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Protein engineering of cellobiose dehydrogenase from *Phanerochaete chrysosporium* in yeast *Saccharomyces cerevisiae* InvSc1 for increased activity and stability

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Highlights

Phanerochaete chrysosporium cellobiose dehydrogenase was cloned and expressed in *S.cerevisiae*.

Enzymatic assay in microtiter plates based on 2,6-dichloroindophenol was optimized.

Several mutants of cellobiose dehydrogenase with increased activity were found.

Recombinant cellobiose dehydrogenases were purified and characterized.

Abstract

Cellobiose dehydrogenase (CDH) can be used in industry for lactobionic acid production, as a part of biosensors for disaccharides and in wound healing. In fungi it is involved in lignocellulose degradation. CDH gene from *Phanerochaete chrysosporium* has been cloned in pYES2 plasmid for extracellular expression and protein engineering in yeast *Saccharomyces cerevisiae* InvSC1 for the first time. A CDH gene library was generated using error-prone PCR and screened by spectrophotometric enzymatic assay based on 2,6-dichloroindophenol reduction detection in microtiter plates. Several mutants with increased activity and specificity towards lactose and cellobiose were found, purified and characterized in detail. Recombinant CDH enzymes showed a broad molecular weight between 120 and 150 KDa due to hyper-glycosylation and the best S137N mutant showed 2.2 times increased k_{cat} and 1.5 and 2 times increased specificity constant for lactose and cellobiose compared to the wild type enzyme. pH optimum of mutants was not changed while thermostability of selected mutants improved and S137N mutant retained 30% of it's original activity after 15 minutes at 70^{o}C compared to 10% of activity that the wild type enzyme retained. Mutants M65S and S137N showed also 1.6 and 1.5 times increased productivity of hydrogen peroxide in the presence of 30mM lactose compared to the wild type.

Keywords: cellobiose dehydrogenase, directed evolution, lactose, Saccharomyces cerevisiae

1. Introduction

Cellobiose dehydrogenase (CDH; E.C. 1.1.99.18; is an extracellular oxidoreductase enzyme produced by many white-rot fungi [1], including well explored *Phanerocaete chrysosporium.* The fungus secretes CDH when cellulose is the main nutrient carbon source [2]. CDH oxidizes the reducing end of cellobiose, a product of cellulose biodegradation and cello-oligosaccharides to their corresponding 1,5-lactones, which are subsequently hydrolyzed to the carboxylic acids in aqueous environments using variety range of electron acceptors. CDH, in addition to cellobiose, oxidizes a few other sugars, mostly β-1, 4-linked disaccharides with a β-glucose moiety at their reducing end [3]. CDH is an enzyme with one subunit that has two distinct domains, one that contains FAD and cytochrome b type heme domain connected to short linker region [4].

The function of CDH is not completely understood. CDH can enhance both cellulose and lignin degradation even though the enzyme is not an essential component of the lignocellulose-degrading enzyme complex [3, 5]. CDH has specificity for β-1,4-linked disaccharides that could enable a range of applications in biosensors, bioremediation [1] or biocatalysis. CDH can also be used as a coupling enzyme in colorimetric assays [6] and in amperometric biosensors for lactose detection [7].

Studies for production of recombinant CDH enzyme in *Pichia pastoris* showed that this yeast is suitable for high-level enzyme production [8]. Aside from protein heterologous expression in *Pichia pastoris* there is a report of successful production of recombinant CDH`s flavin domain in *Escherichia coli* [9]. Professor Sode and his team showed that by expression of CDH`s flavin domain in *E.coli* protein engineering studies of CDH can be performed in prokaryotes as well.

Heterologous expression of cdh gene in. *P. pastoris* was done, but use of *P. pastoris* in directed evolution of CDH and high-throughput screening is not favorable because of a low transformation efficiency [8]. Due to higher transformation efficiency, *Saccharomyces cerevisiae* was used instead of *P. pastoris* as an expression system for directed evolution of enzymes that cannot be functionally expressed in prokaryotic organisms like glucose oxidase [10], α-amylase [11], laccase [12] and peroxidase [13]. Successful use of *S.*

cerevisiae expressing *Myriococcum thermophilum* CDH in directed evolution was recently reported by Sygmund et al [14].

Saccharomyces cerevisiae is a model system for heterologous expression of eukaryotic proteins. *E. coli* can be used as the host for many eukaryotic proteins, but proteins produced from eukaryotic genes in *E.coli* can differ from the normal gene product and lack biological function or they can be insoluble until some chemical modification is applied [15]. *S.cerevisiae* has an advantage over bacteria because it has a secretion system similar to higher eukaryotic systems [16], which can be manipulated to produce heterologous proteins. The ease of genetic manipulation as well as its accessibility as a production organism, has made *S. cerevisiae* a preferred organism for production of many eukaryotic proteins. Directed evolution involves iterative rounds of diversity generation and screening that allows the selection of enzymes with desired improved properties [17, 18]. Due to the above mentioned properties, the most desired host for directed evolution of eukaryotic proteins is *S. cerevisiae* [19].

