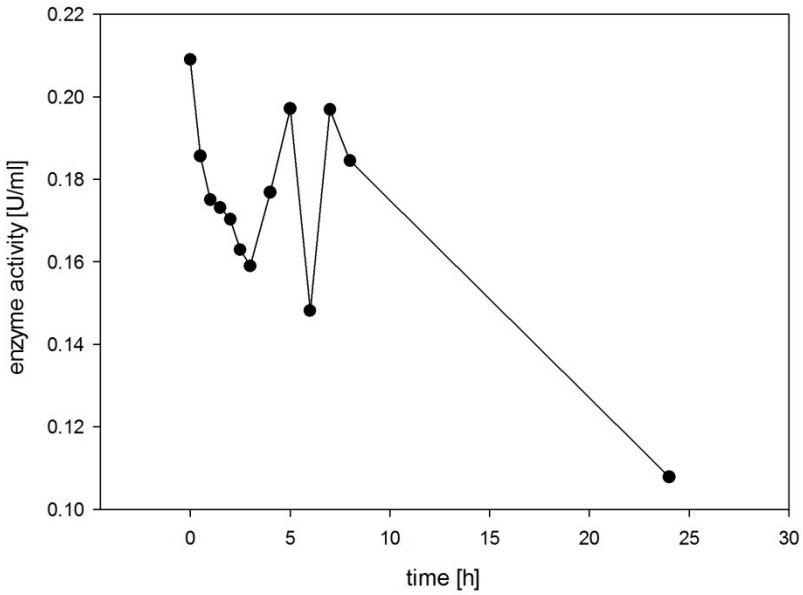


Fig. 39: Determination of enzyme activity with DCIP assay during the converting process from lactose into lactobionic acid using *Mt*CDH in the reactor without pH regulation.

The following figure shows that the enzyme activity continuously decreased in the reactors with glucose, maltose and cellobiose. The activity in the reactions decreased continuously within 24 hours. An enzyme activity of 0.22 U/ml was measured in the reactor with

cellobiose at the beginning. After 24 hours the activity decreased to 0.09 U/ml. In the reactor with maltose an activity of 0.23 U/ml was determined before the implementation. The activity decreased to 0.11 U/ml within 24 hours. In the reactor with glucose the enzyme activity decreased from 0.25 U/ml to 0.20 U/ml.

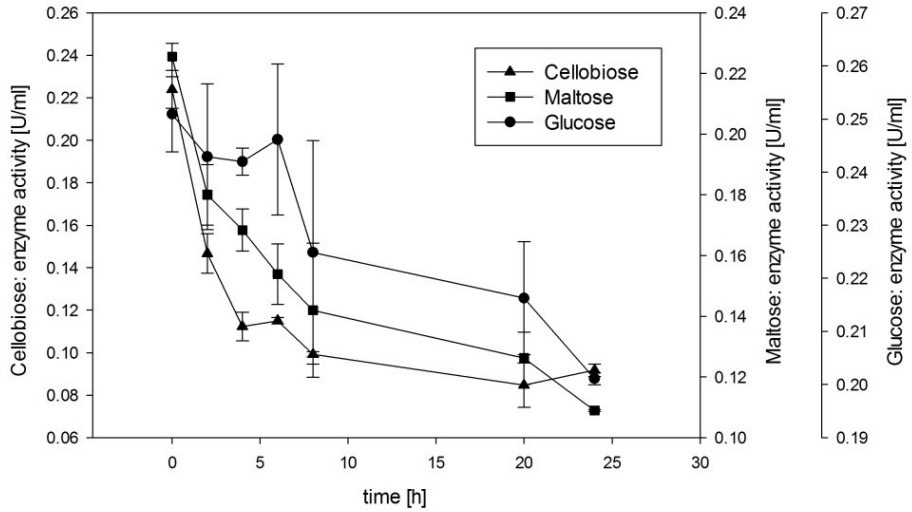


Fig. 40: Enzymatic activity measured with DCIP during the production process of cellobionic acid, maltobionic acid and gluconic acid

During the production process of lactobionic acid in the Sixfors, samples were also regularly taken to determine the hydrogen peroxide concentration by using the FOX assay and also to measure catalase assays.

For the measurement the FOX reagent and methanol were mixed into a cuvette and incubated at room temperature for 30 min. Before the measurement in the photometer started the sample was added. The absorption was noted at 560 nm. There are no significant differences of the H_2O_2 concentrations between the reactor without pH regulation and with pH regulation during the reaction process. At the beginning there was hardly any H_2O_2 measured, but it increased after two hours. In the end point measurement the concentration of hydrogen peroxide decreased again because *A. oryzae* CDH II was consumed.

