


Article

# Directed Evolution of Cellobiose Dehydrogenase on the Surface of Yeast Cells Using Resazurin-Based Fluorescent Assay

Marija Blažić<sup>1</sup>, Ana Marija Balaž<sup>1</sup>, Olivera Prodanović<sup>2</sup>, Nikolina Popović<sup>3</sup>, Raluca Ostafe<sup>4</sup>, Rainer Fischer<sup>5,6</sup> and Radivoje Prodanović<sup>3,\*</sup> 

<sup>1</sup> Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Njegoševa 2, 11000 Belgrade, Serbia; marijabbl@gmail.com (M.B.); anam@chem.bg.ac.rs (A.M.B.)

<sup>2</sup> Institute for Multidisciplinary Studies, University of Belgrade, Kneza Višeslava 1, 11030 Belgrade, Serbia; oliverap@imsi.rs

<sup>3</sup> Faculty of Chemistry, University of Belgrade, Studentski trg 12, 11000 Belgrade, Serbia; nikolina@chem.bg.ac.rs

<sup>4</sup> Molecular Evolution Protein Engineering and Production facility (MEPEP), Purdue University, 207 S. Martin Jischke Dr., West Lafayette, IN 47907, USA; rostafe@purdue.edu

<sup>5</sup> Indiana Bioscience Research Institute, Single Cell Analytics Center, 1345 W. 16th St. Suite 300, Indianapolis, IN 46202, USA; rfischer@indianabiosciences.org

<sup>6</sup> Institute of Molecular Biotechnology, RWTH Aachen University Worringerweg 1, 52074 Aachen, Germany

\* Correspondence: rprodano@chem.bg.ac.rs; Tel.: +381-11-333-6660

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**Featured Application:** Developed fluorescent assay and expression system can be used for obtaining improved cellobiose dehydrogenase whole cell biocatalysts for lactobionic acid production and building of biosensors and biofuel cells.

**Abstract:** Cellobiose dehydrogenase (CDH) from *Phanerochaete chrysosporium* can be used in lactobionic acid production, biosensor for lactose, biofuel cells, lignocellulose degradation, and wound-healing applications. To make it a better biocatalyst, CDH with higher activity in an immobilized form is desirable. For this purpose, CDH was expressed for the first time on the surface of *S. cerevisiae* EBY100 cells in an active form as a triple mutant tmCDH (D20N, A64T, V592M) and evolved further for higher activity using resazurin-based fluorescent assay. In order to decrease blank reaction of resazurin with yeast cells and to have linear correlation between enzyme activity on the cell surface and fluorescence signal, the assay was optimized with respect to resazurin concentration (0.1 mM), substrate concentration (10 mM lactose and 0.08 mM cellobiose), and pH (6.0). Using optimized assay an error prone PCR gene library of tmCDH was screened. Two mutants with 5 (H5) and 7 mutations (H9) were found having two times higher activity than the parent tmCDH enzyme that already had improved activity compared to wild type CDH whose activity could not be detected on the surface of yeast cells.

**Keywords:** cellobiose dehydrogenase; resazurin; fluorescent assay; flow cytometry; yeast surface display

## 1. Introduction

The cellobiose dehydrogenase (CDH; EC 1.1.99.18) is a monomeric, an extracellular hemoflavoprotein with two domains, flavin and heme domain, connected via flexible peptide linker [1]. CDH catalyzes the oxidation of the reducing end of cellobiose and other  $\beta$ -1,4-linked disaccharides

with a  $\beta$ -glucose moiety, to their corresponding lactones [2]. Different electron acceptors can reoxidize the reduced enzyme, such as 2,6-dichloro-indophenol (DCIP), or metal ions, for example Fe(III) or even oxygen, albeit the latter is a very poor electron acceptor [2]. Although diverse of white-rot and other fungi have been reported to secrete CDH, and a lot have been purified and characterized, the *in vivo* function of CDH is not sufficiently understood [3]. It has been suggested that CDH plays a role in lignocellulosic biomass hydrolysis, such as relief of product inhibition of cellulases by oxidation of cellobiose, generation of peroxide for inhibition of opposing microbes, and regeneration of redox metal ions (such as  $\text{Fe}^{2+}$ ) to sustain the Fenton-type chemistry that produces hydroxyl radicals who can modify polysaccharides causing partial hydrolysis and swelling of insoluble fibers which helps breakdown of lignocellulose by glycoside hydrolase [1–3]. Due to its properties, CDH has been applied in biosensors for detection of lactose, in enzymatic biofuel cells as anode catalyst [4,5], for lactobionic acid production [6], dye removal [7], and bioremediation [1].

Improvement of activity and stability of CDH would benefit all of these applications and directed evolution based on random introduction of mutations resulting in a protein library, where mutants having desired properties can be screened is used very often [8]. In order to make screening process more efficient during directed evolution, various expression systems that keep connection between genotype and phenotype are developed, such as phage [9], bacterial [10], yeast [11], and mammalian cell display [12]. The advantage of yeast display is the existence of eukaryotic protein production machinery that is essential for post-translational modifications of expressed proteins [8] and compatibility with flow cytometric analysis, which allows quantitative measurements of equilibrium binding constants, dissociation kinetics, and specificity of the displayed proteins without requisite of soluble protein expression and purification [13]. Screening methods usually involve phenotypic selection based on colorimetric or fluorometric assays in microtiter plates (MTPs) [14] and solid-phase screening on agar plates or membranes [15]. Regardless, during colony screening on solid-phase, or individual clones in MTPs, usually  $10^3$ – $10^5$  clones and seldom more than  $10^6$  can be screened with sophisticated automated systems [16]. One of emerging ultra-high-throughput screening systems to this day is fluorescence-activated cell sorting (FACS) which can routinely sort  $>10^7$  cells/h, if there is an appropriate fluorescence assay [17,18].

