Korean J. Chem. Eng., 33(12), 3434-3441 (2016)
DOI: 10.1007/s11814-016-0205-4

pISSN: 0256-1115
eISSN: 1975-7220

Production and characterization of cellobiose dehydrogenase from *Phanerochaete chrysosporium* KCCM 60256 and its application for an enzymatic fuel cell

Han Suk Choi*, Dong Sup Kim*, Laxmi Prasad Thapa*, Sang Jun Lee*, Sung Bong Kim*, Jaehoon Cho, Chulhwan Park***, and Seung Wook Kim*,†**

*Department of Chemical and Biological Engineering, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Korea
**Green Manufacturing Process R&D Group, Korea Institute of Industrial Technology (KITECH), Chonan 31056, Korea
***Department of Chemical Engineering, Kwangwoon University, Nowon-gu, Seoul 01897, Korea
(*Received 23 April 2016 • accepted 12 July 2016*)

Abstract−The enzyme cellobiose dehydrogenase (CDH), with high ability of electron transport, has been widely used in enzymatic fuel cells or biosensors. In this study, the cellobiose dehydrogenase gene from *Phanerochaete chrysosporium* KCCM 60256 was amplified and expressed in the methylotrophic yeast *Pichia pastoris* X-33. The recombinant enzyme (PcCDH) was purified using a metal affinity chromatography under non-denaturing conditions. The purified enzyme was analyzed by SDS-PAGE, confirming a corresponding band about 100 kDa. The enzyme activity of this purified PcCDH was determined as 1,845 U/L (65 mg/L protein). The enzyme showed the maximum activity at pH 4.5 and high activity in broad ranges of temperature from 30 °C to 60 °C. Moreover, the application of PcCDH to enzymatic fuel cell (EFC) was demonstrated. Lactose was used as the substrate in the EFC system; anode and cathode were immobilized with PcCDH and laccase, respectively. The cell's open circuit voltage and maximum power density of the EFC system were, respectively, determined as 0.435 V and 314 μW/cm^2 (at 0.247 V) with 10 mM lactose.

Keywords: Cellobiose Dehydrogenase, *Pichia pastoris*, *Phanerochaete chrysosporium*, Enzymatic Fuel Cell

INTRODUCTION

Cellobiose dehydrogenase (CDH; E.C. 1.1.99.18) is secreted by various wood-degrading fungi such as white rot, brown rot and soft rot [1]. According to the gene sequence, CDHs can be classified into two major subgroups, Class-I and Class-II. The members of Class-I are produced by basidiomycetes, such as *Trametes villosa* and *Phanerochaete chrysosporium*. This subgroup of CDHs has a shorter sequence due to lack of a C-terminal carbohydrate binding domain, which is highly conserved in the linker sequence between the cytochrome and flavin domains. The members of Class-II are produced by ascomycetous fungi, such as *Myriococcum thermophilum*. In contrast to Class-II, this subgroup of CDHs contains a more complex structure, featuring carbohydrate binding domains in the C-terminal and longer sequences [2].

The CDH structure consists of two distinct domains, including the C-terminal, with catalytic flavin adenine dinucleotide (FAD), and the N-terminal, with cytochrome-b-type heme [3]. This structural information indicates that the electron transformation occurs at the heme-binding domain between the flavin at the C-terminal of CDH and external acceptors [4]. CDH has been expected to be catalytically active in enzymatic reactions, because of its larger domain of flavin dehydrogenase and the smaller cytochrome domain with b-type heme acting as cofactor in electron transformation. In addition, the cytochrome-b-type heme domain at the N-terminal of CDH contains low-spin iron structure representing the enzyme structure with axial ligands of histidine and methionine [5]. The two terminals of CDH with different functions are connected by a Thr-Ser-rich long linker region. Because of the cytochrome b-type heme domain, unusual heme binding through Met/His ligation is presented at the N-terminal. At the C-terminal, the flavin domain binds with FAD, one of the oxidoreductases of the glycose-methanol-choline family. The structure of CDH also estimates a better performance in application of catalytic reactions.

The biological functions of CDH are still being discovered with detailed investigations. Biodegradation and modification of cellulose, hemicelluloses and even lignin have been reported in traditional applications based on the hydroxyl radical-generating mechanism [6]. CDH preferentially catalyzes the oxidation of cellobiose, cellodextrins, or certain other oligosaccharides to the corresponding lactone, and then spontaneously converts to the aldonic acid (e.g., cellobionic acid) [6-8]. In the redoxreaction catalyzed by CDH, electron transformation is performed by carbohydrates acting as the electron donors and phenoxy radicals, ferricyanide or quinones acting as the electron acceptors [6]. The electron acceptors derived from lignin have been suggested as a potential application of lignin biodegradation [9,10]. According to reference survey, various electron acceptors such asquinines, cytochrome c, and metal ions are indispensable for the efficient oxidation of cellobiose, cellooligosaccharides, lactose, and even cellulose by the ping-pong mechanism of CDH catalysis [11]. The ping-pong mechanism of electron transformation in enzymatic reaction has been reported as an effective

†To whom correspondence should be addressed.
E-mail: kimsw@korea.ac.kr
‡This article is dedicated to Prof. Sung Hyun Kim on the occasion of his retirement from Korea University.

