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Expression, purification and characterization of cellobiose dehydrogenase mutants from Phanerochaete chrysosporium in Pichia pastoris KM71H strain

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Abstract: Production of soluble cellobiose dehydrogenase (CDH) mutant proteins previously evolved on the surface of S. cerevisiae yeast cells was established for use in biosensors and biofuel cells. For this purpose, mutant cdh genes tm (D20N, A64T, V592M), H5 (D20N, V22A, A64T, V592M) and H9 (D20N, A64T, T84A, A261P, V592M, E674G, N715S) were cloned to pPICZα plasmid and transformed into Pichia pastoris KM71H strain for high expression in a soluble form and kinetic characterization. After 6 days of expression under methanol induction, CDHs were purified by ultrafiltration, ion-exchange chromatography and gel filtration. SDS electrophoresis confirmed purity and presence of a single protein band at a molecular weight of 100 kDa. Kinetic characterization showed that H5 mutant had the highest catalytic constant of 43.5 s⁻¹ for lactose, while mutant H9 showed the highest specificity constant for lactose of 132 mM⁻¹s⁻¹. All three mutant proteins did not change pH optimum that was between 4.5 and 5.5. Comparing to previously obtained wild types and mutants of CDH from Phanerochaete chrysosporium, the variants reported in this article had higher activity and specificity that together with high protein expression rate in Pichia, makes them good candidates for use in biotechnology for lactobionic acid production and biosensors manufacturing.

Keywords: mutant proteins; yeast; protein purification; kinetic characterisation

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INTRODUCTION

White rot fungi *Phanerochaete chrysosporium* has been known to secrete cellobiose dehydrogenase (CDH, EC 1.1.99.18). Regardless of the fact that the biological role of the CDH is not fully understood, it is known that CDH participates in oxidation of β–1,4–linked disaccharides and oligosaccharides, such as cellulose and hemicellulose as well as cellobiose and lactose. Cellobiose dehydrogenase is a monomeric protein that contains two domains: a catalytically active flavin domain and heme b containing cytochrome domain. These two domains are connected with each other via a long and flexible linker. Oxidation of substrates, catalysed by CDH, involves the reduction of FAD to FADH₂ and the flavin domain is shown to be responsible for electron uptake during the substrate oxidation directly transferring electrons to two-electron acceptors. The role of the heme domain is identified as a significant enhancement of activity towards one-electron acceptors. Since CDH can oxidize both lactose and cellobiose using wide range of electron acceptors, but not oxygen, it is used in biosensors and biofuel cells, for dye removal, bioremediation and lactobionic acid production.

Variants of CDH that would have increased activity would benefit these applications. Directed evolution is often used to generate them via iterative rounds of genetic diversification and library screening. Directed evolution became one of the most useful and widespread tools in basic and applied biochemistry. Expression systems, mainly used for this purpose, are *E. coli* and *S. cerevisiae* due to high transformation efficiency that is needed for creation of large gene libraries.

When it comes to production of improved enzyme variants at a high level the use of methylotrophic yeast *Pichia pastoris* is preferable despite low transformation efficiency. *Pichia pastoris* has many beneficial characteristics for production of recombinant proteins, high cell density growth, the ability to secrete large amounts of the desired protein and post-translational modifications which are characteristic for eukaryotic organisms. Therefore, compared to bacterial expression systems like *E. coli*, *P. pastoris* has become a powerful expression system for recombinant eukaryotic proteins.

In this article three new mutant forms of CDH from *Phanerochaete chrysosporium*, that we discovered during directed evolution on the surface of *S. cerevisiae* EBY100 cells, with increased activity and specificity, were expressed for the first time in a soluble form in *Pichia pastoris* KM71H, purified and kinetically characterized to test if they are promising biocatalysts for use in biotechnology.

EXPERIMENTAL

Cloning cdh gene in pPICZαA vector

Cdh gene (U46081.1) from *Phanerochaete chrysosporium* (synthesized by GenScript USA Inc.) was amplified using forward primer *EcoRI fp CBDH* (5’ – ATGAATTCAGAGTGCCACAGTTTACC – 3’) and reverse primer *XbaI rp CBDH_2* (5’ – ATCTAGA-
TCAAGGACCTCCCGAAG – 3'). The following PCR protocol with Taq polymerase was used for gene amplification (1 cycle – 4 min at 94 ºC; 30 cycles – 1 min at 94 ºC, 1 min at 55 ºC, 2 min 15 sec at 72 ºC; 1 cycle – 10 min at 72 ºC). Obtained PCR products and pPICZαA vector (Invitrogen BV, Groningen, The Netherlands), were both digested with EcoRI and XbaI restriction enzymes and ligated. E. coli XL10 gold strain was used as a host for cloning the recombinant plasmid. For transformation of E. coli cells CaCl₂ transformation protocol was used.