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) is a tetrazolium-based, non-toxic, water-soluble dye that can be reduced by cellular metabolic activity to the highly fluorescent resorufin [19]. Resazurin also changes color from blue to pink upon reduction [20]. This reduction causes the loss of an oxygen atom loosely bound to the nitrogen of the phenoxazine nucleus. Atmospheric oxygen cannot reverse this change and it is mostly independent of both oxygen content and reduction potential [19]. Resazurin has been used to examine the viability of microorganisms (non-destructively) [21], for biofilm quantification [22], and anti-biofilm disinfectant testing of clinical devices [23]. Several intracellular oxidoreductases, for instance diaphorases and NADH dehydrogenase, were reported to be responsible for the reduction of resazurin to resorufin during cell viability assay [24].

In this study, fluorescent assay was developed for detection of CDH expressed on the surface of yeast cells, using resazurin as an electron acceptor. For the first time CDH was expressed in an active form on the surface of yeast cells as a triple mutant (tmCDH), while wild type CDH activity could not be detected. Fluorescent assay was optimized and used for directed evolution of tmCDH on the surface of yeast cells.

## 2. Materials and Methods

### 2.1. Materials and Strains

*S. cerevisiae* strain EBY100 and pCTCON2 vector were kindly provided by Prof. Dane Wittrup [25]. Protocols and media for EBY100 were described in Invitrogen (Carlsbad, CA, USA) manual. All chemicals used were of analytical reagent grade or higher and obtained from Sigma-Aldrich

Chemie GmbH, Taufkirchen, Germany. Resazurin sodium salt was obtained from Invitrogen, Carlsbad, CA, USA. Enzymes and nucleotides were purchased from Fermentas, Waltham, MA, USA, if not stated otherwise. Soluble CDH was wild type CDH expressed and purified from *Pichia pastoris* KM71 using published procedure [26].

## 2.2. Enzymatic Assays

Fluorescence measurements were obtained using FluoroMax-4 (Horiba Scientific, Kyoto, Japan) and Tecan Infinity 200 pro fluorimeter (Tecan Group Ltd, Mannedorf, Switzerland). Excitation wavelength was set on 540 nm with 590 nm as emission wavelength. Hellma fluorescence cuvettes (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) were used for development of the assay. Greiner cellstar 96-well plate (Greiner Bio One GmbH, Frickenhausen, Germany) was used for library screening.

Fluorescence-based assay was optimized in 1.4 mL Hellma fluorescence cuvette, using FluoroMax-4 in final reaction volume of 1 mL. Fluorescence-based assay for cells was performed in 96-well FLUOTRAC 600 clear, flat bottom, black plate using Tecan Infinity 200 pro fluorimeter in a total reaction volume of 200  $\mu$ L for 10 min. A mix of resazurin (0.1 mM; 7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt;  $\lambda_{ex}$ 540 nm;  $\lambda_{em}$ 590 nm) and lactose (10 mM) or cellobiose (8 mM) in sodium phosphate buffer (0.1 M; pH 5.5) was used for cuvette and MTP measurements, if not stated otherwise. All measurements were done in triplicate.

Spectrophotometric assay for measuring CDH activity was done using 0.3 mM DCIP (dichlorophenol-indophenol), 30 mM lactose, and 0.1 M sodium acetate pH 4.5, at 25 °C in total reaction volume of 1 mL. Reaction was started by adding purified CDH and reduction of DCIP was followed by measuring absorbance decrease at 520 nm ( $\epsilon_{520} = 6.80 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ) [27]. One unit of enzyme activity is defined as the amount of enzyme that reduces 1  $\mu$ mol of DCIP per minute under the abovementioned conditions.

## 2.3. Cloning of *cdh* Gene in pCTCON2

A thermal cycler (Mastercycler personal; Eppendorf, Hamburg, Germany) and thin-wall PCR tubes (Multiply-Pro tubes; 0.2 mL; Sarstedt, Germany) were used for all PCRs. Volume for PCR was 50  $\mu$ L. The concentration of DNA was quantified using a NanoDrop photometer (Thermo Fischer Scientific, Waltham, MA, USA).

The *Pharenochaete chrysosporium* *cdh* gene (U46081.1) for mature enzyme was synthesized and cloned in pUC57 plasmid by GenScript, Piscataway, NJ, USA. The PCR reaction (94 °C for 4 min, 1 cycle; 94 °C 1 min/50 °C 1 min/72 °C for 2.45 min, 30 cycles; 72 °C for 10 min, 1 cycle) contained *Pfu* DNA polymerase (0.04 u/ $\mu$ L), dNTP mix (0.2 mM), forward primer (0.5  $\mu$ M)(5'-ATGCTAGCCAGAGTGCCTCACAGTTTACC-3'), reverse primer (0.5  $\mu$ M)(5'-ATGGATCCTCAAGGACCTCCCGCAAG-3'), template CDH-pUC57 (30 ng). The purified fragments were restriction digested with *Nhe*I and *Bam*HI enzyme and ligated in the pCTCON2 vector. The ligated gene was used to transform *Echerichia coli* DH5 $\alpha$ . Gene length was checked with DNA electrophoresis and EBY100 yeast cells were transformed with CDH-pCTCON2 construct.

## 2.4. Library Construction

Plate Thermo-Shaker thermostat PST-60HL (Biosan, Riga, Latvia) was used for library growth in microtiter plates, and for flask fermentation a thermos-shaker Incubator Unimax 1010 (Heidolph GmbH, Schwabach, Germany) was used.

The PCR reaction (94 °C for 4 min, 1 cycle; 94 °C 1 min/50 °C 1 min/72 °C for 2.45 min, 30 cycles; 72 °C for 10 min, 1 cycle) contained *Taq* DNA polymerase (0.04 u/ $\mu$ L), dNTP mix (0.2 mM), 0.05 mM Mn<sup>2+</sup>, forward primer (0.5  $\mu$ M)(5'-ATGCTAGCCAGAGTGCCTCACAGTTTACC-3'), reverse primer (0.5  $\mu$ M)(5'-ATGGATCCTCAAGGACCTCCCGCAAG-3'), template CDH-pCTCON2 (30 ng). The purified fragments were restriction digested with *Nhe*I and *Bam*HI enzyme and ligated in the pCTCON2 vector.