Table 1. Strains, plasmids and primers used in this study

Strains, plasmid andprimers	Genotype, characteristics or sequence	Source or reference
Strains		
Phanerochaete chrysosporium KCCM 60256	Wild type	KCCM
Pichia pastoris X-33	Wild type	Invitrogen
Pichia pastoris/P*ccdh*	*Cdh* expressed in *Pichia pastoris*	In this study
Plasmids		
PLUG-TA vector	Cloning vector, Amp^r	Intronbiotechnology
pPICZαA	Expression vector, Zeo^r	Invitrogen
pPICZαA /P*ccdh*	Recombinant plasmid containing *cdh* gene	This study
cdhF	TTTGAATTCTGCTAGGTCGATCGTTACTTGCGCTTC	This study
cdhR	TTTGAATTCAGGACCTCCCGCAAGCGCG	This study
AOXF	GACTGGTTCCAATTGACAAG	This study
PCRcdhR	GAATTCAGGACCTCCCGCAAGCGCG	This study

KCCM (Korean Culture Center of Microorganisms)

catalysis method, and has received high attention in electrochemical applications such as biosensors and enzymatic fuel cell (EFC) [6,12]. Some researchers reported that the third generation of EFC should focus on development of remarkable enzyme functions and structures. The heme domain of CDH, as a built-in mediator, is considered as an advanced structure and function of enzyme for direct electron transfer to electrode surfaces (DET) in EFC applications, which has been still interested in recent researches. In addition, CDH belongs to the restricted number of oxidoreductases, which shows high performance of electron transfer. It would be an advantage on EFC application with efficient electron transfer between the active site and electrode surface. Also, FAD domain of CDH structure also indicates the feasibility of enzyme reaction with various sugars as substrate [13].

Furthermore, these new applications of CDH are also based on its high productivity of enzyme, indicating that recombinant CDH production would be an effective path to achieve. In general, CDH is produced under cellulolytic conditions such as when cellulose is the major carbon source. In batch culture with cellobiose as the carbon source, little or no activity was obtained. However, Szabo et al. [13] reported the production of high amounts of CDH in fed batch culture of *P. chrysosporium* by adding limited amounts of cellobiose or mixtures of cellobiose and glucose. Thus, CDH is induced by low concentrations of cellobiose, but it is also subjected to catabolite repression by excessive concentrations of cellobiose or glucose.

Our main objectives aimed at cloning and heterologous expression of the *cdh* gene from *P. chrysosporium* KCCM 60256 in *Pichia pastoris* X-33 for reliable and efficient production of enzyme and the possibility to produce the recombinant enzyme. The recombinant CDH (PcCDH) was purified using metal affinity column, and the CDH activity of the purified enzyme was characterized with various substrates. In addition, PcCDH was applied to enzymatic fuel cell.

MATERIALS AND METHODS

1. Microorganisms and Culture Conditions

The fungus *P. chrysosporium* KCCM 60256 was obtained from the Korea Culture Center of Microorganisms. This strain was cultured on PDA (4 g/L Potato, 20 g/L dextrose and 15 g/L agar) plate at 28 °C for 3 days. *Pichia pastoris* X-33 is a component of the Pichia Easy Select Expression System, and was obtained from Invitrogen (Carlsbad, CA). All resulting strains and plasmids used in this experiment are listed in Table 1.

2. *cdh* Amplification and Construction of Recombinant Plasmid

P. chrysosporium KCCM 60256 was cultured in PDB (4 g/L potato and 20 g/L dextrose broth) media at 28 °C for three days. The cells were collected by centrifugation at 15,000 g and shock frozen in liquid nitrogen. The complementary DNA (cDNA) of *P. chrysosporium* KCCM 60256 was extracted by using the RNeasy plant mini kit (Cat. No. 74104). The *cdh* gene, 2.313 kb in size, was then amplified using the primer pair *cdhF* and *cdhR* (Table 1). Thirty cycles of PCR were performed at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 2 min and 30 s. The resulting PCR product was sequenced by Macrogen Inc. (Seoul, South Korea), and further analyzed. The P*ccdh* homologous was identified using the basic local alignment search tool (BLAST). The expression vector pPICZαA/P*ccdh* was then constructed by cloning of the P*ccdh* gene into the pPICZαA plasmid using *Eco*RI restriction enzyme at both sites (Fig. 1). The construction of the recombinant plasmid, pPICZαA/P*ccdh* was confirmed by colony PCR using *AOXF* and *PCRcdhR* primers (Table 1).

3. P*ccdh* Overexpression in *P. pastoris* X-33

The recombinant plasmid pPICZαA/P*ccdh* was linearized by using *pme*I restriction enzyme and it integrated into the *P. pastoris* X-33 genome by electroporation under the control of the methanol-inducible promoter. Transformants were then selected on YPDA (20 g/L yeast extract, 40 g/L peptone, 20 g/L dextrose and 40 g/L agar) plates containing 100 μg/mL Zeocin (Biobasic, USA), and *P. pastoris*/*cdh* confirmed by colony PCR using forward and reverse AOX primers. The recombinant strain *P. pastoris* was cultured for protein expression in a 250 ml Erlenmeyer flask containing 25 mL BMGY (Buffered glycerol-complex Medium: 1% (w/v) yeast extract, 2%(w/v) peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, 1% (v/v) glycerol) at 30 °C in an shaking incubator (250 rpm) until a culture turbidity (OD600) of 3.0 was attained The protein expression was induced by trans-

