International Journal of GEOMATE, Feb., 2017, Vol. 12, Issue 30, pp. 140-145
Special Issue on Science, Engineering & Environment, ISSN:2186-2990, Japan
DOI: http://dx.doi.org/10.21660/2017.30.2654

ORGANIC SOLVENT-RESISTANT PROPERTIES OF PROTEINS ADSORBED ONTO BIOMASS CHARCOAL POWDER

*Hidetaka Noritomi[1], Jumpei Nishigami[1], Nobuyuki Endo[2], Satoru Kato[1] and Katsumi Uchiyama[1]

[1]Department of Applied Chemistry, Tokyo Metropolitan University, Japan; [2]EEN Co., Ltd., Japan

*Corresponding Author, Received: 14 June 2016, Revised: 30 Nov. 2016, Accepted: 12 Dec. 2016

ABSTRACT: We have found out that biomass charcoal powder (BCP), which is prepared from plant biomass wastes by pyrolysis at low temperatures under nitrogen atmosphere, imparts organic solvent resistance to proteins by using BCP as a protein carrier. α-Chymotrypsin (α-CT), which was used as a model protein, was sufficiently adsorbed onto the surface of BCP. When free α-CT and BCP-adsorbed α-CT were immersed in acetonitrile containing 5%(v/v) water, they catalyzed the transesterification of *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) with *n*-butanol (BuOH) to produce *N*-acetyl-L-tyrosine butyl ester (*N*-Ac-Tyr-OBu), which did not proceed in an aqueous solution, where α-CT worked as a hydrolase. The initial rate of transesterification catalyzed by BCP-adsorbed α-CT was strongly dependent upon the kind of BCP, and was about fifty times higher than that catalyzed by free α-CT when bamboo charcoal powder was used as a carrier.

Keywords: Biomass Charcoal Powder, Protein, Adsorption, Organic Solvent Resistance, α-Chymotrypsin

1. INTRODUCTION

The utilization of biomass wastes for energies and functional materials is one of the most important challenges to establish a recycling society [1], [2]. However, plant biomass wastes such as forestry residue have hardly been used. To enhance the utilization of plant biomass wastes, plant biomass charcoal has been prepared by pyrolysis [3], [4]. Moreover, the development in the higher value-added function of plant biomass charcoal has been desired to provide the multiple effective utilization system of plant biomass wastes.

On the other hand, the application of proteins to environmentally benign processes such as biotransformation, biosensor, biofuel cell, and so on in aqueous and non-aqueous media has recently attracted much attention in pharmacy, biotechnology, chemical industry, and so on, since proteins exhibit their outstanding biological activities and specificities under mild conditions [5]-[7]. Especially, the biotransformation catalyzed by an enzyme, which is a kind of proteins, in non-aqueous media has been applied to numerous synthetic processes because of the following benefits [8]: 1. The solubility of non-polar reactants and products is improved. 2. Synthetic reactions can take place by use of a conventional hydrolase without an expensive energy substance such as adenosine triphosphate (ATP). 3. The stereoselectivity of enzymes is markedly altered. 4. The thermal stability of enzymes is highly improved. 5. Enzymes are easily recycled by recovering them with the filtration or the centrifugation. 6. The product is easily recovered with the evaporation when using the volatile organic solvent as a reaction medium. 7. The contamination due to the growth of microorganisms is inhibited by organic solvents. However, the enzyme tends to show the low activity in organic solvents, compared with that in water, since an organic solvent in general works as a denaturant of proteins [9]. In order to improve the performance of enzymes in organic solvents, there have been some modes of enzyme preparation such as entrapped enzymes, lyophilized enzyme powders, lipid-coated enzymes, cross-linked enzyme crystals, and so on [10]-[15]. However, those preparation modes need expensive reagents, fine techniques, or a special apparatus. On the other hand, the adsorption of proteins onto various carriers has been widely used from the laboratory scale to the industrial scale because of the simplest and most economical method of stabilizing proteins [16], [17]. The physical and chemical surface properties of carriers strongly affect the performances of adsorbed proteins such as activity, specificity, and stability. Accordingly, it is possible that the suitable selection of carriers makes enzymes exhibit their desired performances in organic solvents. However, there have been few reports regarding the performances of adsorbed enzymes in organic solvents.