The ligation mixture was transformed to DH5 $\alpha$  *E. coli* cells. Size of the library in *E. coli* was determined by plating one tenth of the transformed *E. coli* DH5 $\alpha$  cells and counting number of colonies on agar plates. The rest of the transformed *E. coli* cells was grown in liquid culture and mini prep plasmid DNA of gene library mixture was prepared. The total number of the *E. coli* transformants in the library was determined to be around 20,000. Afterwards, the gene library mixture was transformed into *S. cerevisiae* EBY100 competent cells, using the Gietz protocol [28]. Transformed yeast cells were grown in YNB-CAA glucose liquid culture and one tenth of the yeast library was plated on YNB-CAA glucose agar plates for library size determination. The size of yeast library was around 10,000 and CDH positive cells in the library were detected using the YNB-CAA galactose agar plates with 1 mM dichlorophenolindophenol (DCIP). Around 8000 colonies were prescreened using DCIP agar plate assay and around 2000 colonies that were positive for CDH activity were transferred to MTP for enzyme expression and screening.

Transferred cells were first grown in sterile MTPs (24 h, 30 °C, 200 rpm) in liquid YNB-CAA glucose media to saturation, centrifuged and resuspended in YNB-CAA galactose media (0.8 O.D. 600 nm) and incubated for 24 h (25 °C, 200 rpm) for induction of enzyme expression. Aliquots of cells suspension from MTPs were tested for CDH enzyme activity using resazurin assay. Mutants having higher activity than the tmCDH were kinetically characterized, for  $K_m$  value.

### 2.5. Flow Cytometry Assay

Flow cytometry analysis was performed using a BD FACSDiVa flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ, USA). For emulsification, a MICCRA D-1 dispenser (ART Prozess- & Labortechnik GmbH & Co. KG, Müllheim, Germany) was used.

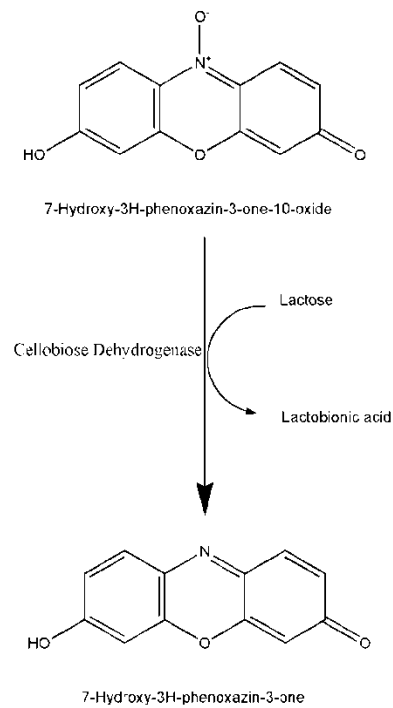
In vitro compartmentalization of *S. cerevisiae* EBY100 cells was done by modified protocol of Aharoni et al. [17]. After 24 h of expression in Gal media, yeast cells were washed and together with the components required for the reaction (25  $\mu$ L), were added to a 250  $\mu$ L ice cold oil phase of 1.5% (v/v) ABIL EM 90 (Evonik Tego Chemie GmbH, Essen, Germany) in Light Mineral Oil. The two phases were homogenized on ice in a 2-mL round-bottom cryotube (3 min, 8000 rpm using a MICCRA D1 homogenizer. The second water phase (250  $\mu$ L) comprising 1.5% (w/v) carboxy-methylcellulose sodium salt (CMC), 1% (v/v) Triton X100 in phosphate-buffered saline (PBS) was added to the primary emulsion and homogenized on ice (3 min, 7000 rpm). Emulsions were diluted in PBS and analyzed using a BD FACSDiVa flow cytometer. The trigger parameter was set to forward scattering. The analysis rate was around 8000 events/s. We used 568 nm laser for excitation and 630/22 nm bandpass filter for emission detection.

## 3. Results

### 3.1. Development of Fluorescent Assay

In order to develop screening system based on fluorescence for detection of CDH activity on the surface of yeast cells we tested resazurin as a substrate for soluble CDH enzyme. Resazurin was used previously as a probe for assessing cell viability during which it was reduced to fluorescent resorufin [24] and in this article it was tried as an electron acceptor in CDH-catalyzed oxidation of lactose, Scheme 1.

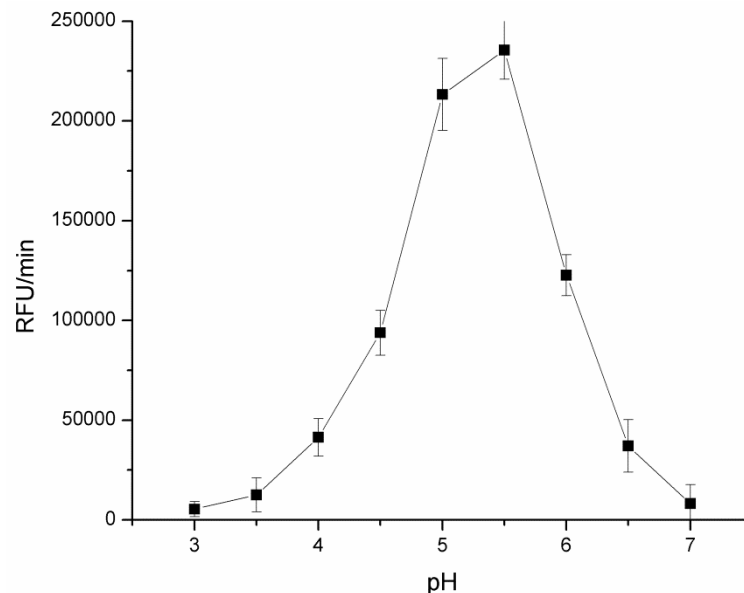
In order to have the most sensitive detection and linear correlation between enzyme activity and fluorescence signal, we optimized assay conditions with respect to the pH, resazurin and substrate concentrations. The assay was optimized for detection of CDH expressed on the surface of yeast cells.



