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Original scientific paper

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, the CDHs were purified by ultrafiltration, ion-exchange chromatography and gel filtration. Sodium dodecyl sulfate electrophoresis confirmed the purity and presence of a single protein band at a molecular weight of 100 kDa. Kinetic characterization showed that the H5 mutant had the highest catalytic constant of 43.5 s^{-1} for lactose, while the mutant H9 showed the highest specificity constant for lactose of 132 mM^{-1} s^{-1}. All three mutant proteins did not change the pH optimum that was between 4.5 and 5.5. Compared to the 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 *P. pastoris*, makes them good candidates for use in biotechnology for lactobionic acid production and biosensor manufacture.

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).[1] Regardless of the fact that the biological role of CDH is not fully understood, it is known that CDH participates in the oxidation of *β*-1,4-linked disaccharides and oligosaccharides, such as cellobiose and lactose.[2] Cellobiose dehydrogenase is a monomeric protein that contains two domains, *i.e.*, a catalytically active flavin domain and heme *b* containing a cytochrome domain. These two domains are connected with each other *via* a long and flexible linker.[1] Oxidation of substrates, catalysed by CDH, involves the reduction of FAD to $FADH_2$, and the flavin domain is shown to be responsible for electron uptake during the substrate oxidation directly transferring electrons to two-electron acceptors.[3–5] The role of the heme domain has been identified as a significant enhancement of activity towards one-electron acceptors.[6] Since CDH can oxidize both lactose and cellobiose using a wide range of electron acceptors, but not oxygen, it is used in biosensors and biofuel cells,[7,8] for dye removal,[9] bioremediation[10] and lactobionic acid production.[11,12]

Variants of CDH having increased activity would benefit these applications. Directed evolution is often used to generate them *via* iterative rounds of genetic diversification and library screening.[13,14] Directed evolution became one of the most useful and widespread tools in basic and applied biochemistry. Expression systems, mainly used for this purpose, are *Escherichia coli* and *Saccharomyces cerevisiae* due to high transformation efficiency that is needed for creation of large gene libraries.[13,15,16]

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.[17] *P. 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.[18] Therefore, compared to bacterial expression systems such as *E. coli*, *P. pastoris* has become a powerful expression system for recombinant eukaryotic proteins.[19,20]

In this article, three new mutant forms of CDH[21] from *P. chrysosporium*, which were 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 *P. 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 the forward primer *Eco*RI_fp_CBDH (5′-AT**GAATTC**-CAGAGTGCCTCACAGTTTACC-3′) and the reverse primer *Xba*I_rp_CBDH_2 (5′-AT-

TCTAGATCAAGGACCTCCCGCAAG-3′). The following polymerase chain reaction (PCR) protocol with *Taq* polymerase was used for gene amplification (1 cycle – 4 min at 94 °C; 30 cycles – 1 min at 94 °C, 1 min at 55 °C, 2 min 15 s at 72 °C; 1 cycle – 10 min at 72 °C). The obtained PCR products and pPICZαA vector (Invitrogen BV, Groningen, The Netherlands) were both digested with *Eco*RI and *Xba*I 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, the $CaCl_2$ transformation protocol was used.

Construction of cellobiose dehydrogenase variants

Mutant proteins previously discovered 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 material to this paper, Table S-I), and QuickChange lightning site-directed mutagenesis kit (Agilent Technologies). *E. coli* XL10 gold competent cells were transformed using constructs and plasmid DNA was isolated using Macherey–Nagel plasmid DNA kit (Düren, Germany).