The main goal of this article was to clone *Phanerochaete chrysosporium* cdh gene in *S. cerevisiae* for heterologous expression, so that it can be used in directed evolution of CDH enzyme in order to increase its activity and specificity, making it more attractive as a biocatalyst for applications in biosensors and biocatalysis.

2. Materials and methods

2.1. Microbial strains and vectors

S.cerevisiae strain INVSc1 (MATa his3D1 leu2 trp1-289 ura3-52) was purchased from Invitrogen (Invitrogen BV, Groningen, The Netherlands). Protocols and media for INVSc1 were described in pYES2 manual [20, 21]. *E.coli* DH5α strain was chemically transformed. PCR amplified products were cloned in pYES2 vector (Invitrogen).

2.2. *Cloning of cdh gene in vector*

The *P. chrysosporium* CDH gene (U46081.1) was synthetized by GenScript USA Inc. PCR was used for gene amplification using forward primer EcoRI_fp_AF (5`-ATC GAA TTC ATG AGA TTT CCT TCA ATT TT-3`) and reverse primer XbaI_rp_CBDH1 (5`-ATC TCT AGA TCA AGG ACC TCC CGC AAG CG-3`). Before insertion in vector, PCR product and pYES2 vector (Invitrogen) were both digested with enzymes EcoRI and XbaI. *E.coli* DH5α strain was used as a host for cloning the recombinant vector.

2.3. *Expression of recombinant enzyme*

S.cerevisiae INVSc1 cell was transformed with pYES2-cdh plasmid DNA. The vector pYES2 without insert was also used for transformation of competent *S.cerevisiae* cells and used as a control. Transformed colonies were picked from YNB-CAA plates supplemented with glucose (Glc) and transferred to YNB-CAA plates supplemented with galactose (Gal). Positive colonies identified in the DCIP overlay assay were inoculated into YNB-CAA /Glc liquid medium and incubated for 48h at 30^{o}C, 250rpm. Expression of CDH was induced by diluting grown cells till OD 0.4 [20] with induction media (YNB-CAA/Gal medium) and incubating the culture as above for various durations to optimize the expression protocol. Samples were analyzed hourly for CDH activity using DCIP assay.

2.4. *Agar plate assay*

YNB-CAA /Gal plates with grown colonies were used and 20mL of molten 2% agar containing 0.15mM cellobiose, 0.78mM $K_3[Fe(CN)_6]$ and 0.16mM $NH_4Fe(SO_4)_2$ in 0.1 M Na-acetate buffer (pH 4.0), cooled to 40^{o}C was added [22]. After cooling, the plates were incubated 4h at room temperature and monitored for

the development of a blue halo around yeast colonies. Another agar plate assay was used. YNB-CAA / Gal plates with grown colonies were overlaid with cooled to 40°C, 20mL of molten 2% agar containing 30mM lactose, 0.3mM 2,6-Dichloroindophenol (DCIP; Sigma chemicals) in 0.1M Na-acetate buffer (pH 4.5). CDH producing transformants were identified by the presence of colorless halo around the *S.cerevisiae* colonies on plates overlaid with DCIP, resulting from CDH activity.

2.5. Enzyme purification

Recombinant CDH produced by *S.cerevisiae* was harvested 16h after induction. Culture was grown in 1L, centrifuged (3000xg for 30 min) and cell free supernatant was concentrated on Vivaflow50 ultrafiltration cell with cut off 50kDa (Vivaflow50, Sartorius AG, Goettingen, Germany) and buffered with 10mM sodium phosphate pH 6.0. The concentrate was dialyzed against same the buffer and applied to HiTrap DEAE FF 5mL column equilibrated with the same buffer. wtCDH was eluted with 0-0.6M NaCl linear gradient in the same buffer. Fractions containing wtCDH activity were pooled and concentrated, and further purified on the same column with the same conditions. Fractions with wtCDH activity were pooled and dialyzed against 10mM sodium phosphate pH 6.0 buffer and applied to HiTrap DEAE FF 5mL column equilibrated with the same buffer. Elution was done as with previous chromatography and fractions were pooled and stored at 4°C.

2.6. Enzyme activity assay

Assay for measuring CDH activity was accomplished with modified DCIP assay [23]. Reaction was set with 0.3mM DCIP, 30mM lactose, and 0.1 M sodium acetate pH 4.5, at 25°C in total reaction volume of 1mL. Reaction was started by adding purified CDH and reduction of DCIP was followed by measuring absorbance decrease at 520nm (ε_{520}=6.80 mM^{-1} cm^{-1}). Linear range of the slope was used for CDH activity

determination. One unit of enzyme activity is defined as the amount of enzyme that reduces 1µmol of DCIP per minute under the above mentioned conditions [23].

2.7. Polyacrylamide gel electrophoresis and zymography analysis

The molecular mass and homogeneity of the enzyme preparation were determined by polyacrylamide (10%) vertical gel electrophoresis in 0.1% SDS [24] containing 2-mercaptoethanol. Proteins were visualized after staining with Coomassie Brilliant Blue R-250 and silver nitrate [25] and were compared to molecular weight standards (Thermo Fisher Scientific, MA, USA). Native electrophoresis was carried out under same conditions in 10% polyacrylamide gels lacking SDS and 2-mercaptoethanol. For zymography, the gels were supplemented with 0.1M sodium acetate buffer (pH 4.5) containing 0.3mM DCIP, 30mM lactose.