**Scheme 1.** Mechanism of the resazurin-based fluorescent assay for cellobiose dehydrogenase (CDH).

### 3.2. pH Optimization

Since resorufin fluorescence is higher at alkaline pH values [29] due to an acidic pH optimum of CDH [1] we tested influence of pH on fluorescence signal of resorufin produced by soluble CDH enzyme, Figure 1.



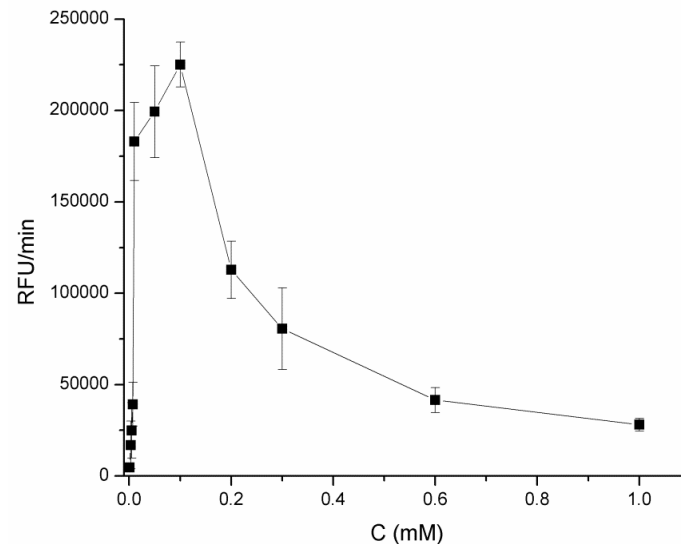
**Figure 1.** Dependence of fluorescence signal (RFU/min) on pH in 0.1 M sodium phosphate buffer with 10 mM lactose, 0.1 mM resazurin and 0.29 IU/mL of CDH enzyme. Fluorescence measured at ex/em 540/590 nm.

Maximal fluorescence signal was detected around pH 5.0–6.0 which is higher than reported pH optimum of the enzyme at 4.5. This was result of higher fluorescence emission of resorufin at

more alkaline pH values. Therefore, in further optimization experiments we used pH 5.5 for CDH activity measurements.

### 3.2.1. Optimization of Resazurin Concentration

Since the optimal pH for the assay was obtained, we tested influence of different resazurin concentrations (0.01–1 mM) on fluorescence signal (Figure 2).

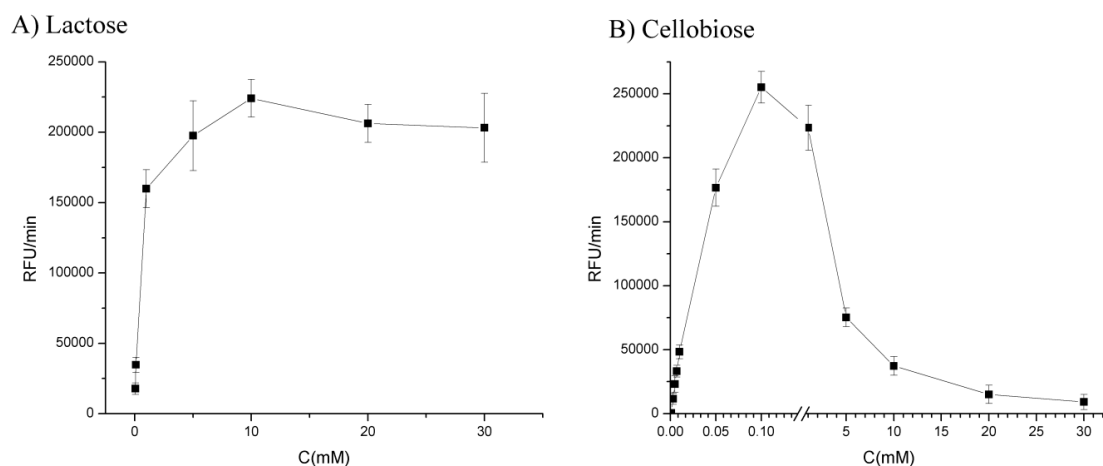


**Figure 2.** Dependence of fluorescence signal (RFU/min) on resazurin concentration in 0.1 M sodium phosphate buffer pH 5.5 with 10 mM lactose and 0.29 IU/mL of CDH enzyme. Fluorescence measured at ex/em 540/590 nm.

The highest enzymatic activity and fluorescence signal was obtained for 0.1 mM resazurin concentration and that concentration was used for further experiments.

### 3.2.2. Optimization of Substrate Concentration

In this assay we used lactose and cellobiose as substrates. Concentrations of lactose varied from 0.1 mM till 30 mM, and cellobiose from 0.001 mM till 0.1 mM (Figure 3).