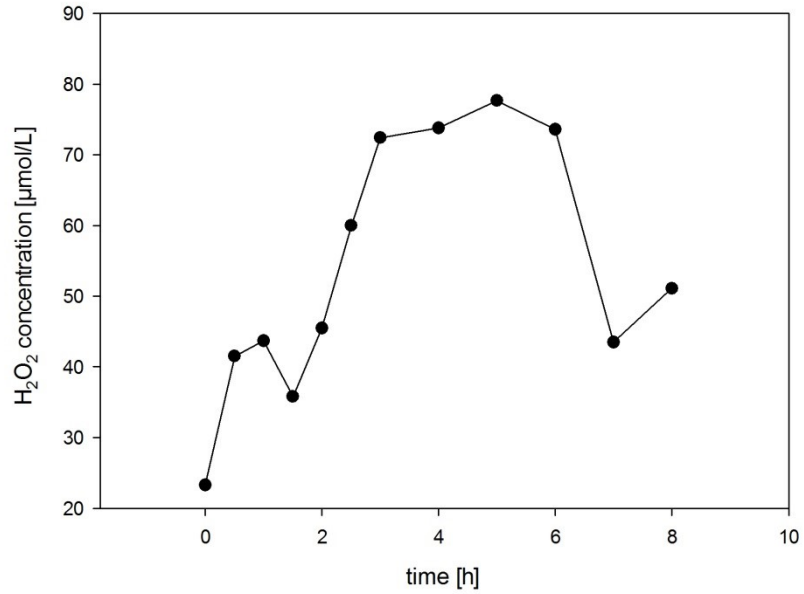


Fig. 41: Measurement of H_2O_2 concentration by FOX assay during the converting process from lactose into lactobionic acid using *Mt*CDH in the reactor without pH regulation.

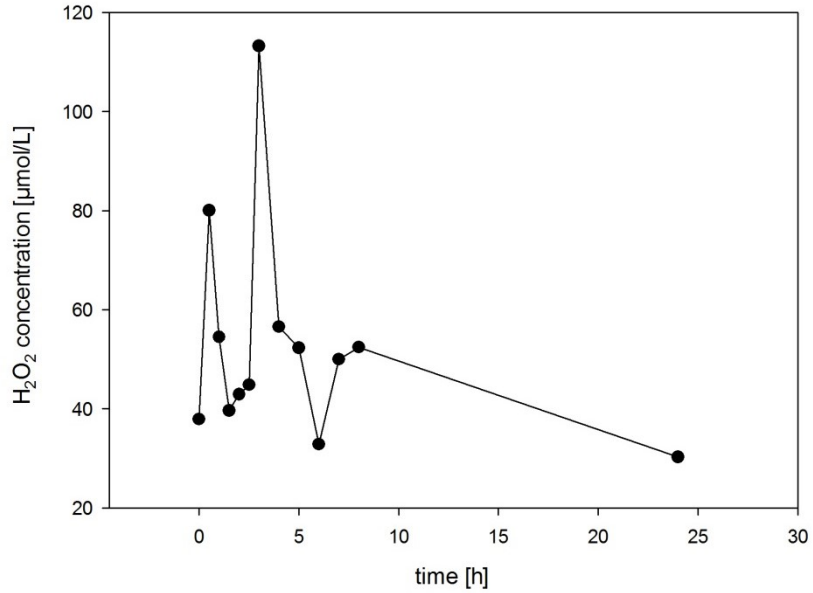


Fig. 42: Measurement of H_2O_2 concentration by FOX assay during the converting process from lactose into lactobionic acid using *Mt*CDH in the reactor with pH regulation.

Catalase was added to prevent the accumulation of hydrogen peroxide. Catalase was measured by using the catalase assay.

The enzyme activity was measured over a time period of 24 hours. 2.90 ml of hydrogen peroxide solution [0.036 % w/w] was pipetted into a cuvette. For each cuvette monitor the A 240 until constant and then 100 µl of catalase was added and mixed well. The time required for the A 240 was recorded while it decreased from 0.45 to 0.40 absorbance units. It was modulated so that one reading per second for 180 seconds was taken.

In the beginning catalase was hardly measured with the assay. While lactose was converted into lactobionic acid catalase increased. However, the catalase should have decreased.