2.8. Mutagenesis and creation of the library

EpPCR of cdh was performed using pYES2 vector and before mentioned primers EcoRI_fp_AF and XbaI_rp_CBDH1. Different concentrations of $MnCl_2$ (0-0.1mM) were used to obtain higher mutation rates with Taq polymerase. The library had thermal cycling parameters 94°C for 4 min (1 cycle), 94°C for 1 min, 55°C for 1 min, 72°C for 2.15 min (30 cycles), and 72°C for 10 min (1 cycle) as a final extension. The PCR products were purified with GeneJET plasmid miniprep kit (Thermo Fisher), digested with EcoRI and BamHI enzymes and cloned into pretreated pYES2 vector (digested with the same enzymes) using EcoRI and BamHI enzymes. Ligated products were used to transform *E.coli* DH5α competent cells. The plasmid isolated from *E.coli* library was used to transform competent *S.cerevisiae* INVsC1 cells. Transformants were selected on YNB CAA (GAL) agar plates and transferred into 96-well microtiter plates (MTPs).

2.9. Screening of gene libraries in microtiter plates

Fermentation was done in MTP and after 16h of induction activity was measured with DCIP assay. DCIP activity assay for microtiter plates(MTPs) was modified from the initial recipe [23] by not adding sodium fluoride, reaction volume in each well was 200µL with 0.3mM DCIP, 30mM lactose, 0.1M sodium acetate buffer and assay had been started with addition of enzyme. Absorbance decrease was observed at 520nm at LKB 5060-006 microplate reader, during half an hour at 25°C.

2.10. Characterization of mutants

mCDH activity as a function of pH for DCIP was measured using McIlvaine`s [26] citrate-phosphate buffer in the range 2.0-9.0 at 30°C. mCDH activity as a function of the temperature for DCIP was measured in the range 25-90°C in 0.1M sodium acetate, pH 4.5, with lactose as a substrate. Thermal stability of mCDH was determined by incubation at 50, 55 and 60°C for up to 3h, depending on the temperature and monitoring activity at different time-points using DCIP as the electron acceptor and lactose as a substrate. Protein concentration used for k_{cat} calculations of wt and mutants of CDH was determined by measuring absorbance at 280nm and using published molar extinction coefficient for CDH from *Phanerochaete chrysosporium* at 280nm of ε_{280nm}=217 $mM^{-1}cm^{-1}$ [8].

2.11. Peroxide production

2.12. Peroxide production was measured with modified 2,2´-azino bis (3 – ethylbenzthiazoline-6-sulfonate) (ABTS) – based assay done by Sygmund et al [15]. Reaction mixture (50µL) containing 60mM cellobiose in 100mM sodium acetate buffer pH 4.5 was added to 50mL of the sample for the production of peroxide. The reaction was incubated at 30°C for 4h before CDH was inactivated at 90°C for 10 minutes. This does not influence the peroxide concentration. The addition of 100µL of ABTS reagent containing

2mM ABTS and 5.7U/mL peroxidase in 100mM sodium acetate buffer pH 5.5 had started the colorimetric reaction. The increase in absorbance was followed by a plate reader on room temperature for 5 min. The stoichiometry for this reaction is two since for one mol of peroxide two mol of the green ABTS cation radical are formed. The enzymatic activity is given in units (IU) which corresponds to the production of 1µmol cellobionic acid or 1µmol peroxide per minute.

2.13. Structural analysis

Posible N-glycosylation sites were predicted using NetNGlc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), while O-glycosylation sites were predicted using NetOGlyc 3.1 Server (http://www.cbs.dtu.dk/services/NetOGlyc-3.1/). Distances of mutated residues from active site and glycosylation sites within each domain were calculated using UCSF Chimera software and published crystal structures in UniProt database for heme (1D7C) [27] and flavin domain (1NAA) [28] of CDH from *Phanerochaete chrysosporium* (Fig. 7.). For multiple alignment of 28 CDH sequences NCBI COBALT (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?CMD=Web) server was used (Supplementary Fig. S13).

3. Results

3.1. Cloning of cdh gene in vector and expression of recombinant enzyme

Primers containing EcoRI and XbaI restriction sites were used for gene amplification. The 2.5kb PCR product (verified by agarose electrophoresis) was digested with EcoRI and XbaI and inserted into the corresponding sites in pYES2 vector. The inserts were verified by colony PCR and by digesting isolated plasmid DNA with EcoRI. Length of the obtained PCR product was 2.6 kbp. Gene length of cdh from *P.*

chrysosporium is 2307 bp, while length of α-factor secretion necessary for secretion of mature protein is 267 bp, and in total, length of fusion gene is 2574 bp (Supplementary; Fig. S1). Recombinant enzyme was cloned into yeast *S.cerevisiae* and expression of wtCDH was followed by measuring activity. The highest activity was achieved after 16h of induction in galactose containing medium. After 16h of induction wtCDH activity was slowly decreasing in the fermentation medium (Supplementary; Fig. S2).

