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Short Communications

A Glucose/O_2 Biofuel Cell Using Recombinant Thermophilic Enzymes

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The utilization of recombinant thermophilic enzymes to construct a glucose/O_2 biofuel cell (BFC) workable in a wide temperature range with enhanced thermostability using thermophilic pyrroloquinoline quinone (PQQ) dependent glucose dehydrogenase (Tth-PQQGDH, active at even 100℃) and laccase (Tth-Lcs) has been studied to improve the thermostability of BFC. In this glucose/O_2 biofuel cell system, electrodes were modified with single-walled carbon nanotubes (SWCNTs) based enzyme films. The resulting electrodes demonstrated enhanced biofuel-cell performance in wide temperature range, which allows them to be used at elevated temperatures.

Keywords: Biofuel cell, Thermophilic enzyme, Carbon nanotubes, Thermostability, Electrode modification

1. INTRODUCTION

Biofuel cells (BFCs) in which biological components are incorporate with electrodes in a desired fashion have become of a great interest as one of potential candidates for clean and renewable energy conversion systems[1-5]. The biological components of BFCs, however, limit its operation only at nearly physiological conditions (20 ─ 40 ℃, neutral pH)[6-9]. Thus, the application of BFCs is inherently limited. The improvement in the thermostability of BFCs has been the objective of this study.

Here, we studied the utilization of thermophilic enzymes to construct a glucose/O_2 biofuel cell workable in a wide temperature range with enhanced thermostability using thermophilic pyrroloquinoline quinone (PQQ) dependent glucose dehydrogenase (Tth-PQQGDH) and laccase (Tth-Lcs), which were cloned from genome DNAs of Thermus thermophilus HB[10-13], for the anode and cathode, respectively. In this glucose/O_2 biofuel cell system, electrodes were modified with single-walled carbon nanotubes (SWCNTs) based enzyme films[14,15] and electron-transfer mediators efficiently shuttling electrons between the enzyme active centers and electrodes. Using engineered[16] and recombinant[17,18] proteins instead of native ones have advantages on the preparation of uniform and substantial amount of purified proteins, as well as the improvement of enzymatic properties[19,20]. To overcome the limitation of thermostability of BFCs, we focused our attention on obtaining recombinant thermophilic GDH as the anode catalyst. The genome sequencing study of a thermophilic bacterium thermus thermophilus HB8 (http://www.thermus.org/) revealed the presence of the hypothetical structural gene of PQQGDH in the organism (an open reading frame of locus tag TTHA0570). GDH harboring PQQ as its prosthetic group has been often used in biosensor[21] and BFCs[22] owing to its oxygen insensitivity and high catalytic efficiency[23]. We cloned the GDH gene fragment from genome DNA of Thermos thermophilus HB8 by PCR, expressed the GDH heterogeneously and the GDH was purified as a single protein utilizing affinity tag system.

The resulting electrodes demonstrated enhanced biofuel-cell performance in the temperature range, which allows them to be used at elevated temperatures. Obtaining thermostable enzymes opens up the opportunity for realizing a high thermostability of BFCs.

2. EXPERIMENTAL

2.1. Preparation and Biochemical Assay of Tth-PQQGDH

Genome DNA of Thermus thermophilus HB8 was purchased from Takara Bio, Japan. The whole genome sequence data was publicly available as GenBank AP008226. The hypothetical Tth-PQQGDH gene fragment, which corresponds to an open reading frame of locus tag TTHA0570, was amplified from its genome DNA with following primers: primer 1, 5'-ACATTCATATGGACCGGAGGCGCTTTCTCGT-3', primer2, 5'-ATACCAAGCTTGCCAAGGAGGCGTAGCACCC GGTC-3' and cloned between the restriction sites NdeI and HindIII of pET22b (Novagen, http://www.). Heterogeneous expression of Tth-PQQGDH in Escherichia coli BL21 (DE3) was carried out by overnight culture at 37 ℃ in auto-inducible expression medium containing 0.05 % glucose, 0.4 % lactose and 0.1 mg L^{-1} ampicillin. Harvested cells were disrupted by ultrasonic treatment and the supernatant was further subjected to nickel column chromatography to purify His-tagged enzyme. The yield of purified Tth-PQQGDH was determined by Bradford assay (Quick Start Kit, Bio-Rad). Temperature specificity of Tth-PQQGDH activity was measured by temperature-controlled electrochemical cell containing 20 mM HEPES-NaOH (pH7.5), 100 mM NaCl, 10 μM dichloroindophenol, 0.1 mM pyrroloquinoline quinone (PQQ), 1 mM $CaCl_2$, 100 mM glucose and 300 μM Tth-PQQGDH. Signals were detected