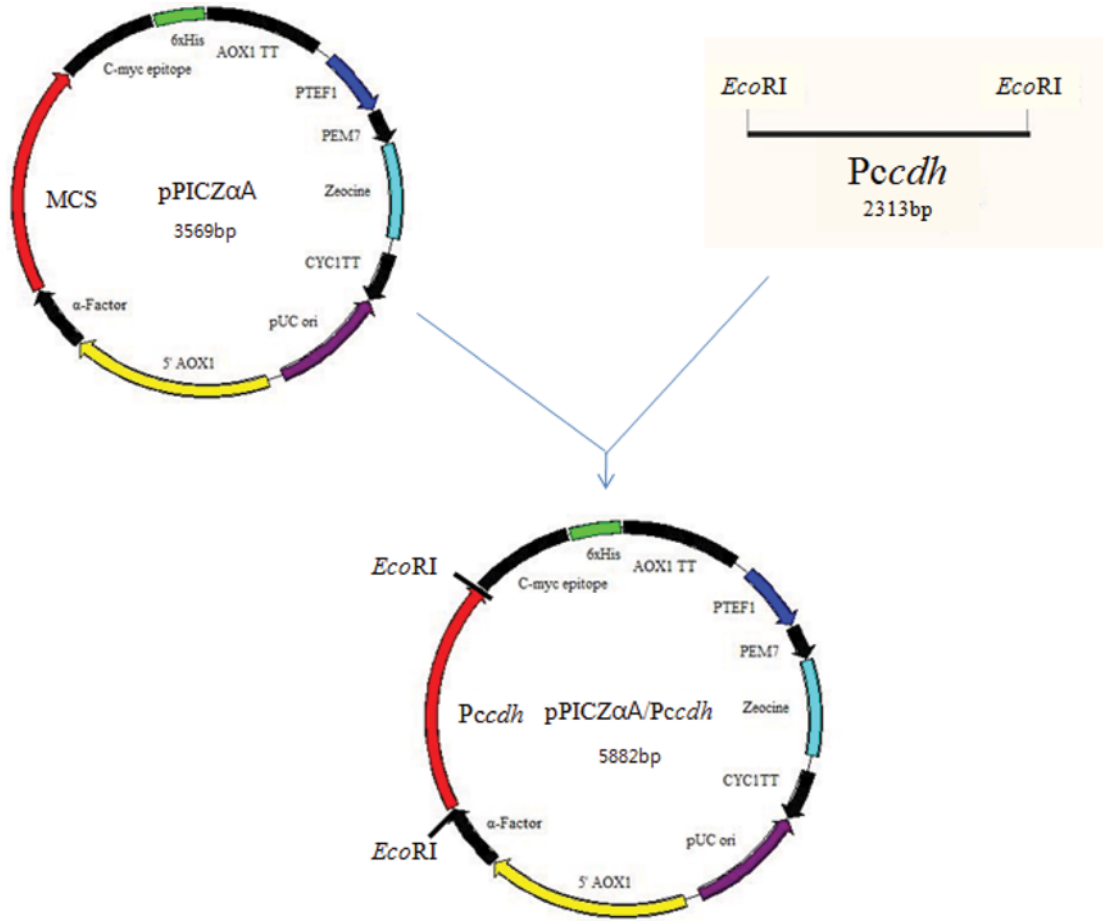

Fig. 1. Strategy for the construction of recombinant plasmid pPICZαA/P*ccdh*.

ferring cells into 50 mL of BMMH (Buffered minimal methanol: 100 mM potassium phosphate buffer, pH 6.0, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, 0.5% (v/v) methanol) in 500 mL flask. Methanol was supplemented at 1% (v/v) concentration every 24 h during the induction period.

4. Enzyme Purification

The PcCDH was purified by using metal affinity chromatography. The poly histidine-tagged CDH fusion mutants were loaded onto a ProBond resin (Ni^{2+}) column. To minimize untagged protein and remove this non-specific binding, the column was washed twice with ten column volumes of binding buffer which contained 50 mM potassium phosphates, 300 mM KCl and 20 mM imidazole at pH 7.0. The resin with specific binding was conducted in batch mode at 4 °C and then washed twice with 5-8 mL Tris-HCl (10 mM Tris) at pH 4.0.

5. SDS-PAGE Analysis

SDS-PAGE was carried out by protein electrophoresis (Bio-Rad Laboratories, Inc., Hercules, California, USA) using a precast 12% Tris-glycine gel to analyze the purified CDH protein. The proteins were stained with coomassie brilliant blue G250 with 50% (v/v) methanol and 7.5% (v/v) acetic acid, and then destained with 30% (v/v) methanol and 7.5% (v/v) acetic acid. The molecular mass of CDH under denaturing conditions was then determined with reference standard proteins (Intron Biotechnology, South Korea).

6. Enzyme Assays

The enzyme activity of CDH was measured by monitoring the decrease in absorbance of the electron accepter 2,6-dichlorophenolindophenol (DCPIP, Bio-Basic, USA) using a UV spectrophotometer (Thermo scientific, USA) at 520 nm (ε_{520}=6.8×103 $M^{-1}\cdot cm^{-1}$), pH 4.5 and 30 °C. The enzyme assay was carried out with 100 μL DCPIP (3 mM in water containing 10% ethanol), 100 μL substrates in 100 mM sodium acetate buffer and 800 μL sodium acetate buffer (pH 4.5) at 30 °C. After temperature adjustment, the reaction was started by the addition of 10 μL purified enzyme, and the decrease in absorbance was monitored for the initial 5 min [14].