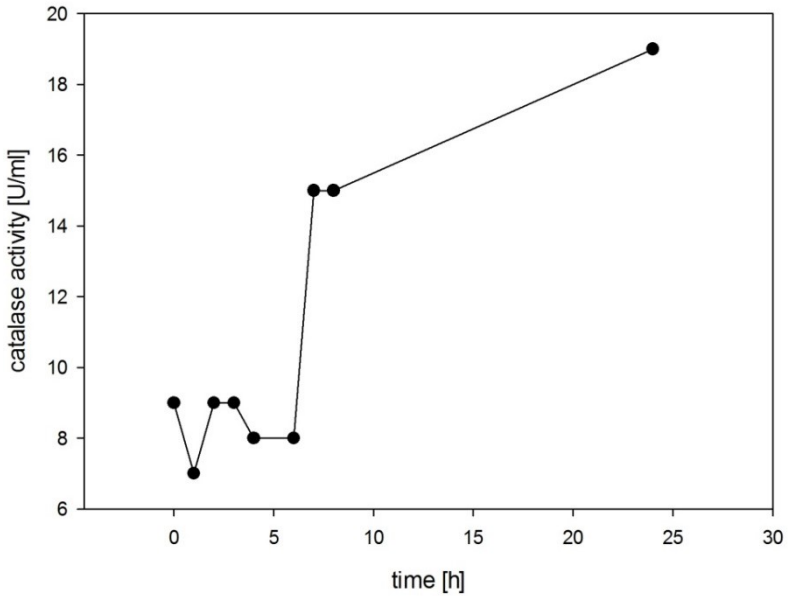


Fig. 43: Catalase assay during the converting process from lactose into lactobionic acid using *Mt*CDH in the reactor with pH regulation.

In the reactor without pH regulation the pH of the initial solution was measured at 4.07. After 24 hours the pH was determined at pH 3.18. Because of the conversion from lactose into lactobionic acid, the solution becomes more acidic with the course of the reaction. The enzymatic process stops when the pH is less than 3.0, because the enzyme is not active at that milieu.

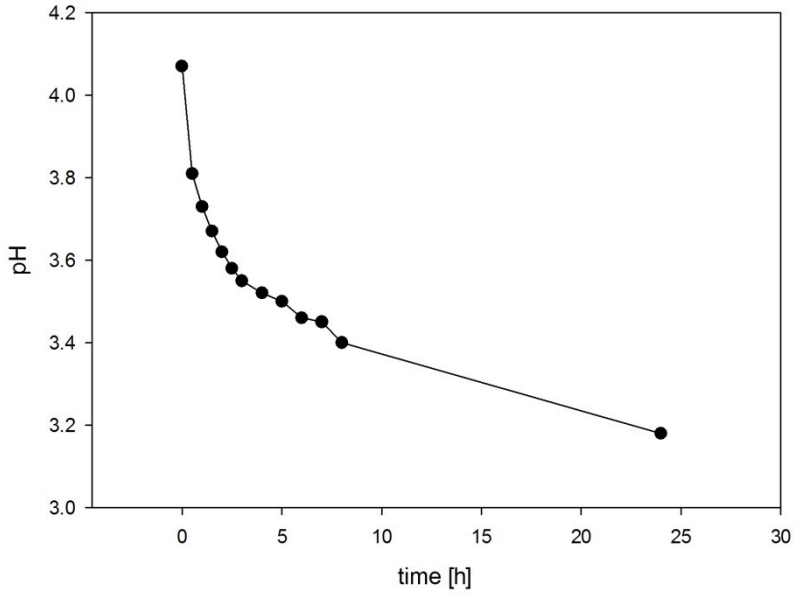


Fig. 44: PH change during the converting process of lactose into lactobionic acid using *Mt*CDH in the reactor without pH regulation.

4.2.4 Enzymatic production of yoghurt

The *Mt*CDH variant with mutations at positions N700 and N748 was used as a biocatalyst to produce bacteria free yoghurt from lactose. The advantage of the usage of that enzyme is that it improves the H_2O_2 production without the usage of a mediator and laccase.

To find out the optimal conditions for the production of enzymatic yoghurt, pre experiments were done in petri dishes.
At the beginning a pH of 6.78 was measured in all prepared petri dishes. The higher the incubation temperature was, the faster the pH values decreased. At pH 4.97 the milk clotted and it was not possible to measure further pH values. With the incubation temperature at 25 °C it took three days to reach this pH, which is twice as long as in the incubation room with 4 °C. The pH values in the attempts, which contained catalase, decreased about 4 to 5 hours faster than in the attempts without catalase.

The *Mt*CDH catalysed oxidation of lactose in milk was performed at 30 °C using the bioreacter system Sixfors. The reactor was equipped with standard probes for the

measurement and controller of the temperature and oxygen. The reaction system was stirred and dissolved oxygen was automatically held constant at 20 % saturation with pure oxygen. The pH values were not held constant during the process in the reactor with milk. While *Mt*CDH converts lactose into acid the pH values changed. At beginning a pH of 6.62 was measured. After 24 hours the conversion of lactose into the acid was finished and a pH of 4.33 was measured.