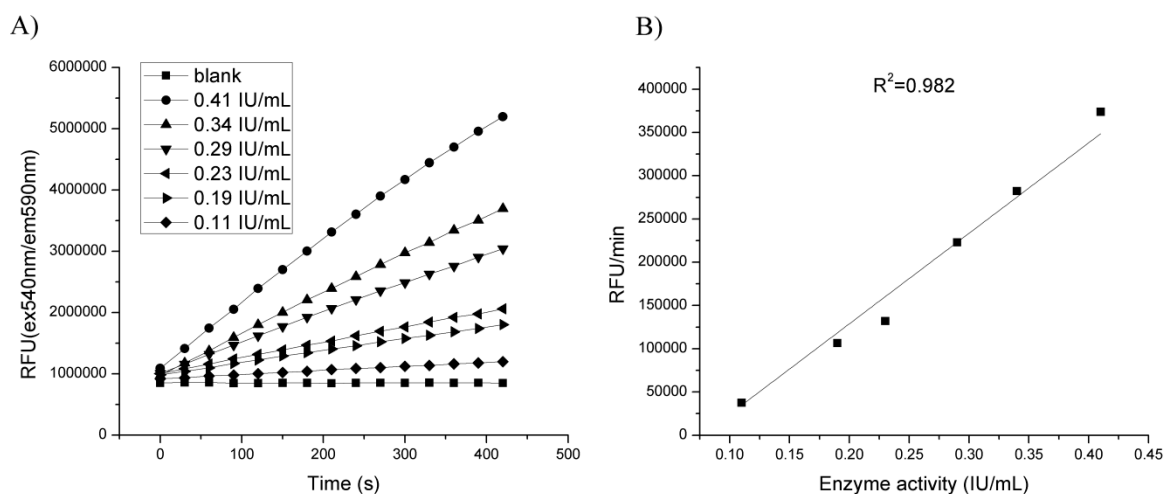
**Figure 3.** Dependence of fluorescence signal (RFU/min) on (A) lactose (B) cellobiose concentration in 0.1 M sodium phosphate buffer pH 5.5 with 0.1 mM resazurin and 0.29 IU/mL of CDH enzyme. Fluorescence measured at ex/em 540/590 nm.



It could be seen that the highest fluorescence signal was obtained for 10 mM lactose concentration, while in the case of cellobiose there was maximum of fluorescence signal between 0.05 and 0.1 mM more precisely at 0.08 mM cellobiose concentration and therefore for all further experiments optimal concentrations of both substrates were used.

### 3.2.3. Influence of Enzyme Amount

Different amounts of CDH were added to reaction mixture and the fluorescence signal was measured. The assay showed a linear response to CDH enzyme concentrations in the range of 0.1–0.45 IU/mL (Figure 4).



**Figure 4.** Dependence of fluorescence (RFU) change in time for different amounts of enzyme in 0.1 M sodium-phosphate at pH 5.5 and 10 mM lactose and 0.1 mM resazurin. (A) Reaction kinetics in time; (B) Calibration curve. Fluorescence measured at ex/em 540/590 nm.

There was no increase of fluorescence in control reaction, and by increasing enzyme amount there was also increase in the slope of fluorescence change. This was confirmation that resazurin assay could be used for detection of CDH mutants with different ranges of activities.

### 3.3. Optimization of Resazurin Fluorescence Assay with CDH Expressed on the Surface of Yeast Cells

#### 3.3.1. Expression of CDH on the Surface of Yeast Cells

Cellobiose dehydrogenase gene was cloned in pCTCON2 vector for expression as a chimera with Aga2 protein on the surface of yeast cells. This was verified by plasmid restriction mapping (see Supplementary material; Figure S1), colony PCR and DNA sequencing. We screened colonies on several agar plates for CDH activity and none of them having wild type (wt) CDH gene showed any activity. After that one round of epPCR was performed using wtCDH gene and after screening several agar plates we found a clone that showed measurable activity on the surface of yeast cells. Sequencing results showed that the clone had three mutations within *cdh* gene (tmCDH): D20N, A64T, and V592M. In order to confirm agar plate screening results fermentations in liquid cultures were done for cells having wtCDH-pCTCON2, tmCDH-pCTCON2, and empty pCTCON2 vector. Only tmCDH showed measurable activity on the cell surface during fermentation. Fermentation conditions for maximum production of CDH activity were at 25 °C after 24–36 h galactose induction (see Supplementary material; Figure S2).

#### 3.3.2. Influence of Substrate Concentration

Since enzymes immobilized on the surface of yeast cells can have higher  $K_m$  values than their soluble counterparts, we have tested influence of substrate concentration on fluorescence signal

(see Supplementary material; Figures S3 and S4). It could be seen that maximal enzymatic activity was also detected around 10–20 mM lactose, while with cellobiose maximal activity was detected at slightly higher substrate concentration of 0.1 mM compared to 0.08 mM for the soluble enzyme.

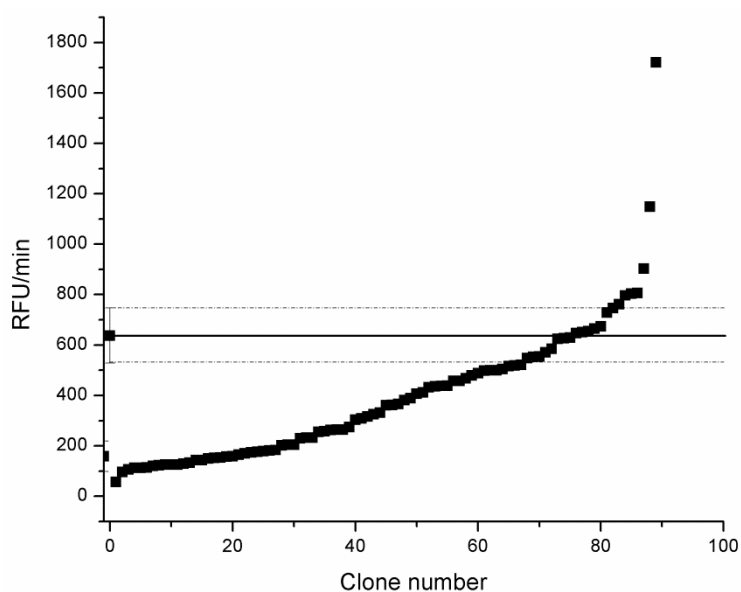
### 3.3.3. Influence of Yeast Cells Density

Having established optimized reaction conditions, we tested assay in a cuvette using *S. cerevisiae* EBY100 cells expressing tmCDH at high and low concentrations per reaction volume. We used EBY100 cells transformed with the empty vector as a negative control. After 24 h of induction, different amounts of cells were added to the reaction mixtures so that final OD<sub>600nm</sub> was 0.1, 0.2, and 0.4 ( $1.28 \times 10^6$ ,  $2.55 \times 10^6$ , and  $5.3 \times 10^6$  cells, respectively).