using CHI600C (CHI Instruments) with three-electrode system: Ag|AgCl|NaCl(3M) as a reference, Pt wire as a counter electrode and glassy carbon (diameter: 3 mm) as a working electrode. Constant potential of 0.1 V was loaded on the working electrode and the current value from which the background current was subtracted was calculated as catalytic activity. Me-PQQGDH (GLD-321. origin: Acinetobacter calcoaceticus) was purchased from Toyobo, Japan for the comparison with Tth-PQQGDH. Temperature dependence of Tth-PQQGDH activity was investigated by the conventional technique. The relative values of Tth-PQQGDH activity at various temperatures were measured.

2.2. Preparation and Biochemical Assay of Tth-Lcs

Genome DNA of Thermus thermophilus HB27 was purchased from NBRC (National Institute of Technology and Evaluation Biological Resource Center). Gene fragment of Tth-Lcs was introduced between NdeI and HindIII restriction sites of expression vector pET22b (Novagen) followed by the amplification from the genome DNA with primers (primer 1: 5' TAATA CGACT CACTA TAGGG 3', primer 2: 5' TAGTT ATTGC TCAGC GGTGG 3') along with PrimeStar kit (Takara bio), so that the laccase had C-terminal 6 x His tag. Expression of Tth-Lcs was carried out with BL21 (DE3) host cell having pET22b-laccase. The cell was cultured in LB medium with antibiotics at 37 ℃ and induction of the expression was started by adding 0.5 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) at optical density of 0.7 (600 nm) and the culture was continued for additional 4 hours. Harvested cell was disrupted by sonication and the soluble fraction containing recombinant Tth-PQQGDH was further purified through nickel column chromatography.

Biochemical activity of Tth-Lcs was measured spectrophotometrically in 20 mM sodium acetate buffer (pH 5.0), 1mM ABTS (2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) and 0.2 mM $CuSO_4$. Enzymatic oxidation of ABTS was monitored by the increase of absorbance at 418 nm (extinction coefficient = 36,000 M^{-1} cm^{-1}). Tth-Lcs was found to be more active at 60 ℃ than lower temperatures, indicating that it was thermophilic enzyme, being consistent with the thermophilic nature of source organism. Calculated turn-over rate (k_{cat}) of the Tth-Lcs was 1.3×10^3 (min^{-1}) at 60 ℃. The enzyme activity of Tth-Lcs at different temperatures was measured.

2.3. Preparation of Bioanode and Biocathode

Prior to use, glassy carbon electrode (GCE, 3 mm diameter) was polished with fine emery paper (600 mesh and 2000 mesh) and aqueous slurries of fine alumina powder (1.0 μm and 0.06 μm). Then it was ultrasonicated in Milli-Q water for 10 min. The SWCNTs suspension was prepared according to the previous report (Gao et al., 2007). A 2 μL of SWCNTs aqueous dispersion was dip-coated onto the clean GCE surface using a pipette and dried at ambient temperature for 1 h. A mixture of 10 μL Tth-Lcs (6.25 units mg^{-1} at 80℃) and 4 μL bovine serum albumin (BSA, 2 wt %) was first coated onto the SWCNTs-modified electrode, and then cross-linked with glutaraldehyde (GA, 2 wt %) by dropping 4 μL GA onto the enzyme-based SWCNTs-

modified GCE surface with a pipette to obtain the biocathode. The resulting electrode (Tth-Lcs/BSA/GA/SWCNTs/GCE) was dried at ambient temperature and rinsed with Milli-Q water prior to use. The Tth-PQQGDH based bioanode (Tth-PQQGDH/BSA/GA/SWCNTs/GCE) was prepared according to a similar procedure to the preparation of the biocathode by replacing Tth-Lcs with Tth-PQQGDH (10 μL, 2×10^3 units mg^{-1} at 80℃).