Construction of cellobiose dehydrogenase variants

Previously discovered mutant proteins during directed evolution of CDH on the surface of S.cerevisiae EBY100 yeast cells were reconstructed using wild type cdh gene in pPICZαA as a template, primers with introduced mutations (Supplementary Table S1), and QuickChange Lightning Site – Directed Mutagenesis Kit (Agilent Technologies). Escherichia coli XL10 gold competent cells were transformed using constructs and plasmid DNA was isolated using Macherey – Nagel Plasmid DNA kit (Duren, Germany).

Expression of recombinant enzyme in Pichia pastoris KM71H strain

Transformation by electroporation of Pichia pastoris strain KM71H (Mut¹, zeocin resistant strain) (Invitrogen BV, Groningen, The Netherlands) with constructs was done using the protocol described in the EasySelect Pichia Transformation Kit. After transformation, expression of single colonies was done using the protocol described in the EasySelect Pichia Transformation Kit, using Buffered Minimal Glycerol (BMGH) and Buffered Minimal Methanol (BMMH) media for growth and expression, respectively. Selection of constructs was done by the addition of 100 μg mL⁻¹ zeocin in the growth media. Growth of the cells was done in BMGH media at 28 °C in an incubator with shaking (250 rpm) until culture OD₆₀₀ was between 2 and 6. Centrifugation for 10 minutes at 3000 rpm was done, cells were separated from the growth media and further resuspended in BMMH media using 1/5 volume of the growth culture volume. Induction was done for 6 days by adding methanol every 24 hours to the final concentration of 0.5 %. Cell free fermentation liquid, including the enzyme, was concentrated on Vivaflow50 ultrafiltration cassette, cut off 50 kDa (Sartorius, Germany), using a peristaltic pump (Heidolph Instruments) and dialyzed versus 10 mM sodium phosphate buffer pH 6.0.

Enzymatic assay

CDH activity was analysed at 20 °C with 0.3 mM 2, 6 – dichloroindophenol (DCIP; Sigma chemicals; λex=520 nm; ε₅₂₀=6.80 mM⁻¹ cm⁻¹) as the electron acceptor in 0.1 M sodium acetate buffer pH 4.5, using 30 mM lactose as substrate. One international unit (IU) of enzyme activity is defined as the amount of enzyme that reduces 1 μmol of DCIP per minute under the above mentioned conditions.

Purification of CDH

Ion exchange chromatography was done on 10 mL Toyopearl DEAE ion exchange column. For equilibration, 10 mM sodium phosphate buffer pH 6.0 was used, elution was done using a linear gradient from 0 to 50% concentration of sodium chloride (1 M NaCl) in the same buffer. Fractions were tested for CDH activity using DCIP solution (30 mM lactose, 0.3 mM DCIP in 0.1 M Na – acetate buffer pH 4.5).

Size exclusion chromatography was used to further purify CDHs, which were dialyzed after ion exchange chromatography versus 20 mM sodium phosphate buffer pH 6.0. Purification was done on 80 mL Toyopearl HF55 size exclusion column in 20 mM sodium
phosphate buffer pH 6.0. Fractions were tested for CDH activity using DCIP solution (30 mM lactose, 0.3 mM DCIP in 0.1 M Na – acetate buffer pH 4.5).

**Polyacrylamide gel electrophoresis**

Purity and size of the protein was determined using denaturing sodium dodecyl-sulphate polyacrylamide gel, with 4 % stacking gel and 10 % separating gel. Protein bands were visualized using Coomassie Brilliant Blue R – 250 and the protein size was determined using molecular weight standards PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (Thermo Fisher Scientific, MA, USA).