3.2. Optimization of DICP enzymatic assay in microtiter plates

Fermentation of 96 samples of wtCDHs was done in MTP and after 16h of induction, activity of wtCDHs in 96 wells was measured. Activity of wtCDHs in individual wells of microtiter plates is represented by black dots, Fig 1.

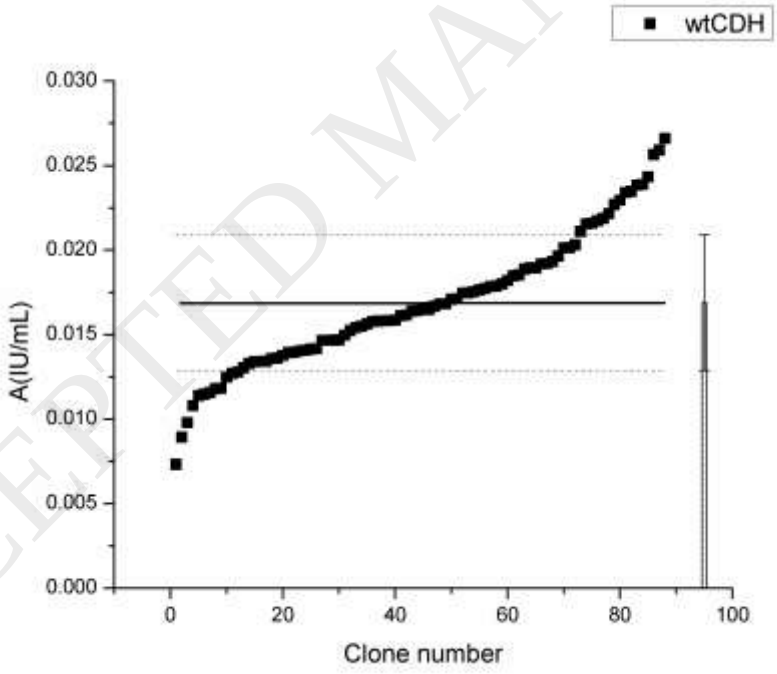

Fig. 1. Performance of DCIP microtiter plate assay on wtCDH. *S. cerevisiae* cells were transformed with plasmid pYES2-wtCDH. 96 individual colonies of wtCDH transformants were cultivated under inducing

conditions. Centrifuged supernatants were used for the DCIP-based assay. Standard deviation was 23%. Standard deviation and mean value are shown in histogram part of the graph.

After optimizing the fermentation time, substrate concentration and liquids handling standard deviation of the measured 96 samples of wtCDH in one microtiter plate was 23 %.

3.3. Creation and screening of gene libraries in microtiter plates

Libraries of CDH mutants were generated by error-prone PCR using 0.05mM manganese since it gave in average 1 to 2 mutations per cdh gene and about 60% of the clones had active CDH enzyme variants in agar plate assay. The PCR products were purified and cloned into pYES2 vector using EcoRI and BamHI enzymes. Plasmids from epPCR *E.coli* library were transformed into *S.cerevisiae* INvsC1 strain. Positive colonies from agar plate assay (around 60% of population) were picked up and screened in MTP for increased activity. Fig. 2 shows data obtained from one of MTPs during screening process.

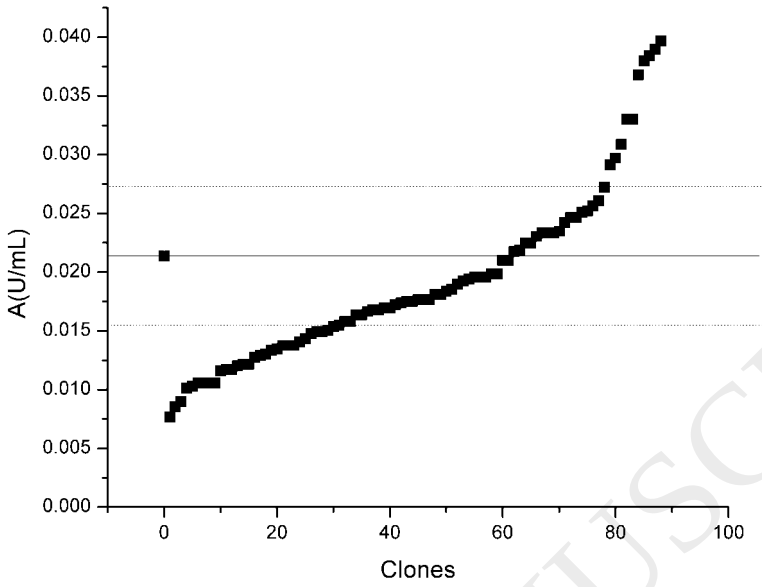

Fig. 2. Screening of an error-prone CDH gene library with DCIP microtiter plate assay in MTP. Dashed lines indicate the standard deviation, while the solid line represents average wtCDH activity. *S. cerevisiae* cells were transformed with plasmid pYES2-mCDH. 96 individual colonies of each transformant were cultivated under inducing conditions. Centrifuged supernatants were used for the DCIP-based assay.