In order to investigate the high value-added function of biomass charcoal powder (BCP) derived from plant biomass wastes by pyrolysis at low temperatures, we have so far examined the usefulness of BCP as a protein carrier in an aqueous solution. We have found out that proteins are effectively adsorbed onto BCP [18], [19], and BCP-adsorbed proteins exhibit the enhanced storage

stability and the high heat stress resistance in an aqueous solution, compared to free proteins [20]-[23].

In our present work, we have assessed whether BCP can effectively enhance the organic solvent resistance of proteins by using BCP as a carrier of proteins to improve the activity of proteins in an organic solvent. We have employed bovine pancreas α-chymotrypsin as a model protein, since it is well investigated regarding its structure, functions, and properties [24].

2. MATERIALS AND METHODS

2.1. Materials

α-Chymotrypsin (EC 3.4.21.1 from bovine pancreas) (type II, 52 units/mg solid)(α-CT) was purchased from Sigma-Aldrich Co. (St. Louis, USA). *N*-Acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) and *N*-acetyl-L-tyrosine (*N*-Ac-Tyr-OH) were also from Sigma-Aldrich Co. (St. Louis, USA). All solvents used were of guaranteed grade, commercially available, and were used without further purification. Before acetonitrile was used as a reaction solvent, it was dried by storing it over dry 0.3 nm molecular sieves (Wako Chemical Co.) for at least 24 h.

2.2. Preparation of Biomass Charcoal Powder (BCP)

Under nitrogen atmosphere, dumped bamboos were dried at 180 ℃ for 2 hr, were pyrolyzed at 450 ℃ for 2 hr, were carbonized at 350 ℃ for 3 hr, and then were cooled at 100 ℃ for 1 hr by pyrolyzer (EE21 Pyrolyzer, EEN Co. Ltd., Japan). Bamboo charcoal powder was obtained by grinding the resultant bamboo charcoal with jet mill (100AS, Fuji Sangyo Co. Ltd., Japan). Adzuki bean charcoal powder and wood charcoal powder were prepared by the same method.

2.3. Characterization of Biomass Charcoal Powder

The SEM micrograph was obtained using a scanning electron microscope (JSM-7500FA, JEOL, Japan) operating at 15 kV. The sample for SEM was prepared on a carbon tape without vapor deposition.

All samples were outgassed at 300ºC for 8 h prior to the nitrogen adsorption measurements. The specific surface area of BCP was calculated with the use of the Brunauer-Emmett-Teller (BET) method using a micropore system (BELSORP-mini II, BEL JAPAN, INC.).

The surface of BCP was analyzed by X-ray photoelectron spectroscopy (XPS) (Quantum-2000, ULVAC-PHI Co. Ltd.) operating at an x-ray beam size of 100 μm.

2.4. Adsorption of α-Chymotrypsin onto Biomass Charcoal Powder

As a typical procedure, 5 mL of 0.01 M phosphate buffer solution at pH 7 containing 300 μM α-CT and 3 g/L BCP was placed in a 10-mL test tube with a screw cup, and was incubated at 25 ℃ and 120 rpm for 24 h. After adsorption, the mixture was filtrated with a membrane filter (pore size: 0.1 μm, Millipore Co. Ltd.). The amount of α-CT adsorbed onto BCP was calculated by subtracting the amount of α-CT included in the supernatant liquid after adsorption from the amount of α-CT in the aqueous solution before adsorption. The amount of α-CT was measured at 280 nm by UV/vis spectrophotometer (UV-1800, Shimadzu Co. Ltd.).