6-1. Effect of Temperature and pH on Enzyme Activity of PcCDH

The thermostability of purified PcCDH represented as enzyme activity, which was determined at temperatures ranging from 20 to 80 °C with 100 mM sodium acetate buffer at pH 4.5. The effect of pH on enzyme activity was evaluated with 100 mM sodium acetate buffer pH range from 3.5 to 6.5 at 30 °C. The enzyme activity of PcCDH was measured as the same method in the section of enzyme assays, previously.

6-2. Enzyme Kinetics of PcCDH

To obtain the steady-state kinetic parameters of various substrates of purified CDH, the reduction of 100 μL DCPIP was measured with the following: cellobiose (50-1,000 μM), lactose (100-1,000 μM) and glucose (200-2,000 μM). The kinetic parameters were

calculated by- nonlinear least-squares regression, fitting the observed data to the Michaelis-Menten equation [15].

7. Chemicals and Enzyme for Electrodes

Chitosan, cobalt (II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and graphite were obtained from Sigma-Aldrich Co. (USA). Laccase from *Trametes vesicolor* was obtained from Sigma-Aldrich. Co. (USA) for immobilized redox.

8. Preparation of Enzymatic Fuel Cell Electrodes

The electrode in this study was cleaned by a mixture of H_2SO_4 and H_2O_2 with ratio 3 : 1 followed by a micro-polishing kit (Digi-Ivy, USA). Then, the impurities on the electrode surface of electrode were removed by ultrasonication. The electrode was prepared to use when its electrostatic adsorption was performed at 19 V with Au electrodes charged at 0-50 V in the adsorption process [16]. The purified PcCDH and laccase were, respectively, immobilized on the prepared Au electrode. The enzyme immobilization was carried out with sodium acetate buffer (100 mM, pH 4.5) containing 1 g/L of PcCDH for the anode and 0.5 g/L of laccase for the cathode. Then, the Au electrode was treated with 20 mM of EDC and 5 mM NHS to assemble the enzymes in 0.05 M of sodium phosphate buffer (pH 4.5) for 8 h. Amine groups were also deposited on the electrodes and coupled immobilized enzymes [17].

9. Fabrication of EFC and the Electrochemical Device

The EFC system was established by three electrodes: the cathode (counter electrode), the reference electrode and the anode (working electrode), which were comprised of graphite oxides immobilized on the modified Au surface with enzymes (Fig. 2).

The electrochemical activity of the enzyme electrode that immobilized with purified CDH was measured by using a VersaSTAT 3 device (AMETEK, Princeton Applied Research, USA) before cyclic voltammetry (CV) test.

RESULTS AND DISCUSSION

1. Analysis of P*ccdh* Sequence and Construction of Plasmid pPICZαA/Pccdh

Potential primers for gene were designed from the highly similar *cdh* gene of *Phanerochaete chrysosporium* (GenBank: X88897.1). Then the *cdh* gene was amplified using the genomic DNA of *P. chrysosporium* KCCM 60256 as a template. The sequence analysis revealed an open reading frame consisting of 2,313 bp, encoding 770 amino acids. These results obtained from P*ccdh* sequencing are in complete agreement with the expected results. The recombinant plasmid pPICZαA/P*ccdh* was constructed by cloning P*ccdh* gene into the expression vector (pPICZαA) with alcohol oxidase (AOX), α-factor secretion signal peptide and His6-tag at C-terminal.

2. Overexpression and Purification of CDH

Heterologous expression is more advantageous for obtaining large amounts of CDHs without contamination by wild type CDH or other cellulolytic enzymes [18]. Therefore, we decided to construct a recombinant strain for the heterologous expression of *P. chrysosporium* KCCM 60256 *cdh* gene using *P. pastoris* X-33. *P. pastoris* is easier to genetically manipulate and can be grown to high cell densities. It has been successfully used for the expression of several genes, including other hemoflavo enzymes, and nitric oxide synthase [18-20], which encouraged us to choose *P. pastoris* X-33 as the host organism for the expression of *cdh* from *P. chrysosporium* KCCM 60256.

The linearized recombinant plasmid pPICZαA/P*ccdh* was inserted into the *P. pastoris* X-33 under the control of the methanol-induc-