Around 1000 clones that showed activity on agar plates were screened in MTPs and three mutants with the highest activities compared to the wild type CDH were sequenced and further characterized (S137N, M65S and M685V).

3.4. Enzyme purification

After expression in 1L, cell free fermentation broth was used for purification of the recombinant CDH protein. Ultrafiltration with 50kDa molecular weight cut-off cell enriched CDH by 10-fold. Ultrafiltered CDH was dialyzed and loaded onto DEAE column. The proteins were eluted using 0-0.6M salt gradient in

10mM sodium phosphate buffer. Fractions with CDH activity were pooled and additionally purified by rechromatography on DEAE column (Supplementary; Fig. S3-S16).

Purity of the protein was determined by native polyacrylamide gel electrophoresis and zymography. The silver staining of the gels after native electrophoresis revealed that crude sample, as well as purified sample had protein bands on the polyacrylamide gel that matched the position of the activity band detected by zymography using DCIP and lactose (Fig. 3).

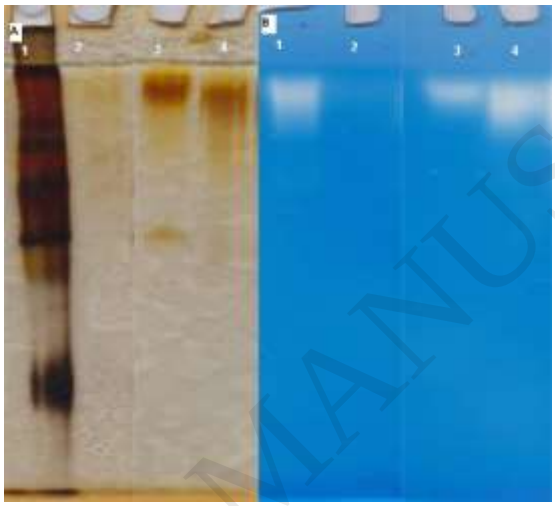

Fig. 3. Native electrophoresis of CDH on 10% gels. 1. wtCDH crude sample, 2. wtCDH sample before IEC , 3. wtCDH purified by IEC, 4. wtCDH purified by rechromatography on IEC. A) Protein bands in gel after silver staining. B) Activity bands in gel after incubation in buffered substrate solution (30mM lactose and 0.3mM DCIP).

Protein and activity band overlapping on zymograme and symmetric protein peak during ion exchange chromatography (IEC) that corresponded to the wtCDH activity confirmed that wtCDH protein was pure. Broad protein band shows that there is microheterogenity of the protein due to hyperglycosylation that occurs in yeast.

The purity and molecular weight of the recombinant CDH was further confirmed by SDS-PAGE, revealing a broad band between 120 and 150kDa for enzyme expressed in *S.cerevisiae* (Fig. 4).

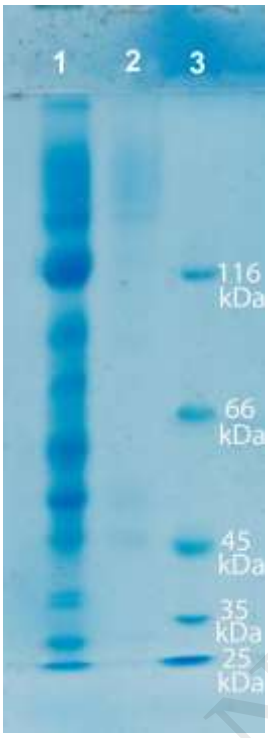

Fig. 4. SDS electrophoresis for samples: 1. wtCDH crude sample; 2. wtCDH purified sample; 3. MM-molecular markers. Gel was done on 8% separating gel, and dyed with Coomassie Brilliant Blue (CBB).

Molecular weight of wtCDH (120-150kDa) is higher than theoretically expected molecular weight for native CDH of 90kDa [29], which is a result of hyperglycosylation that occurs during secretion from *S.cerevisiae* [30]. Because of non-uniform hyperglycosylation that is common for extracellulary expressed proteins in *S.cerevisiae*, a broad band usually can be observed on electrophoresis. Non-uniform hyperglycosylation in *S.cerevisiae* with broad bands on SDS electrophoresis was previously reported for glucose oxidase from *Aspergillus niger* [30], laccase from *Myceliophthora thermophila* [12] and invertase [31]. We also determined pI value of expressed enzyme to be 4.7 by isoelectric focusing (Supplementary Fig. S7) that corresponds to theoretically calculated from sequence to be 4.61.

3.5. Mutants characterization

Activity of purified wtCDH and three purified mutants (Supplementary Fig. S3-S8) was determined in different sugar concentrations, allowing the data to be fitted onto a Michaelis-Menten curve (Supplementary Fig. S9) to calculate the corresponding kinetic parameters [32], Table 1.