3. RESULTS AND DISCUSSION

3.1. Temperature dependence results

Temperature dependence of Tth-PQQGDH activity was investigated by the conventional technique[24]. The relative values of Tth-PQQGDH activity at various temperatures were measured. The activity of mesophilic (non-thermophilic) PQQGDH (Me-PQQGDH) was also measured for comparison and it showed its maximum activity around 50 ℃. Tth-PQQGDH, on the other hand, revealed the highest activity at temperature higher than 80 ℃ at which Me-PQQGDH completely lost its activity. Tth-PQQGDH would be probably still active over 100 ℃ because it holds its maximum activity even at 100 ℃, which is the highest temperature we could investigate the activity because of the experimental limitation. This is the first report on GDH that is active at 100 ℃.

The biocathode catalyst, laccase, which catalyzes the reduction of O_2 to H_2O was chosen from a thermophilic bacterium Thermos thermophilus HB27. The structural gene of Tth-Lcs was cloned from the genome DNA and the enzyme was heterogeneously expressed and purified. The enzyme activity of Tth-Lcs at different temperatures was measured. Its activity increased as the temperature was increased, and it possessed a high activity even at 85 ℃.

3.2. Electrochemical results

Fig. 1 shows a schematic illustration of the glucose/O_2 biofuel cell fabricated using 5-methylphenazinium methyl sulfate (MPMS)-mediated Tth-PQQGDH/BSA/GA/SWCNT/GCE as bioanode and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)-mediated Tth-Lcs/BSA/GA/SWCNT/GCE as biocathode (each area: 0.07 cm^2), respectively. In this case, the enzyme films were fabricated by immobilizing each thermophilic enzyme in the bovine serum albumin (BSA) matrix cross-linked by glutaraldehyde (GA) on the glassy carbon electrode (GCE) on which SWCNTs were adsorbed previously. The catholyte and anolyte compartments of the biofuel cell were saturated with oxygen and argon, respectively. The bioanode system was operated in the absence and presence of electron-transfer mediator, MPMS. The results showed the anodic current obtained in the presence of MPMS (84 μM) was 7 times higher than that obtained in its absence.

Fig. 2a shows cyclic voltammograms of the bioanode in the absence and presence of glucose (curves 1 and 2, respectively). A well-defined biocatalytic oxidation current was observed, when the glucose substrate (40 mM) was present (curve 2).