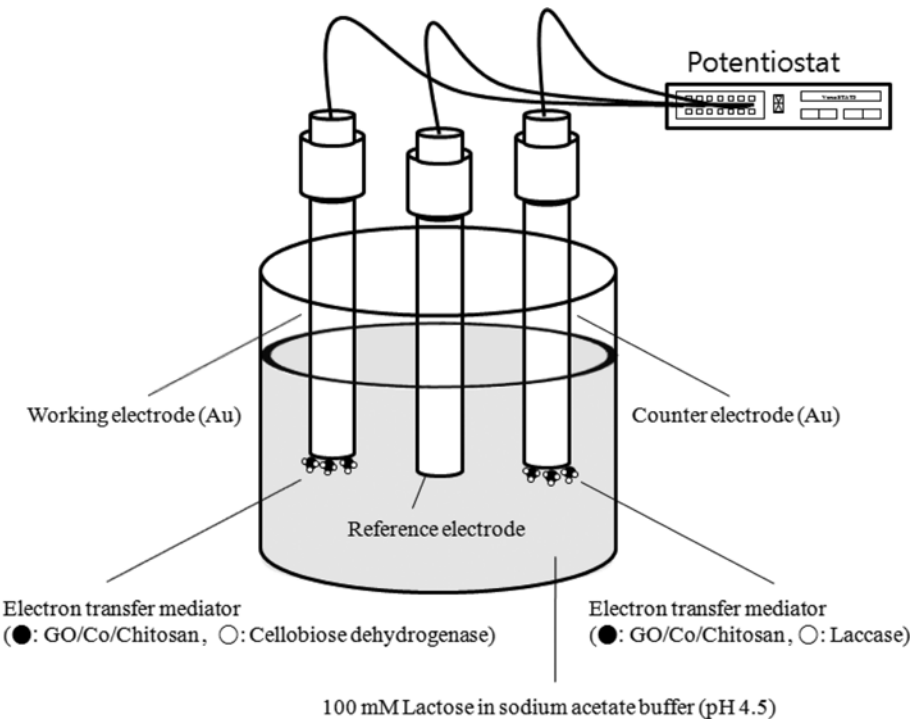

Fig. 2. Experimental scheme of the enzymatic fuel cell. Anode and cathode were composed of CDH and laccase.

ible promoter (AOX1 promoter). Then the multicopy transformants were screened and we selected one recombinant transformant for use in further experiments. The recombinant strain was cultured in 100 mL BMMY medium for four days at 30 °C and the protein overexpression was induced by adding 0.5% (v/v) methanol. SDS-PAGE analysis of *P. pastoris* X-33-P*ccdh* supernatant showed that the recombinant CDH (PcCDH) represented the main secreted protein in the strain. The concentration of the soluble protein in the culture supernatant was measured, reaching to 32 mg/L after four days of cultivation and then decreased (Fig. 3). Similarly, the PcCDH activity in the culture supernatant was measured by DCIP assay and reached a maximum value of 980 U/L after four days of cultivation at 30 °C (Fig. 3). After five days, the cultivation was stopped because of the decrease in the specific activity in the culture supernatant.

The molecular weights of culture supernatants containing PcCDH and purified PcCDH were confirmed by SDS-PAGE. A major single protein band was shown in SDS-PAGE analysis (Fig. 4), which corresponded to a molecular weight of about 100 kDa. Therefore, the *cdh* gene of *P. chrysosporium* KCCM 60256 was successfully expressed in *P. pastoris* X-33.

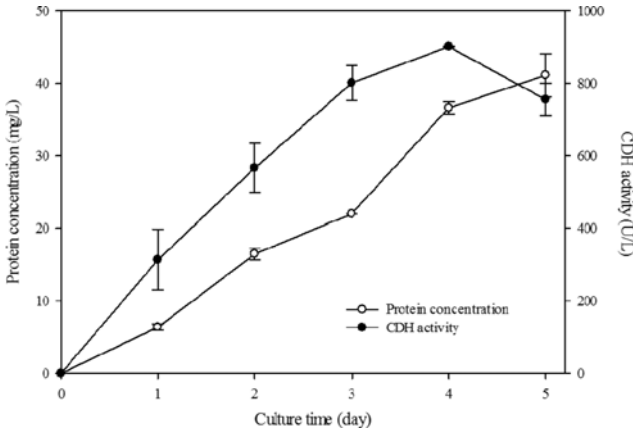

Fig. 3. Time course of CDH activity of PcCDH and concentration of PcCDH protein in the culture supernatant.

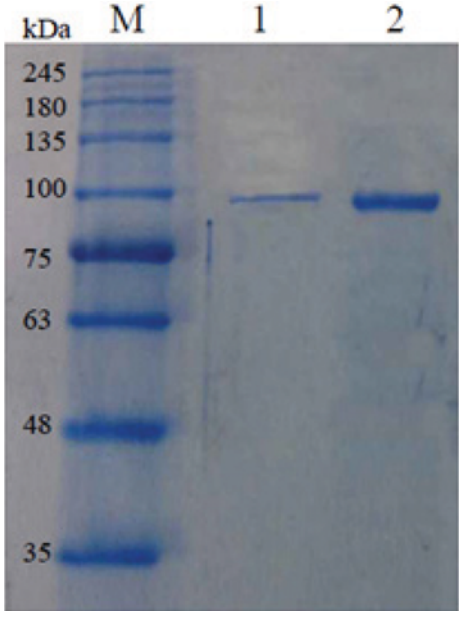

Fig. 4. Analysis via SDS-polyacrylamide gel electrophoresis. M: Marker, Lane 1: PcCDH in BMMH, Lane 2: Purified PcCDH by Ni-NTA chromatography.

Moreover, the recombinant PcCDH containing a His-tag was purified from the supernatant of a four-day induced-culture using a one-step Ni-NTA affinity column chromatogram under non-denaturing conditions. The specific activity of purified PcCDH was found to be 28.37 U/mg. It showed the highest specific activity compared to previous reports of recombinant expression of CDH from *Corynascus thermophilus* (4.1 U/mg) and *Neurospora crassa* (8.37 U/mg) both in *P. pastoris* [25,26].

3. Effects of Temperature and pH on Purified CDH Enzyme

The effect of temperature on the thermostability of purified PcCDH was measured at broad temperature ranges of 20-80 °C at pH 4.5 (Fig. 5). The results revealed that the enzyme was highly stable at 30-60 °C, with maximum activity at 30 °C when incubated for 5 min. Below 30 °C and above 60 °C, the enzyme activity decreased. The relative activity of purified PcCDH decreased at 70 °C. It is reasonable to estimate that the active proteins were broken, leading to denaturation of the protein after a certain temperature and a subsequent reduction in enzyme activity [27]. Thus, an increase in temperature beyond the optimum value may cause reduction in the catalytic rate of CDH, as either the enzyme or substrate become denatured and inactive.