**Kinetic characterization of CDH**

Kinetic properties of CDH variants were determined at 20 °C in 0.1 M sodium acetate buffer pH 4.5 with 0.3 mM DCIP, using lactose and cellobiose as substrates in range from 0.2 mM to 5 mM and from 1 µM to 200 µM, respectively. Results were fitted into Michaelis–Menten hyperbola using Graph Pad Prism 6. Calculation of $k_{\text{cat}}$ was done by using protein concentration determined by measuring absorbance at 280 nm and using published molar extinction coefficient for CDH from *Phanerochaete chrysosporium* at 280 nm of $\varepsilon_{280\text{nm}}=217$ mM$^{-1}$cm$^{-1}$. pH optimum was determined using 30 mM lactose as substrate and 0.3 mM DCIP in citrate – phosphate buffer in pH range from 2.0 to 9.0. Published DCIP extinction coefficients were used for different pH values.

Temperature stability of obtained mutant proteins was established by incubating the enzyme at specified temperatures from 25 to 90 ºC for 15 minutes. The incubation was stopped by transferring the enzyme to the ice, and afterwards residual activity of the enzyme was measured using DCIP solution (30 mM lactose, 0.3 mM DCIP in 0.1 M Na – acetate buffer pH 4.5) at 20 °C.

**RESULTS AND DISCUSSION**

**Cloning CDH in pPICZαA vector**

The genes encoding mutant proteins of CDH (tm, H5 and H9) found during directed evolution of CDH in an immobilized form on the surface of *S.cerevisiae* EBY100 yeast cells have been recloned to pPICZαA vector downstream to alcohol oxidase 1 (AOX1) promotor and α-factor protein secretion peptide, Fig. 1.

![Fig. 1. CDH construct in pPICZαA plasmid.](image-url)
pPICZαA vector has been designed for extracellular expression of proteins in *Pichia pastoris* that was enabled by α factor secretion signal peptide. Expression was governed under methanol induction controlled by AOX promotor. After transformation of *Pichia pastoris* KM71H cells with vector, and selection on zeocine containing plates, transformants were tested for CDH production and the best producers were used for large scale fermentation and production of CDH mutants: tmCDH (D20N, A64T, V592M), H5 (D20N, V22A, A64T, V592M) and H9 (D20N, A64T, T84A, A261P, V592M, E674G, N715S), Fig. 2.

Fig. 2. Presentation of the 3D structure of the FAD (PDB accession code 1NAA) and heme (PDB accession code 1D7C) domain of CDH from *P.chrysosporium* with labelled substitutions: tmCDH (D20N, A64T, V592M), H5 (D20N, V22A, A64T, V592M), H9 (D20N, A64T, T84A, A261P, V592M, E674G, N715S). The picture was made with UCSF Chimera 1.13.1.
Expression of recombinant enzymes in Pichia pastoris KM71H strain

Optimal fermentation time for the highest production of CDH was determined by measuring CDH activity in fermentation broth every 24h from start of the methanol induction, up to 8 days, Fig. 3.

![Fig. 3. Production of tm CDH in fermentation broth by Pichia pastoris KM71H](image)

It could be seen that maximal production of CDH of around 950 IU L

It could be seen that maximal production of CDH of around 950 IU L\(^{-1}\) was achieved after 4 days of methanol induction and CDH activity did not change significantly afterwards. Similar optimal expression time, was also obtained previously with CDH expressed in *Pichia* using pPIC9K vector.\(^{23}\) Therefore, for the expression of mutant CDHs we have used methanol induction for 6 days, in order to have maximal expression yield, and to be in the middle of plateau of CDH activity in fermentation broth. Expression rate that we obtained, was higher than previously obtained 221 IU L

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After induction, fermentation broth was collected and concentrated using membranes with cut-off of 50 kDa. Following ultrafiltration, the enzyme was purified by ion-exchange chromatography on DEAE Toyopearl and gel exclusion chromatography on Toyopearl HF55 (see Supplementary Material, Fig. S1-S8). Purification factor, that was defined by ratio of specific activities after and before purification, was between 7.3 and 16 for different mutant proteins, while yield of purification, that was defined as a percentage of enzyme activity obtained after purification, was between 8 and 29 %. Specific activities for purified wt, tm, H5 and H9 mutant CDHs were 20.4, 14.1, 28.1, and 14.5 IU mg\(^{-1}\) respectively (see Supplementary Material, Table S2). In order to confirm purity, obtained CDH enzymes were analysed by SDS electrophoresis, Fig. 4.
Electrophoresis revealed a single protein band in all preparations with a molecular weight of 100 kDa that was very similar to the molecular weight of native CDH from <i>P. chrysosporium</i> of 97 kDa<sup>26</sup> and the same as 100 kDa for previously recombinantly expressed CDH in <i>Pichia pastoris</i>.<sup>23,25,26</sup> Higher molecular weight of expressed heterologous proteins in <i>Pichia</i> compared to the native ones, is a result of higher glycosylation level. After confirmation of purity, enzymatic kinetic constants both for lactose and cellobiose were determined for all purified proteins by measuring enzyme activity at different substrate concentrations and fitting obtained data directly to Michaelis-Menten equation, Table 1.