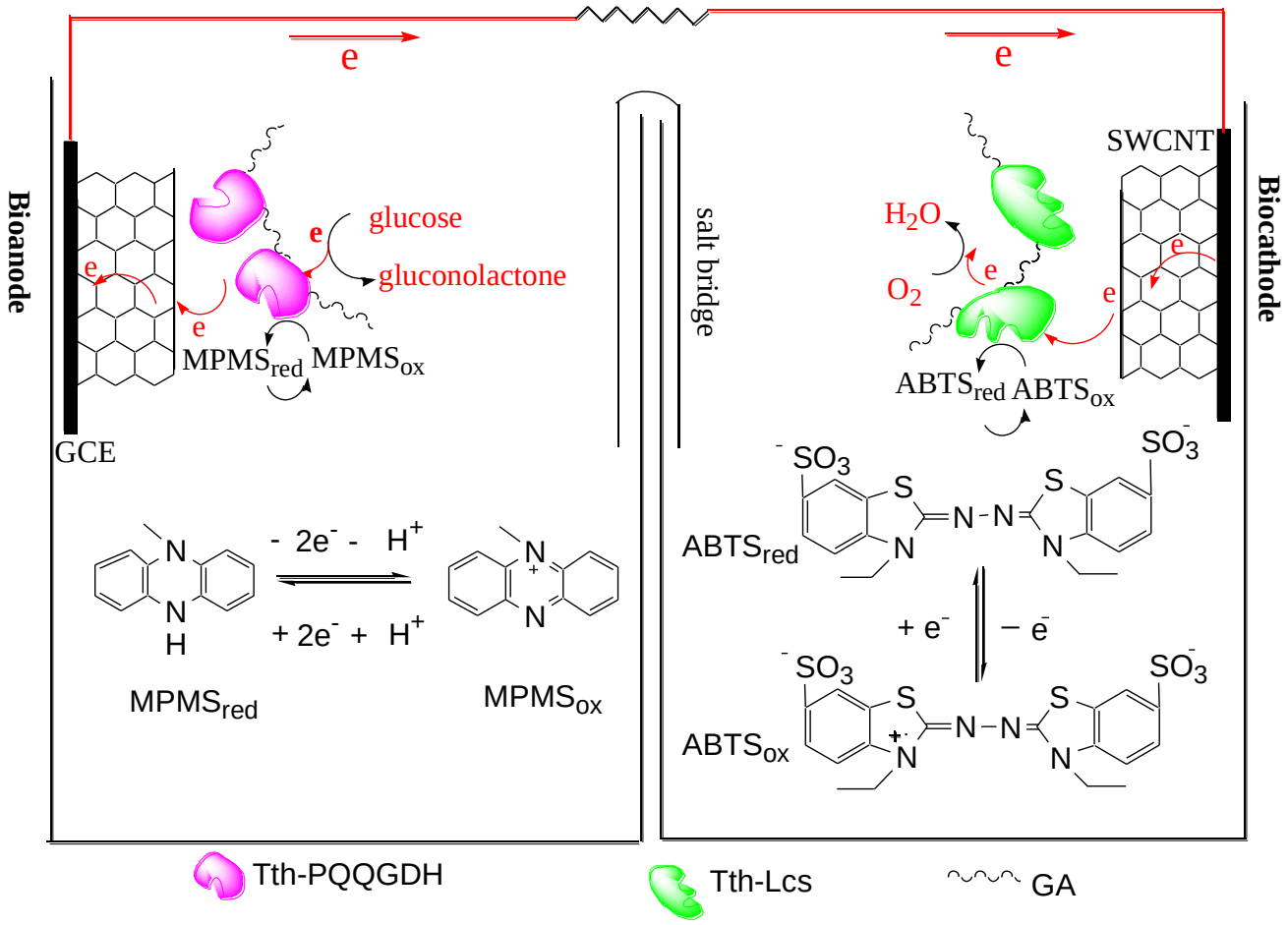

Figure 1. Schematic configuration of a glucose/O_2 biofuel cell employing MPMS-mediated Tth-PQQGDH/BSA/GA/SWCNT/GCE and ABTS-mediated Tth-Lcs/BSA/GA/SWCNT/GCE as bioanode and biocathode, respectively.

There is a pair of redox peaks in curve 1, showing the redox reaction of MPMS. The electrode response of the ABTS-mediated biocathode under the atmosphere of O_2 and Ar is shown in Fig. 2b. ABTS was found to be very efficient in maintaining a relatively high cell voltage output when the current was flowed through the biofuel cell. The biocathodic current can be only observed in the presence of ABTS (curve 1), which implying that the engineered Tth-Lcs requires electron-transfer mediator, ABTS, to efficiently shuttle electrons between the active sites and the electrode. A pair of redox waves of ABTS can be observed in the absence of oxygen (curve 2).