Similarly, the effect of pH on the activity of the purified PcCDH was examined in the pH range 3.5 to 6.5. The activity was measured with DCPIP, and the highest activity was found at pH 4.5 (Fig. 5), followed by a decrease in the CDH activity. The active pH range is considered as a key factor in enzyme application, which was also investigated for this recombinant enzyme production. Enzyme is sensitive to pH conditions and its activity can only be maintained in a limited range. Therefore, defining the optimum pH of recombinant enzyme production for the further application is necessary. The optimal conditions in this study were deter-

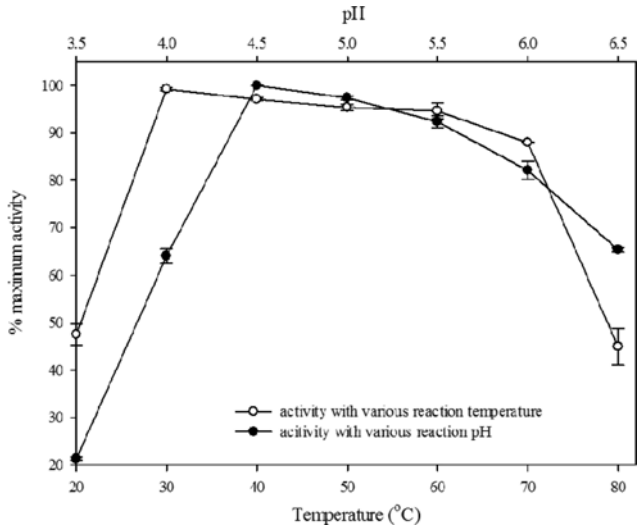

Fig. 5. Effects of temperature and pH on purified PcCDH activity, assayed using DCPIP as an electron acceptor.

mined to be pH 4.5 at 30 °C, which indicated PcCDH needs an acidic environment to be active. However, low relative activity of PcCDH was observed below pH 4.5. It is reasonable to estimate that the low pH condition or the strong proton environment could be able to affect the protein structure of CDH and consequently decline the enzyme activity. The research of Batra and Saxena [29] also supported this view that inactivation or unstable nature of the enzyme could be resulting beyond the optimum culture pH range [28]. Normally, pH effect of enzyme activity is determined by the nature of amino acids on the active site of the enzyme which undergoes major performance of protonation or deprotonation. The conformational changes of the enzyme induced by the ionization of the amino acids also exist with pH changes. The relative activity of purified CDH was decreased when pH was over 4.5 and it was below 80% at 6.5. It is reasonable to believe that ionization of the amino acids results in the inactivation of enzyme with the environment of decreasing protonation. Above pH 6 the enzyme activity was decreased, maybe due to the partial inactivation of heme group of the CDH enzyme of *P. chrysosporium* [29]. Some early studies of CDH application were carried out at pH 6 with slow reduction of heme, and the results in this study were also supported by Jones and Wilson's (1988) research that suggested the heme worked independently of the FAD in enzyme mechanical aspects [30].

4. Enzyme Kinetic Properties

The enzyme kinetics of the purified PcCDH in the oxidization of cellobiose, lactose and glucose at various concentrations was determined by measuring the Michaelis-Menten constant (K_m) and the rate constant (K_{cat}) in the presence of DCPIP as an electron acceptor (Table 2). The result showed a significantly higher value of K_{cat} with cellobiose and lactose.

This result indicated that the substrate structure influences the PcCDH catalysis. The acetal and hemiacetal linkages of cellobiose enhance the strong intramolecular hydrogen bonding, which could also be considered as barriers of the collisions between the enzyme active site and substrate. Moreover, the substrates as the electron donors were divided into two distinctive groups based on the K_{cat} value, one with high values consisting of higher disaccharides of β-1,4 linkage (lactose, cellobiose) and the other with low values consisting of the monosaccharides (glucose) [2]. The highest K_{cat} value of PcCDH catalysis with lactose was almost seven-fold higher than glucose, indicating that PcCDH catalysis was more effective with disaccharides substrate than monosaccharides. According to the CDH catalysis, it was thought that more effective catalysis could be obtained by the electron transformation in the enzymatic reaction. The advantage of lactose as substrate has been reported by researches indicating no inhibition in enzymatic reaction compared to the natural substrate (e.g., cellobiose) [31]. Considering these, lactose was selected as the substrate for the EFC system which was established with PcCDH and laccase immobilized on the anode and cathode, respectively.