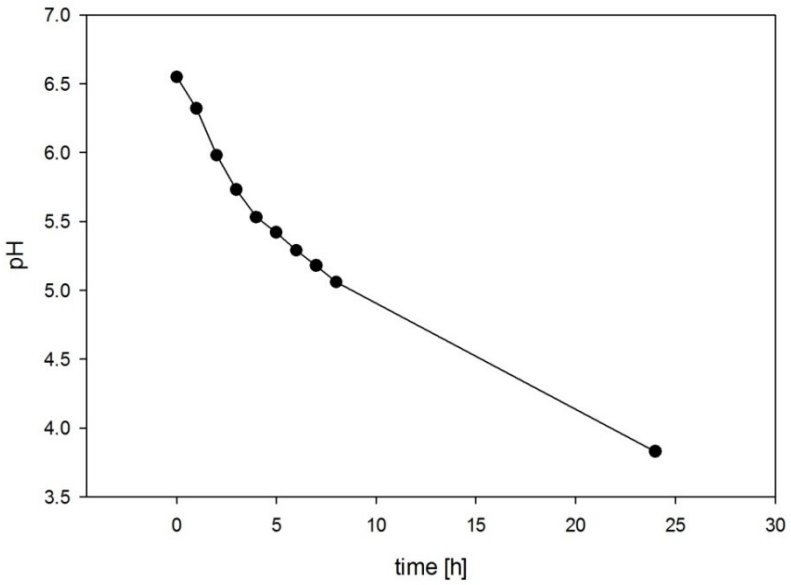


Fig. 45: pH changes while lactose is converted into lactobionic acid using *Mt*CDH in the reactor without pH regulation.

Because the solution with the milk was turbid, it was not possible to measure the activity of the enzyme during the production process.

Lactose and lactobionic acid were analysed by HPLC. At the beginning 22.38 mM lactobionic acid and 134.94 mM lactose were measured. After 24 hours 120.63 mM lactobionic acid and 40.5 mM lactose were analysed by HPLC analysis.

No other product except lactobionic acid could be detected by HPLC, which shows that the *Mt*CDH catalysed oxidation of lactose in milk was worked. In figure 47 the produced yoghurt is shown.

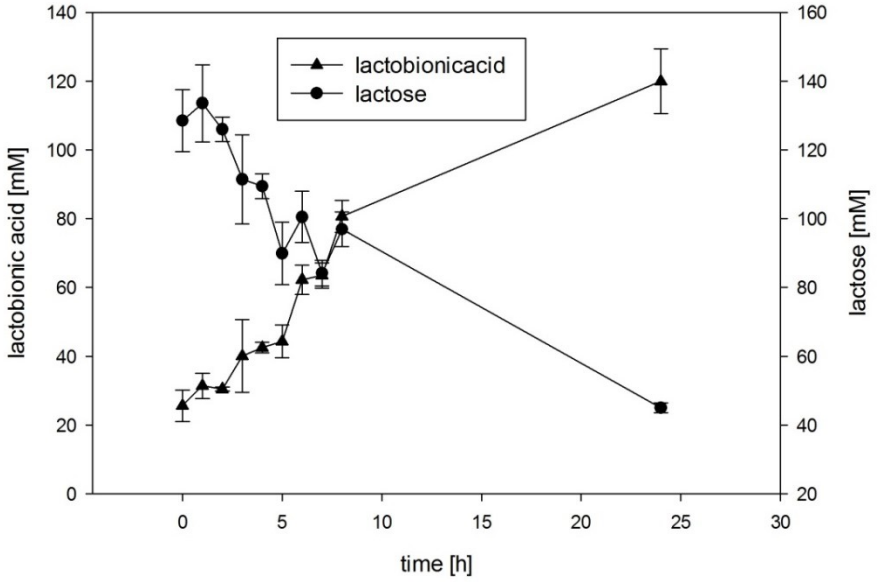


Fig. 46: HPLC analysis during the conversion process from lactose into lactobionic acid using *Mt*CDH in the reactor without controlling pH.