Table 1. Kinetic parameters for the purified wtCDH and its mutants toward lactose (lac) and cellobiose.

	wtCDH	**S137N**	**M65S**	**M685V**	**wtCDH**	**S137N**	**M65S**	**M685V**
	Lactose				Cellobiose			
K_m (mM)	1.08	1.56	1.49	1.17	0.026	0.029	0.054	0.021
k_{cat} (s^{-1})	2.59	5.76	4.06	1.37	1.05	2.3	2.06	1.19
k_{cat}/K_m (s^{-1} mM^{-1})	2.40	3.69	2.72	1.17	39.8	80	38.2	56.9

Recombinant protein had a lower catalytic activity than the native form. The k_{cat} of the wtCDH was 14-fold lower than the native enzyme, and K_m value of wtCDH enzyme was 1.65-fold higher than the native enzyme [33] with cellobiose as substrate. WtCDH showed higher K_m values than has been reported in literature for cellobiose and lactose oxidation and DCIP reduction. The lower catalytic activity of recombinant protein may reflect the impact of the higher molecular weight due to hyper glycosylation [34]. Mutants S137N and M65S had increased k_{cat} compared to the wild type for both cellobiose and lactose as a substrate, while mutant M685V had increased k_{cat} and k_{cat}/K_m only for cellobiose which could mean that it showed higher activity during screening in MTP with lactose due to higher expression rate. Specificity constant for all mutants and both substrates was increased when compared with the recombinant wild type CDH.

McIlvaine buffer was used for measuring pH optima. Due to the spectral characteristics of DCIP, who is dependent on pH of the reaction medium, different extinction coefficients were used for different pH: pH

2.0-4.5 (6.8 $mM^{-1}cm^{-1}$); pH 5 (6.8 $mM^{-1}cm^{-1}$); pH 5.5 (8.2 $mM^{-1}cm^{-1}$); pH 6 (12.7 $mM^{-1}cm^{-1}$); pH 6.5 (15.7 $mM^{-1}cm^{-1}$); pH 7 (16.8 $mM^{-1}cm^{-1}$); and pH 7.5 – 9.0 (17.7 $mM^{-1}cm^{-1}$). The activity of purified wtCDH enzyme and mutants was determined at different pH values. Enzyme showed activity in wide pH range with DCIP as electron acceptor. Activity peak was observed at pH 2.5-5.5, except for S137N whose range went from 3.5-6 pH units, and M65S from 3-5.5 pH units (Fig.5).

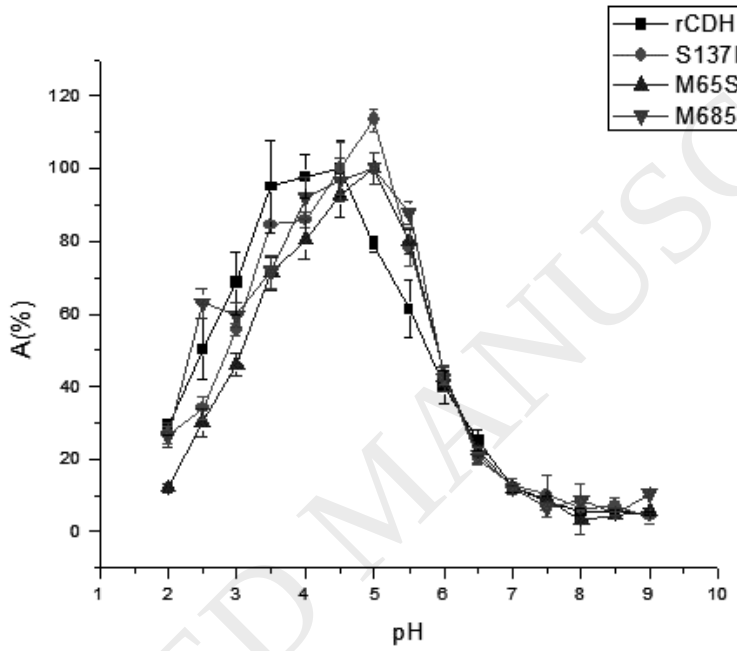

Fig. 5. Effect of pH on wtCDH and mutant activity using 0.3mM DCIP as the electron acceptor, and 30mM lactose in McIlvaine`s buffer pH 2.0-9.0. The values shown are the means of triplicate determinations.

The activity peak was at pH 4.5, and this was the pH used to determine all the activity for other characterizations. Enzyme was stable in the pH range of 2.5-5. The pH optimum for the native CDH was reported to be pH 6, and pH 4 – 4.5 for direct electron transfer [35]. The highest activity in one study was shown to be at pH 4 for flavin domain [9].

The mutants showed higher residual activity than wtCDH with DCIP as the electron acceptor, (Fig.6).