2.5. Measurement of Activity of Free and BCP-Adsorbed α-Chymotrypsin

The standard reaction for transesterification was carried out as follows: Three milliliter of acetonitrile containing 5%(v/v) water, 10 mM *N*-Ac-Tyr-OEt, 1000 mM *n*-butanol, 1 mM acetanilide, and free or BCP-adsorbed α-CT (30 μM) was placed in a 4 mL screw-cap vial, and was incubated at 120 rpm and 25 ºC. The amounts of the reaction components were periodically determined with HPLC (Shimadzu LC-10A) (Shimadzu Co., Kyoto, Japan) using a TSK-GEL ODS-80TM column (Tosoh Co., Tokyo, Japan) eluted with water-acetonitrile (6:4 by volume) at 0.5 mL/min with detection at 270 nm. Acetanilide was used as an internal standard.

2.6. Measurement of Fourier Transform Infrared (FTIR) Spectroscopy

FTIR measurements of free and BCP-adsorbed α-CT were carried out using a Jasco FT/IR spectrometer model FT/IR-4100. A KBr pellet containing 0.5 mg of free or BCP-adsorbed α-CT powder per 100 mg of KBr was prepared, and the measurements were performed using 512 scans under 4.0 cm^{-1} resolution.

3. RESULTS AND DISCUSSION

3.1 Characterization of Biomass Charcoal Powder

We have pyrolyzed plant biomass wastes at low temperatures under nitrogen atmosphere to produce functional groups on the surface of biomass charcoals and to make use of the resultant functional groups as a binding site for the adsorption of proteins. In order to observe the surface of BCP, we

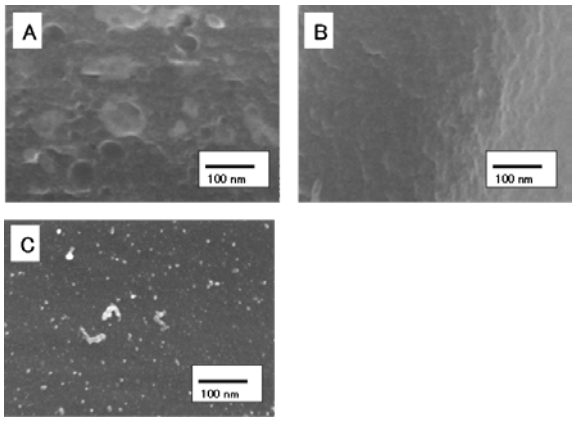

Fig. 1 SEM images of (A) adzuki bean charcoal, (B) bamboo charcoal, and (C) wood charcoal.

Table 1 Specific surface area of BCP and adsorption property of α-CT onto BCP.

Sample	Specific surface area (m^2/g)	Amount adsorbed (μmol/g)	Coating rate (%) end-on	Coating rate (%) side-on
Adzuki bean charcoal	204	17	63	80
Bamboo charcoal	294	9.8	25	32
Wood charcoal	117	21	135	172

have examined SEM images. As seen in Fig. 1, the morphology of BCP was strongly dependent upon the kind of materials. The roughness of BCP was remarkably low, and any pores were not observed at the magnification measured in the present work. The surface of bamboo charcoal was smoother than that of any other charcoal.

Table 1 shows the specific surface area of BCP obtained from low-temperature (-196 ℃) nitrogen adsorption isotherms, the amount of α-CT adsorbed onto BCP, and the coating rate, which is the ratio of the total in the cross-sectional area of α-CT molecules adsorbed on the surface of BCP to the surface area of BCP. The shape of α-CT molecule is a spheroid, the size of which is 5.1 x 4.0 x 4.0 nm [24]. In Table 1, the end-on means that the major axis direction of α-CT molecules contacts with the surface of BCP perpendicularly, while the side-on means that the minor axis direction of α-CT molecules contacts with the surface of BCP perpendicularly. α-CT molecules were sufficiently adsorbed onto the surface of BCP. The coating rate indicates that α-CT molecules are adsorbed onto adzuki bean charcoal and bamboo charcoal at monolayer, since their coating rates are less than 100%, and α-CT molecules are adsorbed onto wood charcoal at multilayer, since its coating rate is more than 100%.