![Fig. 4. SDS electrophoresis of purified CDH enzymes. 1) Molecular weight markers, 2) tmCDH, 3) H5 CDH, 4) H9 CDH, 5) wtCDH](image)

<table>
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<th>Lactose</th>
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Mutant proteins produced in <i>Pichia pastoris</i> had increased $K_m$ values compared to the same variants when expressed in an immobilized form on the surface of <i>S. cerevisiae</i> cells as chimeras with Aga2 protein, but H5 and H9 mutant proteins retained higher $k_{cat}$ compared to their parent tm CDH both when in a
soluble and immobilized form. Reason for increased $K_m$ could be changed conformation of CDH proteins when expressed as chimeras with Aga2 protein.

It could be also seen, that all mutant proteins had increased $k_{cat}$ value for lactose using DCIP as an electron acceptor compared to the previously published $k_{cat}$ values of: 8.2 s$^{-1}$ for wild type CDH from P.chrysosporium KCCM 60256 strain recombinantly expressed in Pichia pastoris X-33, 4.7 s$^{-1}$ (calculated from reported $V_{max}$ of 2.84 IU mg$^{-1}$) for CDH from P.chrysosporium RP78 strain recombinantly expressed in P. pastoris KM71 or 13.4 s$^{-1}$ for native CDH isolated from P. chrysosporium K3 strain. It could be also seen, that all mutant proteins had increased $k_{cat}$ value for lactose using DCIP as an electron acceptor compared to the previously published $k_{cat}$ values of: 8.2 s$^{-1}$ for wild type CDH from P.chrysosporium KCCM 60256 strain recombinantly expressed in Pichia pastoris X-33, 4.7 s$^{-1}$ (calculated from reported $V_{max}$ of 2.84 IU mg$^{-1}$) for CDH from P.chrysosporium RP78 strain recombinantly expressed in P. pastoris KM71 or 13.4 s$^{-1}$ for native CDH isolated from P. chrysosporium K3 strain. It could be also seen, that all mutant proteins had increased $k_{cat}$ value for lactose using DCIP as an electron acceptor compared to the previously published $k_{cat}$ values of: 8.2 s$^{-1}$ for wild type CDH from P.chrysosporium KCCM 60256 strain recombinantly expressed in Pichia pastoris X-33, 4.7 s$^{-1}$ (calculated from reported $V_{max}$ of 2.84 IU mg$^{-1}$) for CDH from P.chrysosporium RP78 strain recombinantly expressed in P. pastoris KM71 or 13.4 s$^{-1}$ for native CDH isolated from P. chrysosporium K3 strain.

H5 mutant protein had increased $k_{cat}$ for lactose while H5 and H9 mutant proteins had increased $k_{cat}$ for cellobiose compared to wtCDH. These data showed, that mutant proteins we have obtained, have much higher activities for lactose than previously reported variants of CDH, and that they could be good candidates for use as biocatalysts in production of lactobionic and cellobionic acid.

Specificity constant ($k_{cat}/K_m$) as one of the most important parameters for an enzyme to be used in biosensors was the highest for mutant H9 both for lactose (132 mM$^{-1}$ s$^{-1}$) and cellobiose (3180 mM$^{-1}$ s$^{-1}$). It could be seen that our mutant H9 had much higher specificity constant both for lactose and cellobiose compared to wild type CDH specificity constant that we have obtained (lactose: 10.2 mM$^{-1}$ s$^{-1}$, cellobiose: 177 mM$^{-1}$ s$^{-1}$) and to the previously published specificity constants for wild type CDH from P. chrysosporium KCCM 60256 strain recombinantly expressed in Pichia pastoris X-33 (lactose: 24.1 mM$^{-1}$ s$^{-1}$, cellobiose: 29.9 mM$^{-1}$ s$^{-1}$), CDH from P. chrysosporium RP78 expressed in P. pastoris KM71 (lactose: 4.1 mM$^{-1}$ s$^{-1}$, cellobiose: 60 mM$^{-1}$ s$^{-1}$) and for native CDH isolated from P. chrysosporium K3 (lactose:12 mM$^{-1}$ s$^{-1}$, cellobiose: 140 mM$^{-1}$ s$^{-1}$). Therefore, H9 mutant is a very good candidate for use in biosensors with increased sensitivity for both lactose and cellobiose.

pH optimum for all mutant CDHs did not change significantly and was between 4.5 and 5.5, Fig. 5.