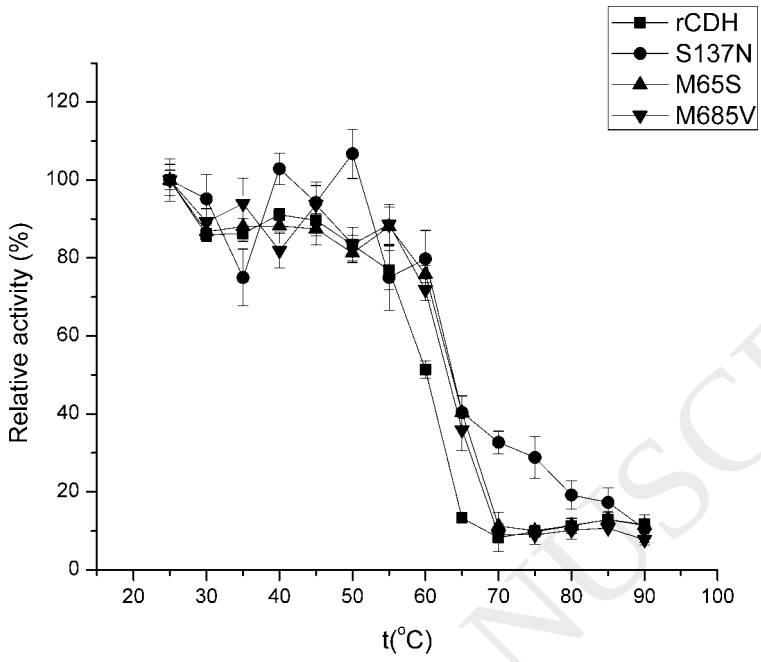

Fig. 6. Effect of temperature on wtCDH and mutant activity with assay carried out in 0.1M sodium acetate buffer pH 4.5 using 0.3mM DCIP as the electron acceptor and 30mM lactose as the substrate. The samples were measured at different temperatures for 15 minutes, and cooled on ice. The values shown are the means of triplicate determination.

Thermal stability studies ranged from 50 to 60°C and were carried out at pH 4.5 (see Supplementary Fig. S9-S11). At 50°C recombinant wtCDH retained 80% of it's original activity after 1h while in the case of CDH from *Trametes versicolor* activity dropped to around 43% of it's original activity. When incubated at 55°C over a period of 1h, residual activity decreased to approximately 40% of the original value for wtCDH, all three mutants had above 90% of residual activity after 2h of incubation, while for CDH from *T.versicolor* activity decreased to approximately 11% [36].

3.6. Peroxide production

One milligram of wtCDH produced 0.273 nmol H_2O_2 per minute, S137N 0.400 nmol H_2O_2 per min, M65S 0.440 nmol H_2O_2 per min and M685V 0.210 nmol H_2O_2 per min. Results are lower than reported production of recombinant CDH from *Myriococcum thermophilum* produced in *P.pastoris* [14].

4. Discussion

Our study showed successful cloning for the first time of cellobiose dehydrogenase gene from *Phanerochaete chrysosporium* in pYES2 vector and heterologous expression in yeast *Saccharomyces cerevisiae* InvSC1 for the purpose of directed evolution. Production rate of wtCDH in yeast *S. cerevisiae* is poorer than the production of enzyme produced in its natural source, from fungus, while the other yeast, *Pichia pastoris,* produces enzyme in higher quantity [8, 37]. The poor transformation rates and difficult recovery process of cloned mutants have made *Pichia*`s use in evolution less appealing. These difficulties have been solved by use of *S. cerevisiae* since it exhibits high transformation efficiency, performs post-translational modifications and possesses a developed machinery for the secretion of proteins into the culture medium. After expressing active CDH in yeast an error prone PCR gene library was created and screened for mutants with increased activity by DCIP enzymatic assay optimized for microtiter plate format. Several mutants of CDH with improved properties were found M65S, S137N and M685V. WtCDH produced in *S.cerevisiae* has k_{cat} values for cellobiose and lactose oxidation lower than the same enzyme when produced in *P.chrysosporium* [38], but this is a very well known fact for yeasts and was explained by excessive glycosylation that occurs in *S.cerevisiae* [10]. Using NetNGlyc and NetOGlyc neural network based servers for analyzing CDH protein sequence, nine putative N and sixteen O-glycosylation sites were predicted and distances of found mutations were calculated using USCF Chimera (see Supplementary Table S1). The analysis showed that the most O glycosylation sites were located in the loop region of CDH connecting heme and flavin domain. Identified mutations were not in this region or close to any of putative

O-glycosylation sites. Only M685V was close to a putative N-glycosylation site at N517 at a distance of 7.385 angstroms, but that mutant did not show such a big improvement in the activity compared to the other two. Analysis of sequences including M65S and S137N mutations showed that glycosylation pattern did not change compared to wild type CDH sequence (see Supplementary). These results showed that CDH protein is heavily non-uniformly glycosylated in yeast at found putative glycosylation sites and that our mutations did not influenced much that glycosylation. Heavy non uniform glycosylation in *S.cerevisiae* that leads to decreased activity compared to homologously expressed proteins was also previously reported for glucose oxidase [30].

Nevertheless, due to easier genetic manipulations *S.cerevisiae* is the preferred host for directed evolution. It was also proved that ratio of activity for different mutants of the same enzyme remains the same when expressed in different hosts [39].