In order to assess the chemical property of the surface of BCP, the measurement on X-ray photoelectron spectroscopy (XPS) was carried out. Figure 2 shows the elemental ratio of the surface of BCP detected by XPS. The main element was carbon atom, and oxygen and nitrogen atoms were also located on the surface of BCP to some extent.

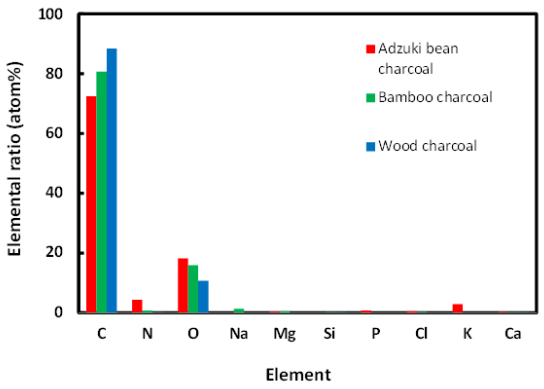

Fig. 2 The elemental ratio of the surface of BCP detected by XPS.

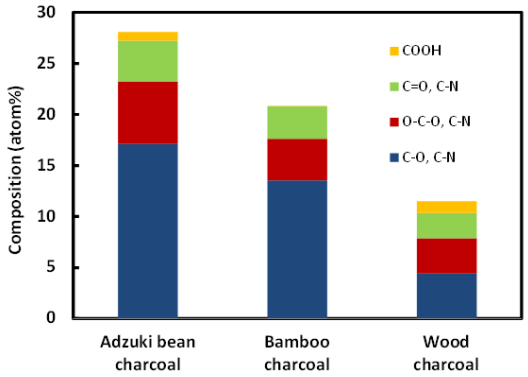

Fig. 3 The chemical bond ratio of BCP obtained from narrow scan spectra of XPS.

The ratios of oxygen and nitrogen atoms in adzuki bean charcoal were greater than those in any other charcoal. From narrow scan spectra of XPS, the chemical states of carbon were mainly C-C and C-H, while as chemical states of carbon with oxygen and nitrogen, C-O, O-C-O, C=O, COOH, and C-N were detected, as shown in Fig.3. Many radical species due to functional groups containing oxygen atoms, which are formed by thermal decomposition of cellulose and hemicelluloses, are detected in charcoals carbonized at 500°C by the measurement of electron spin resonance, and functional groups decrease with increasing carbonization temperature [25],[26]. On the other hand, the ζ-potential of BCP was negative at pH 7 while that of α-CT was positive due to the isoelectric point of α-CT (9.1) [18],[24]. Consequently, it is suggested that α-CT molecules are adsorbed onto the surface of BCP mainly through the electrostatic force which tends to strengthen the adsorption due to the low dielectric constant in acetonitrile, compared to that in water [27].

3.2. Activity of BCP-Adsorbed α-Chymotrypsin

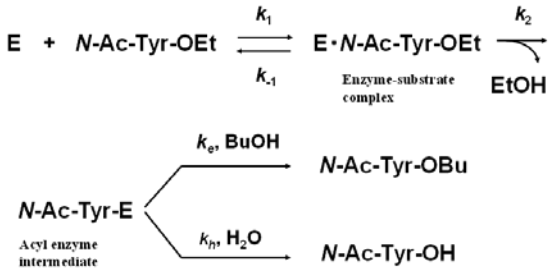

Fig. 4 α-CT-catalyzed transesterification of *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) with *n*-butanol (BuOH) to *N*-acetyl-L-tyrosine butyl ester (*N*-Ac-Tyr-OBu) and competing hydrolysis (*N*-Ac-Tyr-OH).