Expression of recombinant enzyme in Pichia pastoris *strain KM71H*

Transformation by electroporation of *P. pastoris* strain KM71H (Mut^s, zeocin resistant strain, Invitrogen BV, Groningen, The Netherlands) with constructs was realized using the protocol described in the EasySelect *P. pastoris* transformation kit. After transformation, expression of single colonies was performed according to the EasySelect *P. pastoris* transformation kit, using buffered minimal glycerol (BMGH) and buffered minimal methanol (BMMH) media for growth and expression, respectively. Selection of constructs was realized by the addition of 100 μg mL^{-1} zeocin to the growth media. Growth of the cells was performed in BMGH media at 28 °C in an incubator under shaking (250 rpm) until the OD_{600} of the culture was between 2 and 6. The cells were separated from the growth media by centrifugation for 10 min at 3000 rpm and further resuspended in BMMH media using $1/5^{th}$ of the volume of the growth culture. Induction was performed for 6 days by adding methanol every 24 h to a final concentration of 0.5 %. Cell free fermentation liquid, including the enzyme was concentrated on a Vivaflow 50 ultrafiltration cassette with a cut-off of 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; λ_{ex} 520 nm; ε_{520} = 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 min under the above-mentioned conditions.

Purification of CDH

Ion exchange chromatography was performed on 10 mL Toyopearl DEAE ion exchange column. For equilibration, 10 mM sodium phosphate buffer pH 6.0 was used, elution was performed using a linear gradient from 0 to 50 % concentration of sodium chloride (1 M NaCl) in the same buffer. The 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 the CDHs, which were dialyzed after ion exchange chromatography *versus* 20 mM sodium phosphate buffer pH 6.0. Purification was performed 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.[22] Protein bands were visualized using Coomassie Brilliant Blue R-250 and the protein size was determined using molecular weight standards PageRuler^{TM} Plus prestained protein ladder, 10 to 250 kDa (Thermo Fisher Scientific, MA, USA).

Kinetic characterization of CDH

Kinetic properties of the 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 the range from 0.2 mM to 5 mM and from 1 to 200 μM, respectively. The results were fitted into the Michaelis–Menten hyperbola using GraphPad Prism 6. The value of k_{cat} was calculated using a protein concentration determined by measuring the absorbance at 280 nm and using the published molar extinction coefficient for CDH from *P. chrysosporium* at 280 nm of ε_{280nm} = 217 mM^{-1} cm^{-1}.[23] The pH optimum was determined using 30 mM lactose as substrate and 0.3 mM DCIP in citrate–phosphate buffer in the pH range from 2.0 to 9.0. Published DCIP extinction coefficients were used for different pH values.[24] The temperature stability of obtained mutant proteins was established by incubating the enzyme at specified temperatures from 25 to 90 °C for 15 min. The incubation was stopped by transferring the enzyme to ice, and afterwards, the residual activity of the enzyme was measured using a 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)[21] 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.

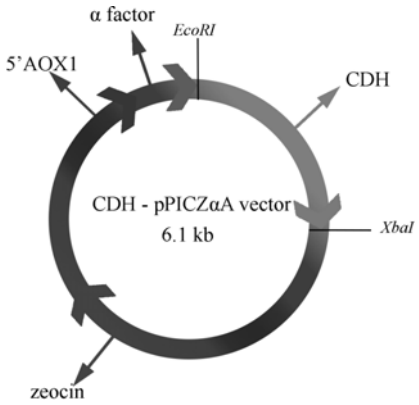

Fig. 1. CDH construct in pPICZαA plasmid.

The pPICZαA vector was designed for extracellular expression of proteins in *Pichia pastoris* that was enabled by *α*-factor secretion signal peptide. The expression was governed under methanol induction controlled by AOX promotor. After transformation of *P. pastoris* KM71H cells with the vector, and selection on zeocin containing plates, the 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.