It could be seen that assay showed a linear response to the amount of added cells confirming that it can be used for detection of the CDH mutants expressed on the surface of yeast cells (see Supplementary material; Figure S5).

### 3.3.4. Screening of tmCDH Gene Library in MTP by Resazurin Assay

Gene library of tmCDH parent gene made by error prone PCR was expressed in *S. cerevisiae* EBY100 cells. Clones were transferred from agar plates into wells of MTP in liquid YNB-CAA Glc medium and grown for 48 h at 30 °C till saturation, diluted 4 times with YNB-CAA Gal medium to induce expression of CDH, grown for 24 h and activity of cell suspension was measured in MTP (Figure 5).



**Figure 5.** Screening of error prone tmCDH-pCTCON2 gene library in MTPs. Activity was measured in 0.1 M sodium phosphate buffer pH 5.5 with 0.1 mM resazurin and 10 mM lactose for 30 minutes. Relative fluorescence units (RFU)/min are at excitation/emission (ex/em) wavelengths of 540/590 nm. ■- Parental gene activity (tmCDH).

From this library, two mutants were found higher activity than the parent tmCDH mutant, H5 and H9.

### 3.4. Kinetic Characterization of CDH Mutants on the Surface of Yeast Cells

Mutants showing higher activity than parent gene were more detailed kinetically characterized in the immobilized form on the surface of yeast cells and their genes were sequenced (Table 1).



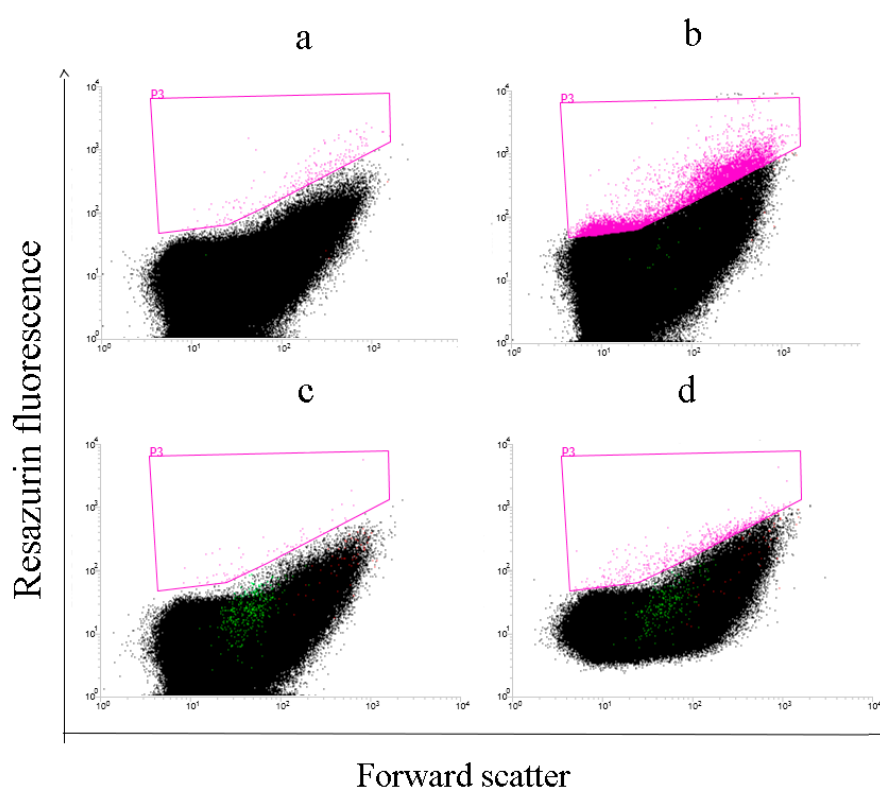
**Table 1.** Kinetics properties of CDH mutants. Enzymatic assay was done with resazurin (0.1 mM), lactose/cellobiose (different concentrations), sodium-phosphate buffer (0.1 M; pH 5.5), and fluorescence measured at ex/em 540/590 nm.

	Lactose	Lactose	Cellobiose	Cellobiose	Mutations
	$V_{\max}$ (RFU $\times$ $10^5$ /OD $\cdot$ min)	$K_m$ (mM)	$V_{\max}$ (RFU $\times$ $10^5$ /OD $\cdot$ min)	$K_m$ ( $\mu$ M)	
tmCDH	1.70 $\pm$ 0.11	0.144 $\pm$ 0.063	2.71 $\pm$ 0.05	2.7 $\pm$ 0.4	D20N, A64T, V592M
H5	2.80 $\pm$ 0.16	0.225 $\pm$ 0.099	3.51 $\pm$ 0.18	5.5 $\pm$ 1.3	D20N, A64T, V592M, V22A, T99T
H9	3.53 $\pm$ 0.24	0.654 $\pm$ 0.245	3.13 $\pm$ 0.16	4.5 $\pm$ 1.2	D20N, A64T, V592M, T84A, A261P, E674G, N715S

It could be seen from kinetic data that H5 and H9 mutants had 1.6 and 2 times increased  $V_{\max}$  compared to the parent tmCDH that already was improved compared to wtCDH since it was showing activity on the cell surface while wtCDH did not have any measurable activity. Both mutants had increased  $K_m$  values, but since screening was performed under high substrate concentrations, they were selected from the library for increased  $V_{\max}$  value.

### 3.5. Detection of CDH by Flow Cytometry

In order to test if there is potential to use developed resazurin assay for detecting CDH activity by flow cytometry in water-in-oil-in-water double emulsions series of enzymatic reactions was set up and analyzed by flow cytometry (Figure 6).