In an aqueous solution, a synthase and an expensive energy substance such as adenosine triphosphate (ATP) are needed to carry out the enzymatic synthetic reaction. On the other hand, in an organic solvent, hydrolases such as lipase, protease, and so on can function as a synthase without energy substances [8]. In the present work, α-CT-catalyzed transesterification was examined as shown in Fig. 4. When *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) is used as a substrate, the transesterification is a kinetically controlled reaction process. This process involves the competitive distribution of the rapidly formed acyl enzyme intermediate (*N*-Ac-Tyr-E) between water (hydrolysis) and *n*-butanol (BuOH) as another nucleophilic reagent (transesterification). Since the nucleophilic reaction is rate-determining step, and k_1, k_{-1}, and k_2 are much greater than k_e and k_h [28], the initial rates are shown as

$$\boldsymbol{V_e = k_e[N-Ac-Tyr-E][BuOH]} \quad (1)$$
$$\boldsymbol{V_h = k_h[N-Ac-Tyr-E][H_2O]} \quad (2)$$

where V_e is the initial rate of transesterification, V_h the initial rate of hydrolysis, k_e the rate constant of transesterification, and k_h the rate constant of hydrolysis. From Eqs. (1) and (2), the selectivity (k_e/k_h) is derivated as the following equation.

$$\frac{\boldsymbol{k_e}}{\boldsymbol{k_h}} = \frac{\boldsymbol{V_e[H_2O]}}{\boldsymbol{V_h[BuOH]}} \quad (3)$$

In an aqueous solution, the activity, specificity, and stability of enzymes adsorbed onto the surface of carriers markedly depend upon the chemical and physical properties in the surface of carriers [16], [17]. In order to evaluate the effect of the surface properties of carriers on the activity of adsorbed enzymes in an organic solvent, we have examined the transesterification catalyzed by α-CT adsorbed onto the different kind of BCP in acetonitrile containing 5%(v/v) water. Figure 5 shows the initial rates of *N*-acetyl-L-tyrosine butyl ester (*N*-Ac-Tyr-OBu) and *N*-acetyl-L-tyrosine (*N*-Ac-Tyr-OH) catalyzed by free and BCP-adsorbed α-CT in acetonitrile containing 5%(v/v) water. Both initial rates of *N*-Ac-Tyr-OBu and *N*-Ac-Tyr-OH catalyzed by BCP-adsorbed α-CT were much higher than those catalyzed by free α-CT. α-CT adsorbed onto bamboo charcoal powder was the most effective of all, with respect to the enhancement in the initial rates of *N*-Ac-Tyr-OBu and *N*-Ac-Tyr-OH. With regard to the transesterification catalyzed by hydrolase, which is characteristic of the non-aqueous enzymology, the initial rate of *N*-Ac-Tyr-OBu catalyzed by α-CT adsorbed onto bamboo charcoal powder was about fifty times higher than that catalyzed by free one. Enzymes are aggregated in an organic solvent, and most of them can not directly come in contact with the bulk organic phase containing substrates, although they are soluble in an aqueous solution. On the other hand, most of the enzymes adsorbed onto BCP are directly in contact with the bulk organic phase, since they are located on the surface of BCP.

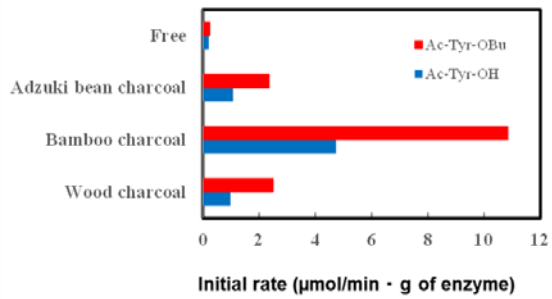

Fig. 5 Dependence of kind of BCP on BCP-adsorbed α-CT-catalyzed transesterification and hydrolysis. Free or BCP-adsorbed α-CT was placed in acetonitrile containing 5% (v/v) water, 10 mM *N*-Ac-Tyr-OEt, 1000 mM BuOH, and 1 mM acetanilide, and the resulting mixture was shaken at 120 rpm and 25 °C.