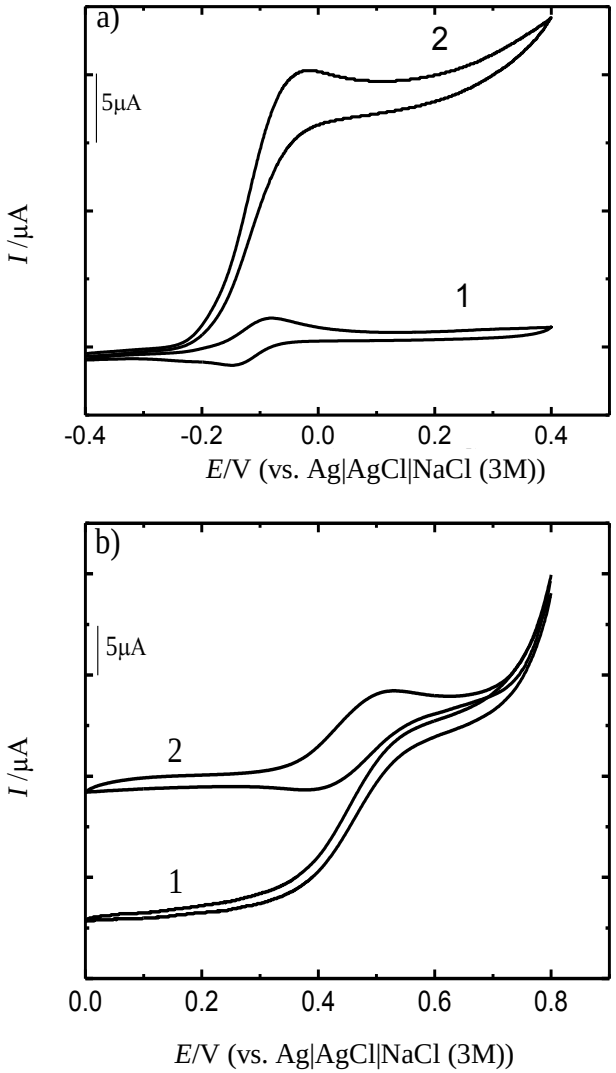

Figure 2. a) Cyclic voltammograms of Tth-PQQGDH/BSA/GA/SWCNT/GCE recorded in MOPS (0.1 M, pH 6.00) containing 0.2 mM PQQ, 4 mM $CaCl_2$ and 84 mM MPMS in the absence and presence of 40 mM glucose (curves 1 and 2, respectively). b) Cyclic voltammograms of Tth-Lcs/BSA/GA/SWCNT/GCE recorded in PBS (50 mM, pH 6.00) containing 0.5 mM ABTS under the atmosphere of O_2 and Ar (curves 1 and 2, respectively). Both were operated at 55 ℃ at a scan rate of 5 mV s^{-1}.

3.3. Evaluation of the glucose/O_2 biofuel cell

A typical current-voltage behavior of the present biofuel cell at different temperatures is shown in Fig. 3a. The enhanced performance was accomplished at elevated temperature (76 ℃) with an open circuit potential of 750－779 mV and a maximum current density of 200 μA cm^{-2}. The current density versus power density plots (Fig. 3b) indicate that the power density increased as the temperature was increased and reached a maximum (35 μW cm^{-2} at 0.3 V) at 76 ℃. The present biofuel cell was not operated at a temperature higher than 76 ℃ as the oxygen solubility decreases dramatically at higher temperatures. If this problem can be solved, e. g., by fabricating the bioelectrode systems of the so-called MEA type as in the usual proton exchange membrane

H_2/O_2 fuel cells[25-27], the present biofuel cell would still exhibit a high performance over 76 ℃ because it held a maximum activity even at 76 ℃.

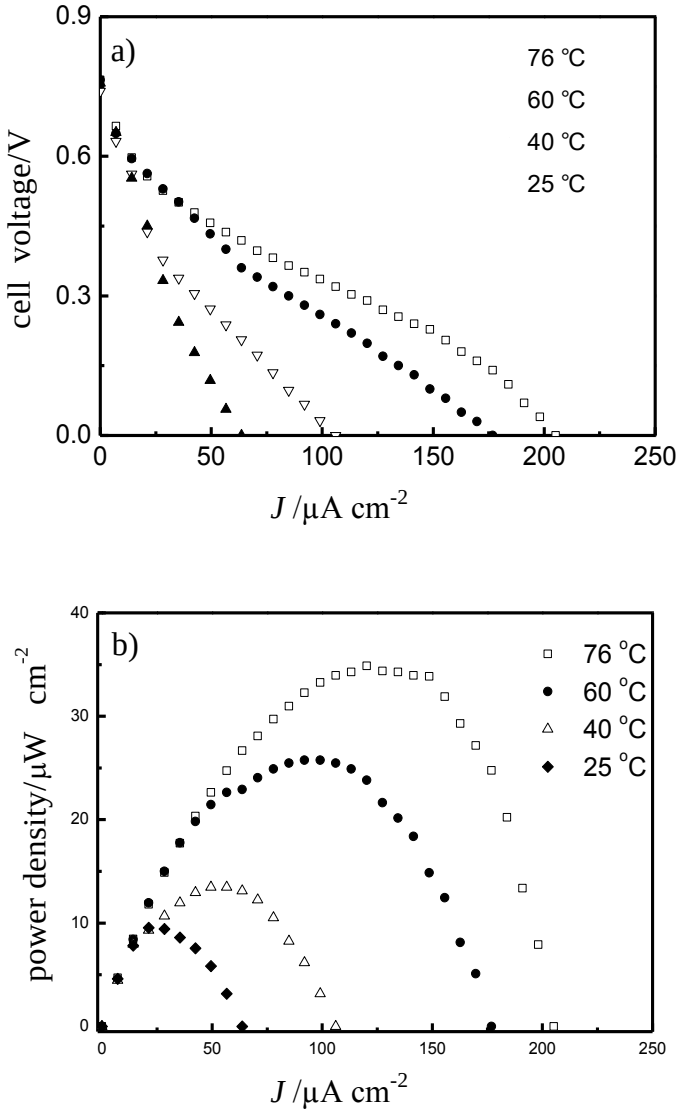

Figure 3. a) Cell voltage-current density plots and b) power density-current density plots of the glucose/O_2 biofuel cell at different temperatures.