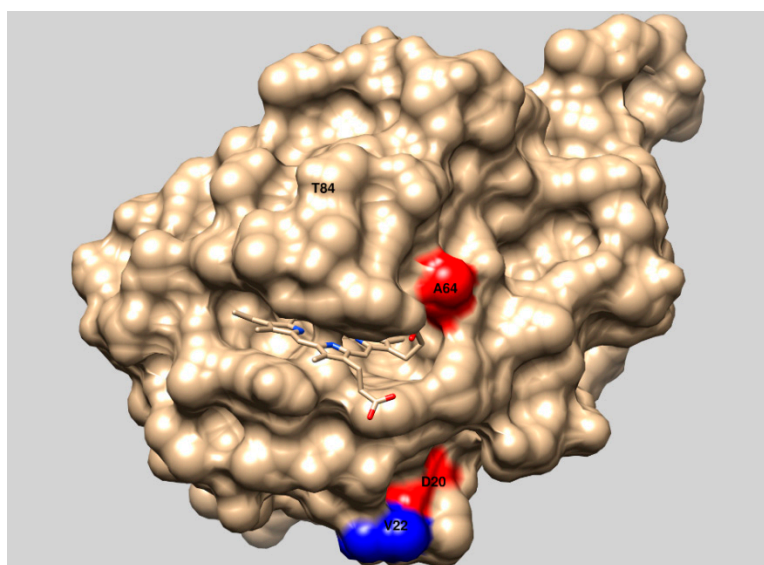


**Figure 6.** Flow cytometry analysis of the resazurin assay for cellobiose dehydrogenase activity in double emulsions. (a) Emulsions with resazurin; (b) resazurin plus soluble enzyme; (c) resazurin plus cells not expressing CDH; (d) resazurin plus cells expressing tmCDH. The plots show bivariate histograms of forward scattering and resorufin fluorescence.

It could be seen that population of droplet with increased red fluorescence was visible in enzymatic reaction mixtures with soluble enzyme compared to the blank reaction. Also, smaller population of droplets with increased fluorescence was observed in the case of cells encapsulated within double emulsion expressing active tmCDH compared with cells without expressed CDH. Cross-boundary reactions during analyzing cells were not significant due to the small number of encapsulated cells compared to the number of double emulsion compartments.

#### 4. Discussion

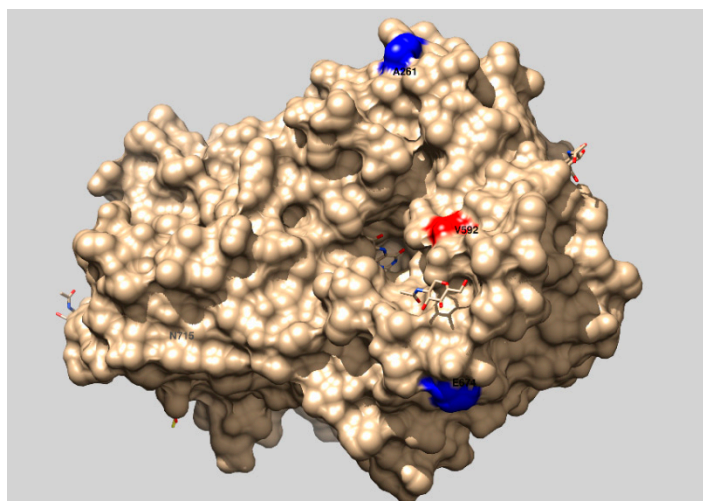
In order to develop CDH expression system suitable for whole-cell biocatalysis and directed evolution by high-throughput screening systems, *cdh* gene from *Phanerochaete chrysosporium* was cloned in pCTCON2 vector for expression as a chimera with Aga2 protein on the surface of *Saccharomyces cerevisiae* EBY100 yeast cells. After expression on the cell surface activity of the wild type CDH could not be detected. Significant decrease in specific activity of enzymes expressed on the surface of yeast cells compared with their soluble counterparts was described previously for other enzymes like glucose oxidase [30]. Since CDH has much lower specific activity compared to glucose oxidase [31] further decrease in specific activity of CDH due to the expression in the immobilized form on the surface of yeast cells could be the reason why we did not detect wild type CDH activity. During agar plate screening of yeast colonies expressing epPCR gene library of wtCDH, a triple mutant (D20N, A64T, and V592M) of CDH that was showing activity was found. All three substitutions were located on the surface of CDH protein, with two of them in heme domain (D20N and A64T) near the active site containing heme, Figure 7.



**Figure 7.** Presentation of the structure of the heme domain of CDH from *P. chrysosporium* (PDB accession code 1D7C) with labelled substitutions. The picture was made with UCSF Chimera 1.13.1. (University of California, San Francisco, CA, US).

Third substitution V592M was within FAD domain and also very close to the entrance of the active site containing FAD in it, Figure 8.

Both A64T and V592M substitutions are in the region where there two domains interact with each other and these interactions are important for the activity of CDH [3]. Any change in this region can significantly influence activity of CDH and these two substitutions could be the reason tmCDH showed measurable activity on the surface of yeast cells, while at the same time wtCDH activity could not be detected.



**Figure 8.** Presentation of the structure of the FAD domain of CDH from *P.chrysosporium* (PDB accession code 1NAA) with labelled substitutions. The picture was made with UCSF Chimera 1.13.1. (University of California, San Francisco, CA, US).

Expression of tmCDH in liquid culture was further optimized and the highest activity was obtained at 25 °C after 24 h of galactose induction (Supplementary material; Figure S2). Optimal temperature for enzyme expression was lower than usually reported 30 °C, while expression time of 24 h was longer than optimal 16 h previously determined for glucose oxidase expression in the same host [32]. This could be result of higher molecular weight of CDH protein compared to GOx that very often requires lower expression rate of protein in order to properly fold. Some other proteins like eGFP when expressed in yeast also require lower expression temperature of 20 °C [33].