Table 2. Enzyme kinetics of PcCDH at 30 °C and pH 4.5, using DCPIP

Substrate	V_{max} ($\mu mols^{-1} \cdot mg^{-1}$)	K_m (mM)	K_{cat} (s^{-1})
Cellobiose	12.54	0.21	6.27
Lactose	16.40	0.34	8.2
Glucose	2.44	1.47	1.22

*All parameters calculated by Michaelis-Menten equation

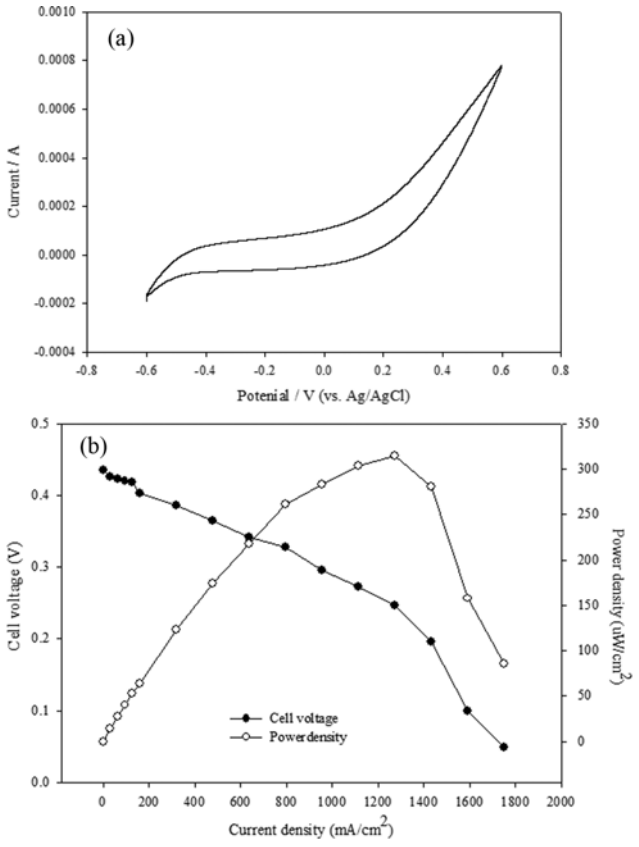

Fig. 6. Cyclic voltammetry obtained on PcCDH modified Au electrode (a). Effect of using modified bioelectrode on cell voltage and power curves in a basic EFC (b).

5. Application in Enzymatic Fuel Cell

Prepared Au electrodes consisting of PcCDH immobilized on GO/Co/chitosan were submerged in sodium acetate buffer solution and the changes of cyclic voltammetry (CV) were measured. The CV of PcCDH/Au electrode showed the oxidation and reduction peak start at 0.2 V and −0.2 V, respectively (Fig. 6(a)). The result indicated the electron was generated by lactose oxidation on PcCDH/Au electrode and then electron transferred to the cathode (laccase) for reduction. Similar tendency of CV result was also shown by previous research of *Phanerochaete sordid* modified pyrolytic graphite electrode working in 0.1 M lactose [24].

The electrode potential was scanned between −0.1 and +0.6 V at a scan rate of 100 mV/s. The performance of this EFC system was evaluated as that cell's open circuit voltage was 0.435 V and the maximum power density was 314 $\mu W/cm^2$ at 0.247 V in sodium acetate buffer with pH 4.5 at 30 °C (Fig. 6(b)). The maximum power density obtained in the EFC with the PcCDH was higher than the results of the reference report as shown in Table 3. Compared to the results reported Salaj-Kosla et al., the power density of this study was seven-times higher than this latest report [25]. The improved mediator based on the previous research [17] and the developed recombinant enzyme (PcCDH) of this study were considered as the reasons to

Table 3. Studies of enzymatic fuel cell based on CDH

Origin of CDH	Anode**	Cathode	Power density*	Reference
Dichomera saubinetii	DsCDH/SG	Laccase/SG	15	[21]
Phanerochaete sordida	PsCDH/SWCNT/PG	Pt black electrode	32	[22]
Myriococcum thermophilum	MtCDH/Os-polymer SWCNT/PG	Pt black electrode	157	[23]
Corynascus thermophilus	CtCDH/Au	Laccase/Au	135	[24]
Phanerochaete chrysosporium	PcCDH/Au	Laccase/Au	314	This study

*Unit of power density: $\mu W/cm^2$

**SG: Spectroscopic graphite; SWCNT: single wall carbon nanotube; PG: pyrolytic graphite

achieve the high power density. The result of high power density also indicated the potential development of biosensor and EFC system based on application of PcCDH. Expecting the development of material science, enzyme engineering and the advance technologies of immobilization, the prospective applications of PcCDH on electrochemical aspects can be estimated.

CONCLUSION

A gene encoding *cellobiose dehydrogenase* (CDH) from *P. chrysosporium* KCCM 60256 was cloned into pPICZαA and heterologously expressed in *P. pastoris* X-33 under the control of the AOX1 promoter. The PcCDH activity produced in BMMH culture broth reached 980 U/L in flask culture under optimized conditions, which is comparable to the level reported for other recombinant CDH produced using *P. pastoris* X-33 as the expression host, achieved in flasks. The purity of the enzyme was examined by SDS-PAGE, and a single band corresponding to a molecular weight of about 100 kDa was observed. The purified CDH specific activity was found to be 28.38 U/mg. The optimal conditions were found to be temperatures between 30 °C and 65 °C, and pH of 4.5. Therefore, the *cdh* from *P. chrysosporium* KCCM 60256 was successfully expressed in *P. pastoris* X-33 and characterized as a *cellobiose dehydrogenase*. The result of high power density also indicated the potential development of biosensor and EFC system based on application of PcCDH. Expecting the development of material science, enzyme engineering and the advance technologies of immobilization, the prospective applications of PcCDH on electrochemical aspects can be estimated.

ACKNOWLEDGEMENTS

This research was supported by a grant from the National Research Foundation of Korea (NRF), funded by the Korean Government (MSIP) (No. 2014R1A2A2A01007321), and by the Advanced Biomass R&D Center (ABC-2011-0031360) of the Global Frontier Project, funded by the Ministry of Science, ICT and Future Planning of Korea.