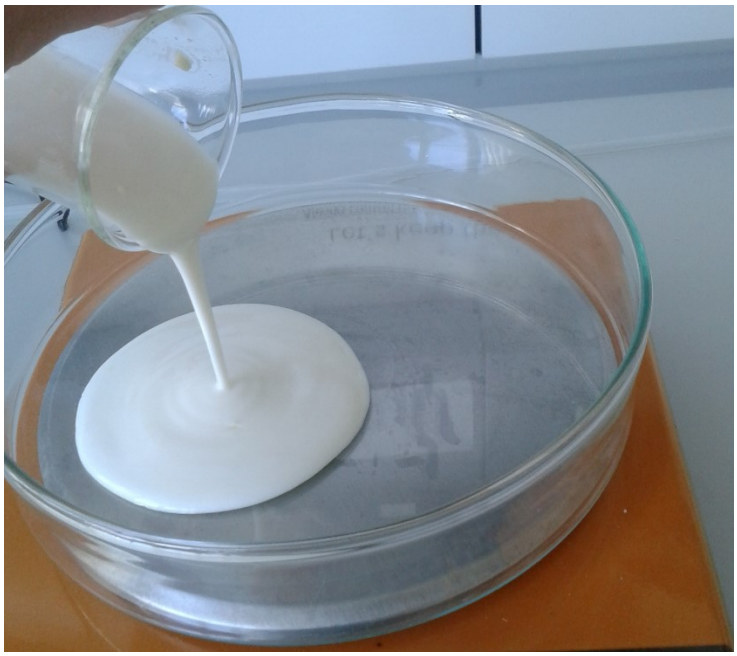


Fig. 47: *Mt*CDH catalysed oxidation of lactose in milk to yoghurt.

To determine the textual properties of the produced yoghurt, a syneresis test was performed and compared with a commercial set and stirred yoghurt from the supermarket. During the syneresis process whey was collecting on the surface of yoghurt.

After 24 hours production of yoghurt in the Sixfors, the product was stored for one week at 4 °C. During that time the texture got firmer. The incurred whey of the enzymatic yoghurt was removed after the storage. The graph shows the syneresis [w/w] in percent at the beginning and after every centrifugation step at 2000 rpm, 3000 rpm and 4000 rpm. After the first step with 2000 rpm of the enzymatic yoghurt 34.98 % whey was removed.

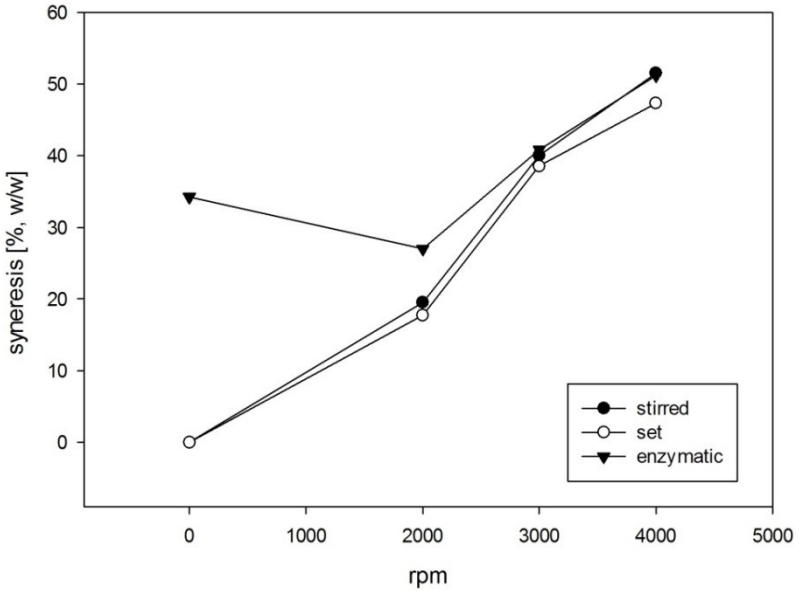


Fig. 48: Determination of the textual properties of a stirred-, set- yoghurt and yoghurt with *Mt*CDH.

5 Conclusions

The aim of this thesis was to characterize class III CDH. Therefore one class III CDH gene of *Aspergillus oryzae* was selected for recombinant production. To compare the characteristics of class III CDH with class II, a class II CDH from *A. oryzae* was expressed and characterized.

A.oryzae CDH III in *Pichia pastoris* was produced in shaking flasks. Purification was done by Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. After chromatography process a total protein yield of 6.91 mg was measured by Bradford assay.
With the purified *A. oryzae* CDH III a substrate screeing with cyt *c* and DCIP assay containing different mono-, di-, tri- and polysaccharides, alcohols and amino acids was performed. There was no substrate, which showed any activity with the class III CDH. It would be of a high scientific interest to try another expression system than *Pichia Pastoris* X33, or to use a CDH class III from a different fungi.
As it was not possible to do more characterization with *A. oryzae* CDH III, it could not be compared with class II from this target organism.