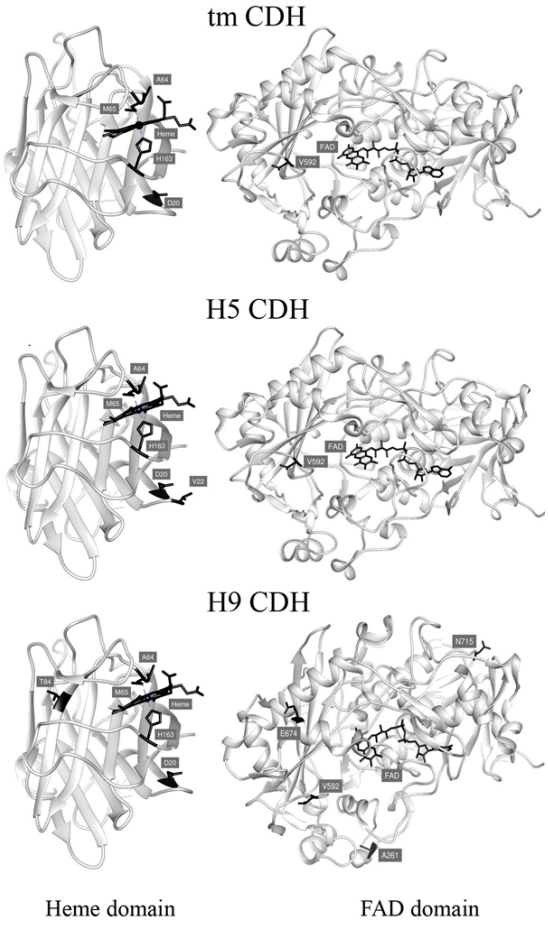

Fig. 2. Presentation of the 3D structure of the FAD (PDB accession code 1NAA) and heme (PDB accession code 1D7C) domain of CDH from *P. chrysosporium* with labelled substitutions: tmCDH (D20N, A64T, V592M), H5 (D20N, V22A, A64T, V592M), H9 (D20N, A64T, T84A, A261P, V592M, E674G, N715S). The picture was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

Expression of recombinant enzymes in P. pastoris *KM71H strain*

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

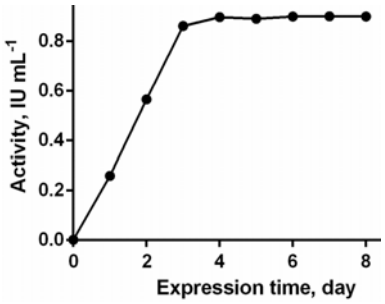

Fig. 3. Production of tm CDH in fermentation broth by *P. pastoris* KM71H.

It could be seen that the maximal production of CDH of around 950 IU L^{-1} was achieved 4 days after methanol induction and the CDH activity did not change significantly thereafter. A similar optimal expression time was obtained previously with CDH expressed in *Pichia* using the pPIC9K vector.[23] Therefore, for the expression of mutant CDHs, methanol induction for 6 days was used in order to have maximal expression yield, and to be in the middle of plateau of CDH activity in fermentation broth. The obtained expression rate was higher than the previously obtained rate of 221 IU L^{-1} for wild type CDH using the same *P. pastoris* KM71H strain.[25] The reason for this could be that a transformant had been found with a multiple integration of plasmid construct into the chromosome.

After induction, the fermentation broth was collected and concentrated using membranes with a cut-off of 50 kDa. Following ultrafiltration, the enzyme was purified by ion-exchange chromatography (see Supplementary Material, Figs. S-1––S-8). The purification factor, which was defined by the ratio of the specific activities after and before purification, was between 7.3 and 16 for different mutant proteins, while the yield of purification, which was defined as the percentage of enzyme activity obtained after purification, was between 8 and 29 %. The 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 S-II). In order to confirm the purity, the 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, which was very similar to the molecular weight of native CDH from *P. chrysosporium* of 97 kDa[26] and the same as 100 kDa for previously recombinantly expressed CDH in *P. pastoris*.[23,25,26] Higher molecular weight of expressed heterologous proteins in *P. pastoris* compared to the native ones is the result of a higher glycosylation level. After confirmation of the purity, the enzymatic kinetic constants for both lactose and cellobiose were determined for all purified proteins by measuring enzyme activity at different substrate concentrations and fitting the obtained data directly to the Michaelis–Menten equation, Table I.