Obtained pH optimum was similar to previously published values for pH optimum between 4 and 6 for native and recombinant CDHs.

Temperature stability was slightly different between mutant proteins with the tm CDH being the most stable, Fig. 6.

Since H5 and H9 mutant proteins had higher activity and were derived from tm CDH parent during directed evolution experiments, lower thermostability of H5 and H9 compared to tm CDH was expected, and in an agreement with
previous findings that increased activity of mutant offspring usually comes at the expenses for stability.\textsuperscript{28}

In the literature was reported that native CDH retained 50\% of activity after 15 min of incubation at 60 °C\textsuperscript{29}, while our wild type CDH retained 75 \% of activity. At the same time H5 and H9 mutant proteins showed decreased thermostability with the exception of tm CDH that was retaining 40 \% of activity after 15 min incubation at 60 °C.\textsuperscript{29}

![Fig. 5. pH optimum of wt and mutant CDHs](image)

![Fig. 6. Temperature stability of wt and mutant CDHs. Enzyme was incubated for 15 min at specified temperature](image)

CONCLUSION

Three CDH mutant proteins (tm, H5 and H9) found during directed evolution of the enzyme on the surface of yeast cells were recloned from pCTCON to pPICZ\textalpha vector downstream of α-factor for extracellular expression
in *Pichia pastoris* KM71H under methanol induction. After 6 days of fermentation, recombinant enzymes were concentrated by ultrafiltration and purified using ion-exchange and gel filtration chromatography. Purity of the mutant proteins was confirmed by SDS electrophoresis and molecular weight determined to be 100 kDa. Kinetic constants for all three CDH enzymes confirmed that obtained purified mutant enzymes have higher activities for both lactose and cellobiose compared to previously described CDH enzyme preparations. High catalytic constant of 43.5 s\(^{-1}\) for H5 mutant makes it a very promising biocatalyst for production of lactobionic acid, while H9 high specificity constant of 132 mM\(^{-1}\) s\(^{-1}\) makes it a very good biocatalyst for use in biosensors. Developed expression system for new CDH mutant proteins that we have described in this article can be of importance for lactobionic acid production, and design of more sensitive biosensors for lactose and cellobiose.

SUPPLEMENTARY MATERIAL

Supplementary Material are available electronically from [http://www.shd.org.rs/JSCS/](http://www.shd.org.rs/JSCS/), or from the corresponding author on request.

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oblicima CDH proteina iz *Phanerochaete chrysosporium*, oblici prikazani u ovom radu imaju veću aktivnost i specifičnost što povезано са високом експресијом протеина у *Pichia* их чини добrim кандидатима за употребу у биотехнологији за производњу лактобионске киселине и биосензора.

(Примљено 20. марта; ревидирано 7. јуна; прихваћено 7. јуна 2019)

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SUPPLEMENTARY MATERIAL TO
Expression, purification and characterization of cellobiose dehydrogenase mutants from Phanerochaete chrysosporium in Pichia pastoris KM71H strain

ANA MARIJA J. BALAŽ¹, MARIJA B. BLAŽIĆ¹, NIKOLINA POPOVIĆ², OLIVERA L. PRODANOVIĆ³, RALUCA V. OSTAFE⁴, RAINER FISCHER⁵ and RAĐIVOJE M. PRODANOVIĆ²

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Table S1. Primers used for creation of triple mutant and error prone library mutants using wtCDH – pPICZαA vector as template

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Fig. S1. Ion-exchange chromatography of tm CDH

Fig. S2. Ion-exchange chromatography of H5 CDH
Fig. S3. Ion-exchange chromatography of H9 CDH

Fig. S4. Ion-exchange chromatography of wt CDH
Fig. S5. Gel filtration of tm CDH

Fig. S6. Gel filtration of H5 CDH
Fig. S7. Gel filtration of H9 CDH

Fig. S8. Gel filtration of wt CDH

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