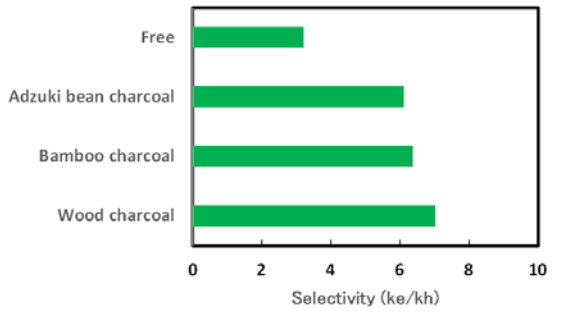

Fig. 6 Dependence of kind of BCP on the selectivity of BCP-adsorbed α-CT-catalyzed transesterification. Free or BCP-adsorbed α-CT was placed in acetonitrile containing 5% (v/v) water, 10 mM *N*-Ac-Tyr-OEt, 1000 mM BuOH, and 1 mM acetanilide, and the resulting mixture was shaken at 120 rpm and 25 °C.

Accordingly, BCP-adsorbed enzymes can effectively proceed the reaction, compared to free enzymes, since mass transfer of substrates and products is rapidly facilitated [29].

Figure 6 shows the selectivity of the reaction catalyzed by free and BCP-adsorbed α-CT in acetonitrile containing 5%(v/v) water. The selectivity corresponds to the transesterification preference. The selectivity of BCP-adsorbed α-CT was superior to that of free one. A hydrophilic organic solvent such as acetonitrile is a good solvent for an amino acid derivative, which is an important intermediate in fine chemistry, but tends to denature proteins strongly [8], [10]. It appears that the structure and microenvironment of the active site in BCP-adsorbed α-CT is suitable for the transesterification, compared to that in free one.

3.3. Secondary Structure of BCP-Adsorbed α-Chymotrypsin

In order to elucidate the influence of adsorption on the secondary structure of α-CT, we have measured the FTIR spectra of free and BCP-adsorbed α-CT. The most sensitive spectral region to protein secondary structural components is amide I (1700 – 1600 cm^{-1}), which is due to the C=O stretching vibrations of the peptide linkages [30]. Table 2 shows the ratio of the absorbance at 1650 cm^{-1} to the absorbance at 1630 cm^{-1} (ABS_{1650}/ABS_{1630}) of free α-CT and α-CT adsorbed onto BCP. The band located at ca. 1650 cm^{-1} is assignable to α-helix, and the band located at ca. 1630 cm^{-1} is assignable to intramolecular β-sheet. The order of the ABS_{1650}/ABS_{1630} ratio was bamboo charcoal-adsorbed α-CT > adzuki bean charcoal-adsorbed α-CT = wood charcoal-adsorbed α-CT > free α-CT. The order of the ABS_{1650}/ABS_{1630} ratio was similar to that of the initial rate of transesterification. The α-helical structure of α-CT molecule is more changeable than β-sheet, since the β-sheet structure is the main backbone of α-CT molecule [24]. Thus, the results indicate that at the higher initial rate the transesterification is catalyzed by α-CT molecules having the secondary structure kept more highly. Moreover, it is suggested that the content of functional groups in bamboo charcoal is suitable to keep the secondary structure of α-CT in acetonitrile, since functional groups contribute to the adsorption of α-CT onto BCP.

Table 2 Ratio of the absorbance at 1650 cm^{-1} to the absorbance at 1630 cm^{-1} (ABS_{1650}/ABS_{1630}) of α- CT provided by the FTIR measurement.

Sample	ABS_{1650}/ABS_{1630} (-)
Free α-CT	1.1
Adzuki bean charcoal-adsorbed α-CT	1.3
Bamboo charcoal-adsorbed α-CT	1.5
Wood charcoal-adsorbed α-CT	1.3

4. CONCLUSION

We have demonstrated that the adsorption of α-CT onto BCP markedly improves the organic solvent resistance of α-CT. BCP exhibited the sufficient adsorption capacity of α-CT. In acetonitrile, the catalytic activity of BCP-adsorbed α-CT was much superior to that of free α-CT. Especially, bamboo charcoal improved the more effective organic solvent resistance of α-CT than any other charcoal. In acetonitrile, BCP-adsorbed α-CT exhibited an excellent transesterification preference, compared to free α-CT. Moreover, the secondary structure of BCP-adsorbed α-CT was greater than that of free α-CT. Consequently, the enhancement in the catalytic activity of BCP-adsorbed α-CT results not only from the facilitation in the mass transfer of substrates and products due to the inhibition of aggregation of α-CT molecules but also from the high secondary structure of α-CT molecules adsorbed onto BCP.