Due to the decrease in specific activity of surface displayed enzymes, we wanted to have more sensitive enzymatic assay than usually used spectrophotometric assays based on 2,6-dichlorophenol-indophenol and methylene green [34] and that could also be used with flow cytometry. Therefore, we tested fluorogenic resazurin dye as a substrate for detection of CDH activity on the surface of yeast cells. Resazurin reduction to resorufin that can be measured by red fluorescence was used previously for measuring viability of living cells [24] and blood glucose concentration [35]. The CDH assay using resazurin was optimized with respect to resazurin, cellobiose/lactose concentration, and pH of the reaction medium.

Since resorufin has the highest fluorescence at pH above 7.5 when it is in anionic form [36] and CDH has an acidic pH optimum around pH 4–5 with only 20% of residual activity at pH 7.5 [37], optimal pH for resazurin assay was determined to be at pH 5.5 (Figure 1). Above this pH resorufin has higher fluorescence but enzyme is less active, while below this pH the enzyme is more active but due to protonation of resorufin fluorescence of that product is decreased. Similar results were obtained also during development of resazurin based assay for dihydroorotate dehydrogenase that also has an acidic pH optimum [38].

Optimal concentration of resazurin dye was determined to be 0.1 mM since at higher concentrations it behaves as an inhibitor of CDH activity (Figure 2). Drop in fluorescence signal produced by enzymatic action at higher concentrations of resazurin (above 0.025 mM) was also previously reported for diaphorase coupled detection of glucose [35]. Drop in the enzyme activity at higher resazurin concentrations were results of substrate inhibition phenomenon that is often caused by nonproductive binding of the substrate for the enzyme active site.

Lactose and cellobiose that were used as CDH substrates in the assay also showed inhibition of fluorescence signal at higher concentrations. Substrate inhibition of CDH at higher concentrations due to non-productive binding of two disaccharide units is probably the result of two binding sites for hexoses that are present in the active site of CDH [39]. Substrate inhibition was observed only

with cellobiose and not for lactose, probably because of much higher affinity of binding subsites for glucose than for galactose, leading to higher probability of non-productive binding of two molecules of cellobiose for one molecule of the enzyme. This difference in affinity was confirmed by much lower CDH  $K_m$  value for cellobiose than for lactose. Therefore, optimal substrate concentration was determined to be 10 mM for lactose and 0.08 mM for cellobiose. The resazurin assay showed linear correlation between amount of added enzyme and fluorescence intensity, showing that it could be used for detection of enzymatic variants with different activities usually created in directed evolution experiments.

The assay was also tested and optimized for measuring CDH activity on the surface of yeast cells that is particularly important for developing high-throughput screening systems based on flow cytometry. Similar for soluble enzyme, substrate inhibition was detected, but due to the diffusional limitations that immobilized enzymes usually show, optimal substrate concentrations were higher, and for lactose it was 20 mM, while for cellobiose was 0.1 mM. Also, with CDH expressed on the cell surface the assay showed a linear response to the number of added cells.

In order to test the resazurin assay as a high-throughput screening system in MTP, a tmCDH gene library was generated by epPCR using 0.05 mM  $MnCl_2$  and around 2000 colonies were screened in MTPs by resazurin assay for higher activity. Two mutants with activity higher than the parent gene were found and characterized by sequencing and measuring kinetic parameters in immobilized form on the surface of yeast cells. The expression rate of Aga1 protein that acts as an anchor for CDH did not change in the CDH gene library and was reported to be  $10^5$  copies per single cell [13]. Therefore, the number of CDH mutants expressed on the surface of yeast cells should remain constant ( $10^5$  per cell) and all kinetic data normalized to cell density of washed cells (OD<sub>600nm</sub>) should not be influenced by mutant expression rate. Mutant H5 showed additional mutation V22A on the surface of CDH heme domain (Figure 7) that was very close to the already mutated D20N position in tmCDH. This could mean that mutations in this region can increase activity of CDH. Mutant H9 showed additional T84A substitution in heme domain that is located inside the protein molecule and A261P, E674G, N715S substitutions on the surface of FAD domain of CDH compared to the parent tmCDH (D20N, A64T, and V592M). Since substitutions in internal part of protein molecule can have profound influence on activity and stability, change of T84 residue to alanine, as one of the most acceptable substitutions after serine based on BLOSUM62 matrix table, can have positive influence on activity of CDH.

The H5 and H9 mutants had higher activity at high lactose concentrations compared to the parent tmCDH and therefore showed potential to be used as a whole-cell biocatalyst for production of lactobionic acid [6].

Resazurin assay in double emulsions could detect activity of CDH by flow cytometry both for the soluble enzyme and for the enzyme expressed on the surface of yeast cells.

## 5. Conclusions

The *cdh* gene from *Phanerochaete chrysosporium* was cloned in pCTCON2 plasmid and triple mutant of CDH (D20N, A64T, and V592M) was expressed in an active form on the surface of yeast cells for the first time, while wtCDH did not show any measurable activity. A resazurin-based fluorescent assay for the detection of CDH activity on the surface of yeast cells was developed and used in MTP for finding mutants with improved activity. The assay also showed potential for detection of CDH activity by flow cytometry. Therefore, developed resazurin assay and expression system could be used for finding better CDH whole-cell biocatalyst in directed evolution experiments. The H5 and H9 mutants that we found showed increased activity for lactose on the surface of yeast cells and could be used as whole cell biocatalysts in biotechnology.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2076-3417/9/7/1413/s1>, Figure S1. DNA electrophoresis on 0.6% agarose gel.; Figure S2. Fermentation graph after galactose induction at 25 °C.; Figure S3. Dependence of fluorescence slope in time with lactose concentration.; Figure S4. Dependence of fluorescence slope in time with cellobiose concentration.; Figure S5. Dependence of fluorescence signal (RFU/min)

on OD at 600 nm of yeast cells expressing cellobiose dehydrogenase.; Table S1. CDH synthetic gene sequence (U46081.1).

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