The best mutant S137N had 2.2 times increased k_{cat} for both lactose and cellobiose compared to wtCDH and also 1.5 and 2 times increased specificity constant for lactose and cellobiose respectively.

Calculating distance of found mutations from the active sites in heme and flavin domain of CDH it could be seen that M65S mutation is coordinating iron in the heme active site at distance of only 11.1 angstroms from other coordinating His163 in the active site [40], (Fig. 7).

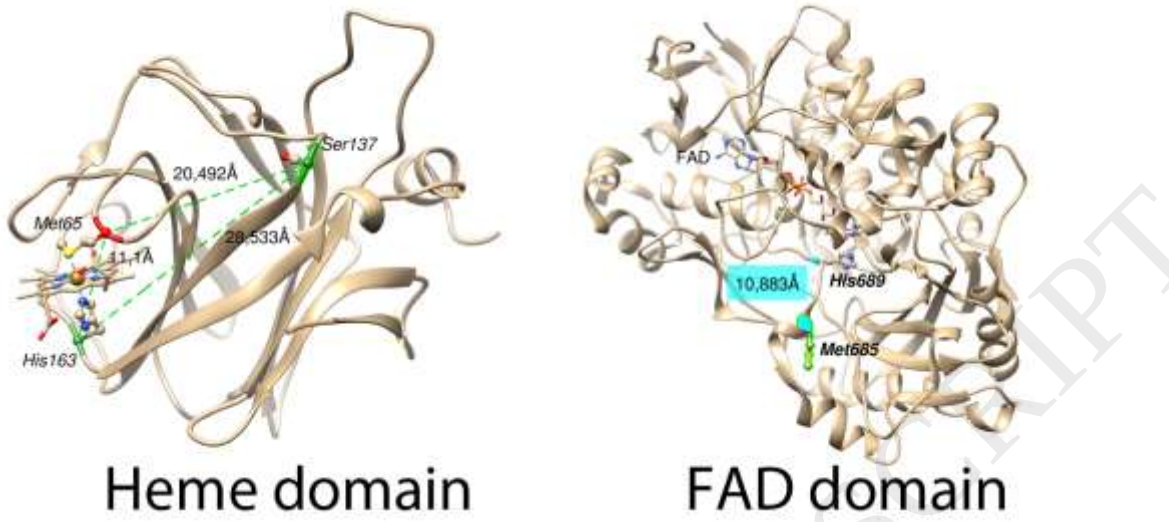

Fig. 7. Presentation of the structure of the two (separated) domains of *P.chrysosporium* CDH with shown distances of found mutations from the active sites.

M65S mutation did not disrupt CDH activity with DCIP even it was coordinating critical cofactor like heme and that was in agreement with results obtained expressing only FAD domain of CDH in *E.coli* that showed even higher activity than native CDH containing both domains [9]. Also, in multiple sequence alignment of 28 CDH sequences (Supplementary Fig. S13) M65T substitution was found in one homolog that can also explain why such mutation is tolerated. Other S137N mutation was also in the heme domain at distance of around 20 angstrems from the heme active sites, while M685V mutations was in the flavin domain relatively close to the catalytic His689 reside at a distance of around 11 angstrems. From this data we could conclude that M65S and M685 could influence activity of the CDH due to the proximity to the active site while influence of S137N mutations is most likely achieved through long distance interactions and as a surface mutation could also contribute to the enzyme stability that was previously shown for surface mutations [41]. Multiple sequence alignment (Supplementary Fig. S13) showed that amino acid residues at positions S137 and M685 are moderately conserved and substitutions could be found in 30-50% of homologues.

pH optimum of mutants was not changed significantly while thermostability of all mutants improved compared to wtCDH. The most active mutant S137N retained 30% of it's original activity after 15 minutes at 70°C compared to 10% of activity that wild type enzyme retained. Also, when incubated at 55°C over a period of 1h, residual activity of wtCDH decreased to approximately 40% of the initial activity while all three mutants had more than 90% of their original activity. Under the similar conditions activity of CDH from *T.versicolor* decreased to approximately 11% of initial activity [36]. Generally, mutations found by screening an epPCR library for increased activity lead to decreased stability, but finding mutations that increase both activity and stability is possible and it is often the case when using consensus approach in directed evolution experiments [42].

When tested for hydrogen peroxide production that can be used in wound healing and laundry, all three mutants showed increased hydrogen peroxide production in the presence of 30mM lactose compared to the wild type. The best mutant for hydrogen peroxide production showed to be M65S and produced hydrogen peroxide at rate that is 1.6 times higher compared to wtCDH. Since M65S is very close to the heme in the active site this could indicate importance of heme iron in CDH for production of reactive oxygen species helping lignocellulose degradation.

All of these results show that we can use *S.cerevisiae* InvSC1 as an expression host for directed evolution of CDH from *P. chrysosporium*. Using the optimized microtiter plate DCIP assay we have obtained CDH mutants with increased activity and specificity towards lactose and cellobiose. They were also more thermostable and were producing higher amounts of hydrogen peroxide than the wild type enzyme. This makes them good candidates for applications in biosensors and biocatalysis.

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