4. CONCLUSIONS

In conclusion, this study has demonstrated a successful application of protein engineering of thermophilic enzymes to biofuel cell technology with a view to constructing enzyme-based biofuel cells that can be operated at elevated temperatures higher than physiological conditions. The use of thermophilic Tth-PQQGDH and Tth-Lcs offered an unprecedented opportunity to obviate the temperature limitation of currently used biofuel cells, probably opening a bright future in the application of protein engineering of thermophilic enzymes for realizing enhanced biofuel cell performance.

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References

1. I.Willner, *Science* 298(2002)2407.
2. A.Heller, *Phys. Chem. Chem. Phys.* 6(2004)209.
3. F.Davis and S.P.J.Higson, *Biosens. Bioelectron.* 22(2007)1224.
4. P.Kavanagh, S.Boland, P.Jenkins and D. Leech, *Fuel Cells* 9(2009)79.
5. S.Tsujimura,K.Kano and T.Ikeda, *J.Electroanal.Chem.* 576(2005)113.
6. D.Wen,L.Deng,M.Zhou,S.Guo,G.Xu and S.Dong, *Biosens. Bioelectron.* 25(2010)1544.
7. R.A.Bullen,T.C.Arnot,J.B.Lakeman and F.C.Walsh, 2006, *Biosens. Bioelectron.* 21(2006)2015.
8. M.J.Moehlenbrock and S.D.Minteer, *Chem. Soc. Rev*. 37(2008)1188.
9. S.C.Barton,J.Gallaway and P.Atanassov,2004, *Chem. Rev.* 104(2004)4867.
10. X.Li,L.Zhang,L.Su,T.Ohsaka and L.Mao, *Fuel Cells* 1(2009)85.
11. K.Miyazaki, *Extrmophiles* 9(2005)415.
12. T.Hoshino,R.Fuji and T.Nakahara, *Appl. Environ. Microb*. 59(1993)3150.
13. J.Miyazaki,N.Kobashi,M. Nishiyama and H.Yamane, *J. Biol. Chem*. 278(2003)1864.
14. X.Li,H.Zhou,P.Yu,L.Su,T.Ohsaka and L.Mao, *Electrochem. Commun*. 10(2008)851.
15. F.Gao,Y.Yan,L.Su,L.Wang and L.Mao, *Electrochem. Commun*. 9(2007)989.
16. E.Katz,I.Willner and A.B.Kotlyar, *J. Electroanal. Chem*. 479(1999)64.
17. T.S.Wong and U.Schwaneberg, *Curr. Opin. Biotechnol*. 14(2003)590.
18. O.A.Raitman,F.Patolsky,E.Katz and I.Willner, *Chem. Commun*.(2002)1936.
19. J.Okudashimazaki,N.Kakehi,T.Yamazaki,M.Tomiyama and K.Sode, *Biotechnol. Lett*. 30(2008)1753.
20. M.J.Cooney,V.Svoboda,C.Lau,G.Martin and S.D.Minteer, *Energy Environ. Sci*. 1(2008) 320.
21. V.Laurinavicius,B.Kurtinaitiene,V.Liauksminas,A.Jankauskaite,R.Simkus,M.Meskys, R.Boguslavsky,L.Skotheim and T.S.Tanenbaum, *Talanta* 52(2000)485.
22. T.Ikeda and K.Kano, *Biochim. Biophys. Acta* 1647(2003)121.
23. T.Hirokawa and K.Sode, *Biomol. Eng*. 21(2004)81.
24. A.Oubrie, *Biochim. Biophys. Acta* 1647(2003)143.
25. M.S.Saleh,T.Okajima,M.Hayase,F.Kitamura and T.Ohsaka, *J.Power Sources* 164 (2007)503.
26. K.Kwon,T.Kim,D.Yoo,S.Hong and J.Park, *J Power Sources* 188(2009)463.
27. J.Larminie and A.Dicks, Fuel Cell Systems Explained, 2nd Ed., John Wiley & Sons Ltd, England(2003).

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