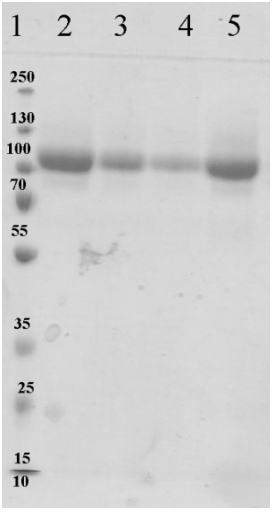

Fig. 4. SDS electrophoresis of purified CDH enzymes. 1) Molecular weight markers, 2) tm CDH, 3) H5 CDH, 4) H9 CDH and 5) wt CDH.

TABLE I. Kinetic constants of purified CDH proteins for lactose and cellobiose with DCIP as the electron acceptor

Protein	Lactose			Cellobiose		
	K_m / mM	k_{cat} / s^{-1}	(k_{cat}/K_m) / mM^{-1} s^{-1}	K_m / μM	k_{cat} / s^{-1}	(k_{cat}/K_m) / mM^{-1} s^{-1}
tm CDH	1.00	19.9	19.8	79.6	28.4	358
H5 CDH	3.25	43.5	13.4	140	34.5	247
H9 CDH	0.17	22.5	132	10.6	33.7	3180
wt CDH	3.49	35.7	10.2	168	29.8	177

Mutant proteins produced in *P. pastoris* had increased K_m values compared to the same variants when expressed in an immobilized form on the surface of *S. cerevisiae* 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.[21] 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 *P. pastoris* X-33,[26] 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[25] or 13.4 s^{-1} for native CDH isolated from *P. chrysosporium* K3 strain.[27] The k_{cat} values for cellobiose for all mutant proteins were also higher than previously published k_{cat} values, such as 6.27 s^{-1} for CDH from *P. chrysosporium* KCCM 60256 strain recombinantly expressed in *P. pastoris* X-33,[26] 3.6 s^{-1} (calculated from reported V_{max} of 2.17 IU mg^{-1}) for CDH from *P. chrysosporium* RP78 strain recombinantly expressed

in *P. pastoris* KM71[25] or 15.7 s^{-1} for native CDH isolated from *P. chrysosporium* K3 strain.[27] The H5 mutant protein had increased k_{cat} for lactose while H5 and H9 mutant proteins had increased k_{cat} for cellobiose compared to wt CDH. These data show that the presently obtained mutant proteins have much higher activities for lactose than the previously reported variants of CDH, and that they could be good candidates for use as biocatalysts in the production of lactobionic and cellobionic acid.[11]

The specificity constant (k_{cat}/K_m) as one of the most important parameters for an enzyme to be used in biosensors[8] was the highest for the mutant H9 for both lactose (132 mM^{-1} s^{-1}) and cellobiose (3180 mM^{-1} s^{-1}). It could be seen that the mutant H9 had a much higher specificity constant for both lactose and cellobiose compared to the specificity constant for the obtained wild type CDH (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 *P. pastoris* X-33 (lactose: 24.1 mM^{-1} s^{-1}, cellobiose: 29.9 mM^{-1} s^{-1}),[26] CDH from *P. chrysosporium* RP78 expressed in *P. pastoris* KM71 (lactose: 4.1 mM^{-1} s^{-1}, cellobiose: 60 mM^{-1} s^{-1})[25] and for native CDH isolated from *P. chrysosporium* K3 (lactose:12 mM^{-1} s^{-1}, cellobiose: 140 mM^{-1} s^{-1}).[27] Therefore, the H9 mutant is a very good candidate for use in biosensors with increased sensitivity for both lactose and cellobiose.