The production- and purification process of *A. oryzae* CDH II was equal with the procedures with *A. oryzae* CDH III. It was possible to produce 3.20 mg pure enzyme. The most effective electron donor for *A. oryzae* CDH II is cellobiose. The enzyme shows similar activity with lactose as substrate. Maltose and Glucose seem not to be convenient as a substrate for *A. oryzae* CDH II.
Some researchers have obtained evidence that most CDHs have a preference for disaccharides and oligosaccharides. Steady state kinetic constants of *N.crassa* class II CDH shows similar results as *A. oryzae* CDH II by using cyt *c* as electron acceptor. The highest catalytic efficiencies for electron donor were found for cellobiose. With glucose the k_M value was determined at 1700 mM, which is an indices that glucose is not appropriate for that enzyme. The reached K_M values for lactose with 0.028 mM and for maltose with 31.0 mM are comparable with the results of *A. oryzae* CDH II. [HARREITHER W., 2012]
H.insolens, which is produced from ascomycetes shows also less activity with maltose and lactose and a high specific activity with cellobiose. [ZAMOCKY M., 2006]

pH Profiles were obtained to determine the pH with the highest enzyme activity for enzymatic reactions. The optimal pH for *A. oryzae* CDH II is at pH 5.0 for cyt *c* and for DCIP and the enzyme is most stable at 55 °C. At higher temperatures the enzymes fully denaturized and no activity remained.
P.chyrsosporium which is an organism from Basidiomycetes, shows the best enzyme activity at pH 6.0 at 25 °C. H.insolens have a higher pH optima, at pH 7.5 and are most stable at 40 °C. [ZAMOCKY M., 2006]
For N.crassa the pH optima found with cyt *c* was at pH 6.0 and DCIP at pH 5.0. [HARREITHER W., 2012]

As cellobiose dehydrogenase can be applied in food science, the production of lactobionic acid and yoghurt with the produced and purified *Myriococcum thermophilum* CDH mutant was another goal of this project.
The dehydrogenase domain of *Mt*CDH with mutations at positions N700 and N748 is a CDH variant with increased oxygen reactivity. This CDH variant was produced to improve the H_2O_2 production *in situ* without the usage of a mediator and laccase to regenerate the system. The production of the enzyme was done in large scale in the fermenter and the purification was done by Hydrophobic Interaction Chromatography and Anion Exchange Chromatography. After purification a total protein yield of 10.7 mg was measured.
For the first time lactobionic acid with *Mt*CDH variant without forming other by-products was produced successfully. It was possible to produce 15.78 g lactobionic acid. The oxidation process of maltobionic acid, lactobionic acid and gluconic acid was also done by adding *Mt*CDH. HPLC data show that the converting process of cellobiose into cellobionic acid had worked successfuly. Further experiments should optimize the production process of gluconic acid and maltobionic acid.

In conventional yoghurt manufacture processes bacteria are used to convert milk sugar into lactic acid. In a further experiment milk instead of lactose by adding *Mt*CDH was used to produce enzymatic yoghurt. Patients who are immunosuppressive could benefit from yoghurt, which is produced with *Mt*CDH mutant. During the production process of yoghurt,

the concentration of lactose decreased and the concentration of lactobionic acid increased in the bioreactor Sixfors system. The product was stored for one week at 4 °C and during that time the texture became firmer. Texture is one of the most essential components of yoghurt quality. Therefore a syneresis test was done to compare the texture of a stirred-, set- and enzymatic yoghurt.

To improve the textual properties of enzymatic yoghurt, it would be interesting to conduct further experiments in which stabilizer like pectin are added.

6 Zusammenfassung

Das Flavoenzym Cellobiose Dehydrogenase (CDH) stammt aus holzzersetzenden Ascomyceten und Basidomyceten und findet vor allem in biotechnologischen Bereichen Verwendung. Da die Erzeugung in Pilzen sehr komplex, mit einem hohen Aufwand, sowie mit enormen Kosten verbunden ist, wurde die Hefe *Pichia pastoris* als Expressionssystem gewählt. Das Enzym wird vorwiegend in der Analytik als Biosensor beispielsweise für die Quantifizierung von Laktose oder anderen Sacchariden eingesetzt. In der pharmazeutischen-, sowie in der Lebensmittelindustrie besteht ein starkes Interesse an der Laktobionsäure, die mittels der CDH produziert werden kann. Bei der Umsetzung aus Laktose wird das Enzym als Biokatalysator eingesetzt. Laktobionsäure hat einen mild-sauren Geschmack und wird in verschiedenen Medizinprodukten eingesetzt.