REFERENCES

1. B. Mathieu, B. Jean-Guy, P. Laetitia and S. Jean-C, *Microb. Cell. Fac.*, **10**, 113 (2011).
2. G. Henriksson, V. Sild, I. J. Szabó, G. Pettersson and G. Johansson, *Biochim. Biophys. Acta*, **1383**, 48 (1998).
3. Desriani, F. Stefano and S. Koji, *Biochem. Biophys. Res. Co.*, **391**, 46 (2010).
4. K. Igarashi, M. F. Verhagen, M. M. Samejima, M. Schülein and K. E. Eriksson, *J. Biol. Chem.*, **274**, 3338 (1999).
5. B. M. Hallberg, T. Bergfors, K. Backbro, G. Pettersson, G. Henriksson and C. Divne, *Structure.*, **8**, 79 (2000).
6. G. Henriksson, G. Johansson and G. Pettersson, *J. Biotechnol.*, **78**, 93 (2000).
7. E. J. Kim, H. S. Choi, S. W. Kang, K. H. Song, S. O. Han, C. Park and S. W. Kim, *Korean J. Chem. Eng.*, **29**, 77 (2012).
8. G. Henriksson, P. Ander, B. Pettersson and G. Pettersson, *Appl. Microbiol. Biot.*, **42**, 790 (1995).
9. A. Leonowicz, A. Matuszewska, J. Luterek, D. Ziegenhagen, M. Wojtaś-Wasilewska, N.-S. Cho, M. Hofrichter and J. Rogalski, *Genet. Biol.*, **27**, 175 (1999).
10. F. Huang, J. Fang, X. Lu, P. Gao and J. Chen, *Chinese Sci. Bull.*, **46**, 1956 (2001).
11. E. Kim, Y. S. Song, H. S. Choi, H. Y. Yoo, S. W. Kang, K. H. Song, S. O. Han and S. W. Kim, *Biopocess. Biotechnol. Eng.*, **17**, 55 (2012).
12. J. Sulej, G. Janusz, M. Osińska-Jaroszuk and P. Rachubik, *Appl. Biochem. Biotechnol.*, **176**, 1638 (2015).
13. R. Ludwig, R. Ortiz, C. Schulz, W. Harreither, C. Sygmund and L. Gorton, *Anal. Bioanal. Chem.*, **405**, 3637 (2013).
14. I. J. Szabo, G. Johansson and G. Pettersson, *J. Biotechnol.*, **48**, 221 (1996).
15. U. Baminger, B. Nidetzky, K. D. Kulbe and D. Haltrich, *J. Microbiol. Meth.*, **35**, 253 (1999).
16. H. U. Lee, H. Y. Yoo, T. Lkhagvasuren, Y. S. Song, C. Park, J. Kim and S. W. Kim, *Biosens. Bioelectron.*, **42**, 342 (2013).
17. D. S. Kim, S. B. Kim, X. Yang, J. H. Lee, H. Y. Yoo, Y. Chun, J. Cho, C. Park, J. Lee and S. W. Kim, *J. Electrochem. Soc.*, **162**, G113 (2015).
18. J. Y. Lee, H. Y. Shin, S. W. Kang, C. Park and S. W. Kim, *Biosens. Bioelectron.*, **26**, 2658 (2011).
19. J. L. Cereghino and J. M. Cregg, *FEMS Microbiol. Rev.*, **24**, 45 (2000).
20. R. Gachhui, A. Presta, D. F. Bentley, H. M. Abu-Soud, R. McArthur, G. Brudvig, D. K. Ghosh and D. J. Stuehr, *J. Biol. Chem.*, **271**, 594 (1996).
21. A. Leber, B. Hemmens, B. Klösch, W. Goessler, G. Raber, B. Mayer and K. Schmidt, *J. Biol. Chem.*, **274**, 58 (1999).
22. V. Coman, C. Vaz-Domínguez, R. Ludwig, W. Herreither, D. Haltrich, A. L. De Lacey, T. Ruzgas, L. Gorton and S. Shleev, *Phys. Chem. Chem. Phys.*, **10**, 6093 (2008).

23. F. Tasca, L. Gorton, W. Harreither, D. Haltrich, R. Ludwig and G. Nöll, *J. Phys. Chem. C.*, **112**, 13668 (2008).
24. F. Tasca, L. Gorton, W. Harreither, D. Haltrich, R. Ludwig and G. Nöll, *Anal. Bioanal. Chem.*, **81**, 2791 (2009).
25. U. Salaj-Kosla, M. D. Scanlon, T. Baumeister, K. Zahma, R. Ludwig, P. Ó. Conghaile, D. MacAodha and D. Leech, *Anal. Bioanal. Chem.*, **405**, 3823 (2013).
26. Z. Ruifu, Z. Fan and K. Takao, *Protein. Expres. Purif.*, **75**, 63 (2011).
27. W. Harreither, A. K. Felice, R. Paukner, L. Gorton, R. Ludwig and C. Sygmund, *Biotechnol. J.*, **7**, 1359 (2012).
28. N. Kannan and R. Aravindan, *E. J. Chem.*, **6**, 979 (2009).
29. A. Batra and R. K. Saxena, *Biochem.,* **40**, 1533 (2005).
30. M. Samejima and K. E. L. Eriksson, *Eur. J. Biochem.*, **207**, 103 (1992).
31. G. D. Jones and M. T. Wilson, *Biochem. J.*, **256**, 713 (1988).