The optimum pH for all mutant CDHs did not change significantly and was between 4.5 and 5.5, Fig. 5. The obtained pH optimum was similar to previously published values for the pH optimum between 4 and 6 for native and recombinant CDHs.[26]

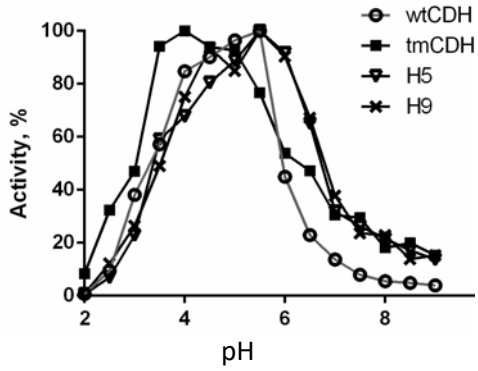

Fig. 5. pH optimum of wt and mutant CDHs.

Temperature stability was slightly different between the mutant proteins with tm CDH being the most stable, Fig. 6. Since H5 and H9 mutant proteins had higher activity and were derived from a tm CDH parent during directed evolution experiments, lower thermostability of H5 and H9 compared to tm CDH was exp-

ected, and is in agreement with previous findings that increased activity of mutant offsprings usually comes at the expense of stability.[28]

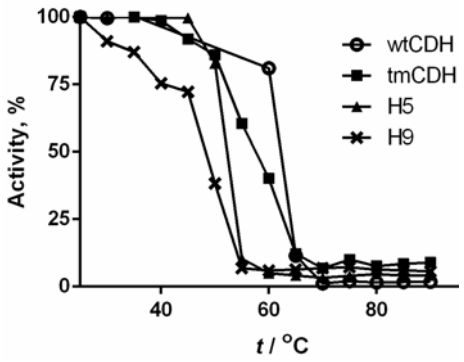

Fig. 6. Temperature stability of wt and mutant CDHs. The enzymes were incubated for 15 min at the specified temperature.

In the literature, it was reported that native CDH retained 50 % of its activity after 15 min of incubation at 60 °C,[29] while the present wild type CDH retained 75 % of its activity. At the same time, H5 and H9 mutant proteins showed decreased thermostability with the exception of tm CDH that retained 40 % of its activity after 15 min incubation at 60 °C.[29]

CONCLUSIONS

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

SUPPLEMENTARY MATERIAL

Additional data are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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И З В О Д

ЕКСПРЕСИЈА, ПРЕЧИШЋАВАЊЕ И КАРАКТЕРИЗАЦИЈА МУТАНАТА ЦЕЛОБИОЗА-ДЕХИДРОГЕНАЗЕ ИЗ *Phanerochaete chrysosporium* У *Pichia pastoris* KM71H СОЈУ

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У циљу употребе у биосензорима и биогоривним ћелијама, успостављена је производња растворних облика целобиоза дехидрогеназе (CDH) претходно еволуираних на површини квашчевих ћелија *S. cerevisiae*. У ту сврху су мутанти CDH, tm (D20N, A64T, V592M), H5 (D20N, V22A, A64T, V592M) и H9 (D20N, A64T, T84A, A261P, V592M, E674G, N715S) клонирани у pPICZα плазмид и трансформисани у *Pichia pastoris* KM71H соj за високу експресију у растворном облику и кинетичку карактеризацију. После 6 дана експресије под индукцијом метанолом, мутанти су пречишћени ултрафилтрацијом, јоноизмењивачком хроматографијом и гел-филтрацијом. SDS електрофореза је потврдила чистоћу уз присуство једне протеинске траке молекулске масе од 100 kDa. Кинетичка карактеризација је показала да H5 мутирани протеин поседује највећу каталитичку константу од 43,5 s^{-1} за лактозу, док је H9 имао највећу константу специфичности за лактозу од 132 mM^{-1} s^{-1}. Сва три мутирана протеина су имала неизмењен pH оптимум који је био у опсегу од 4,5 до 5,5. У поређењу са претходно добијеним природним и мутантним облицима CDH протеина из *Phanerochaete chrysosporium*, облици приказани у овом раду имају већу активност и специфичност, што их, повезано са високом експресијом протеина у *P. Pastoris*, чини добрим кандидатима за употребу у биотехнологији за производњу лактобионске киселине и биосензора.

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