CDH vom Organismus *Aspergillus oryzae* Klasse II und Klasse III wurden erstmals im Rahmen dieser Masterarbeit produziert, gereinigt und charakterisiert.
Mit der CDH Klasse III wurde ein Substratscreening durchgeführt, um heraus zu finden mit welchem Substrat dieses Enzym aktiv ist. Um die besten Bedingungen der gereinigten CDH Klasse II zu bestimmen, wurden ein pH- Wert-, sowie ein Temperaturprofil erstellt. Weiters wurden mittels DCIP und cyt *c* Messungen kinetische Bestimmungen für Maltose, Laktose, Cellobiose und Glucose durchgeführt.
Ein weiteres Ziel dieser Arbeit war es, eine Variante des Organismus *Myriococcum thermophilum* mit Mutationen an den Positionen N700 und N784 zu produzieren. Dieses Enzym wurde gewählt, da es eine erhöhte O_2 Aktivität hat und somit die H_2O_2 Produktion optimieren kann. Die Oxidation von Laktose zu Laktobionsäure durch den Einsatz dieser *Mt*CDH wurde charakterisiert. Es wurde auch getestet, ob eine Produktion von Maltobionsäure, Cellobionsäure und Gluconsäure mithilfe dieses Enzyms möglich ist.
Ein weiteres Ziel war es, das rekombinante Enzym für die Produktion von bakterienfreiem Joghurt einzusetzen. Zur Charakterisierung wurde ein Synärese Test durchgeführt, um die Textur mit einem konventionellen gerührten und einem gesetzten Joghurt zu vergleichen.

Es ist gelungen *A. oryzae* Klasse II und Klasse III zu produzieren. Auf die Ergebnisse der Cha-

rakterisierung der Klasse II wird in dieser Arbeit ausführlich eingegangen. Für die Klasse III wurde im Substratscreening kein Substrat gefunden, welches mit diesem Enzym aktiv ist. Es wäre interessant ein anderes Expressionssystem oder eine Klasse III von einem anderen Pilz zu verwenden.

Analysen während der Umsetzung von Laktose zu Laktobionsäure haben gezeigt, dass die *Mt*CDH aktiv war und dass die Konzentration der Laktose gesunken ist, währenddessen ist aber ist die Konzentration von Laktobionsäure angestiegen. Vergleichbare Ergebnisse konnten bei der Umsetzung von Cellobiose zu Cellobionsäure erzielt werden.
Weitere Experimente für die Produktion von Maltobionsäure und Gluconsäure wären von wissenschaftlichem Interesse.
Es ist gelungen während der Umsetzung von Laktose aus Kuhmilch zu Laktobionsäure mithilfe des Biokatalysator *Mt*CDH enzymatisches Joghurt zu produzieren.

7 References

CAMERON M., AUST S.; Cellobiose dehydrogenase- an extracellular fungal flavocytochrome, Enzyme and Microbial Technology, 2001, 28: 129-138

in the text: [CAMERON M., 2001]

COMPTON S., JONES C., Mechanism of dye response and interference in the Bradford protein assay, Analytic Biochemistry, 1985, 151 (2): 369-74

in the text: [COMPTON S., 1985]

COX M., ROGER M., CHEESMAN M., JONES G., THOMSON A., WILSON M., MOORE G., Spectroscopic identification of the haem ligands of cellobiose oxidase, Federation of European Biochemical Societies, 1992, 2:233-236

CREGG J., MADDEN K., BARRINGER K., THILL G., STILLMAN C., Functional Characterisation of the two alcohol oxidase genes from the yeast Pichia pastoris, Molecular cell biology, 1989, 1316-1323

in the text: [CREGG J., 1989]

DALY R., HEARN M., Expression of heterologous proteins in Pichia pastoris: a useful experimental tool in protein engineering and production, Journal of Molecular Recognition, 2005, 18: 119-138

in the text: [DALY R., 2005]

GUTIERREZ L., HAMOUDI S., BELKACEMI K., Lactobionic acid: A high value added lactose derivative for food and pharmaceutical applications, International Dairy Journal, 2012, 103-109

in the text: [GUTIERREZ L., 2012]

HALLBERG M., HERIKSSON G., PETTERSON G., DIVNE C., Crystal structure of the Flavoprotein domain of the extrazellulär flavocytochrome cellobiose dehydrogenase, Journal of Molecular Biology, 2002, 315, 421-434

in the text: [HALLBERG M., 2002]

HARREITHER W., NICHOLLS P., SYGMUND C., GORTON L., LUDWIG R., Investigation of the pH-dependent electron transfer mechanism of ascomycetous class II cellobiose dehydrogenase on electrodes, American Chemical Society, 2012, 28:6714-6723

in the text: [HARREITHER W., 2012]

HILL H., The development of bioelectrochemistry, Coordination Chemistry Reviews, 1996, 151: 115-123

in the text: [HILL H., 1996]

JOOSTEN V., BERKEL W.; Flavoenzymes, Current Opinion in Chemical Biology, 2007, 11:195-202

in the text: [JOOSTEN V., 2007]

KLIMANT I., MEYER V., KÜHL M., Fiber- optic oxygen microsensor, a new tool in an aquatic biology, American Society of Limnology, 1995, 40: 1159-1165

in the text: [KLIMANT I., 1995]

PDF available: http://onlinelibrary.wiley.com/doi/10.4319/lo.1995.40.6.1159/pdf

LUCEY J., Cultured dairy products: an overview of their gelation and texture properties, International Journal of Dairy Technology, 2004, Vol 57, No 2/3

in the text: [LUCEY J., 2004]

LUDWIG R., HARREITHER W., TASCA F., GORTON L., Cellobiose Dehydrogenase: A versatile catalyst for electrochemical applications, ChemPhysChem, 2010, 11:2674-2697

in the text: [LUDWIG R., HARREITHER W., 2010]

LUDWIG R., ORTIZ R., SCHULZ C., HARREITHER W., SYGMUND C., GORTON L.; Cellobiose dehydrogenase modified electrodes: advances by materials science and biochemical engineering, Anal Bioanal Chem, 2013, 405:3637-3658

in the text: [LUDWIG R., 2013]

LUDWIG R., OZGA M., ZAMOCKY M., PETERBAUER C., KULBE K., HALTRICH D., Continuous enzymatic regeneration of electron acceptors used by flavoenzymes: Cellobiose Dehydrogenase- catalyzed production of lactobionic acid as an example, Biocatalysis and Biotransformation, 2004, Vol. 22 (2): 97-104

in the text: [LUDWIG R., 2004]

MIKKEL N., NIELSON P., VILLADSEN J., Oxidation of lactose to lactobionic acid by a *Microdochium nivale* carbohydrate oxidase: Kinetics and operational stability, Biotechnology and Bioengineering, 2006, Vol. 97: 694-707

in the text: [MIKKEL N., 2006]

OZCAN T., Determination of Yoghurt Quality by using rheological and textural parameters, IPCBEE, 2013, Vol. 53: 118-122
in the text: [OZCAN T., 2013]
RAMACHANDRAN S., FONTANILLE P., PANDEY A., LARROCHE C., Gluconic acid: Properties, Applications and Microbial production, Food Technol. Biotechnol., 2006, 44: 185-195
in the text: [RAMACHANDRAN S., 2006]
SYGMUND C., KRACHER D., SCHEIBLBRANDNER S., ZAHMA K., FELICE A., HARREITHER W., KITTL R., LUDWIG R., Characterisation of the two Neurospora crassa Cellobiose Dehydrogenase and their connection to oxidative cellulose degradation, Applied and Environmental Microbiology, 2012, 18:17: 6161-6171
in the text: [SYGMUND C., 2012]
SYGMUND C., SANTER P., KRONDORFER I., KRONDORFER I., PETERBAUER C., ALCALDE M., HYANHONGO G., GUEBITZ G., LUDWIG R.; Semi-rational engineering of cellobiose dehydrogenase for improved hydrogen peroxide production, Microbial Cell Factories, 2013, 12:38
in the text: [SYGMUND C., 2013]
TAN T., KRACHER D., GANDINI R., SYGMUND C., KITTL R., HALTRICH D., HÄLLBERG M., LUDWIG R., DIVNE C., Structural basis for cellobiose dehydrogenase action during oxidative cellulose degradation, Nature Communications, 2015, 6:7542/DOI: 10.1038
in the text: [TAN T., 2015]
WESTERMARK U., ERIKSSON K.; Cellobiose: Quinone Oxidoreductase, a new wood- degrading enzyme from white-rot fungi, Acta Chemica Scandinavica, 1974, B28: 209-214 in the text: [WESTERMARK U., 1974]
ZAMOCKY M., LUDWIG R., PETERBAUER C., HALLBERG B., DIVNE C,. NICHOLLS P., HALTRICH D.; Cellobiose Dehydrogenase- A Flavocytochrome from wood- degrading, Phytopathogenic and Saprotropic fungi, Current Protein and Peptide Science, 2006, 7: 255-280
in the text: [ZAMOCKY M., 2006]
PDF:
http://www.presens.de/fileadmin/user_upload/products/Sensor_Probes/Oxygen_Microsensors/140401_SP-OxMic-14-01_w.pdf
in the text: [Precision sensing]