5. REFERENCES

[1] Ho YC, Show KY, "A perspective in renewable energy production from biomass pyrolysis-challenges and prospects", Current Organic Chem., Vol. 19, 2015, pp. 423-436.

[2] Straathof AJ, "Transformation of biomass into commodity chemicals using enzymes or cells", Chem. Rev., Vol. 114, 2014, pp. 1871-1908.

[3] Peterson SC, Jackson MA, Appell M, "Biochar: Sustainable and versatile", ACS Symposium Series, Vol. 1143, 2013, pp. 193-205.

[4] Manya JJ, "Pyrolysis for biochar purposes: A review to establish current knowledge gaps and research needs", Environmental Sci. Technol., Vol. 46, 2012, pp. 7939-7954.

[5] Buchholz K, Kasche V, Bornscheuer UT, Biocatalyst and Enzyme Technology 2nd ed. Wiley-Blackwell, 2012.

[6] Silwana B, Horst CVD, Iwuoha E, "Amperometric determination of cadmium, lead, and mercury metal ions using a novel polymer immobilized horseradish peroxidase biosensor system", J. Environmental Sci. Health Part A, Vol. 49, 2014, pp.1501-1511.

[7] Leech D, Kavanagh P, Schuhmann W, "Enzymatic fuel cells: Recent progress", Electrochimica Acta, Vol. 84, 2012, pp. 223-234.

[8] Klibanov AM, "Improving enzymes by using them in organic solvents", Nature, Vol. 409, 2001, pp. 241-246.

[9] Lehninger AL, Nelson DL, Cox MM, Principles of Biochemistry 2nd ed. Worth, 1993, p. 180.

[10] Kise H, Hayakawa A, Noritomi H, "Protease-catalyzed synthetic reactions and immobilization-activation of the enzymes in hydrophilic organic solvents", J. Biotechnol., Vol. 14, 1990, pp. 239-254.
[11] Noritomi H, Almarsson O, Barletta GL, Klibanov AM, "The influence of the mode of enzyme preparation on enzymatic enantioselectivity in organic solvents and its temperature dependence", Biotechnol. Bioeng., Vol. 51, 1996, pp. 95-99.
[12] Kamiya N, Kasagi H, Inoue M, Kusunoki K, Goto M, "Enantioselective recognition mechanism of secondary alcohol by surfactant-coated lipases in nonaqueous media", Biotechnol. Bioeng., Vol. 65, 1999, pp. 227-232.
[13] St. Clair NL, Navia MA, "Cross-linked enzyme crystals as robust biocatalysts", J. Am. Chem. Soc., Vol. 114, 1992, pp. 7314-16.
[14] Wescott CR, Noritomi H, Klibanov AM, "Rational control of enzymatic enantioselectivity through solvation thermodynamics", J. Am. Chem. Soc., Vol. 118, 1996, pp. 10365-10370.
[15] Noritomi H, Sasanuma A., Kato S, Nagahama K, "Catalytic properties of , Biotechnol. Tech. 12 (1998), 467-469.cross-linked enzyme crystals in organic media", Vol. 33, 2007, pp. 228-231.
[16] Elnashar MMM, "Review article: Immobilized molecules using biomaterials and nanobiotechnology", J. Biomaterials Nanobiotechnol., Vol. 1, 2010, pp. 61-77.
[17] Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lorente R, "Improvement of enzyme activity, stability and selectivity via immobilization techniques", Enzyme Microbial Technol., Vol. 40, 2007, pp. 1451-1463.
[18] Noritomi H, Iwai D, Kai R, Tanaka M, Kato S, "Adsorption of lysozyme on biomass charcoal powder prepared from plant biomass wastes", J. Chem. Eng. Jpn., Vol.v46, 2013, pp. 196-200.
[19] Noritomi H, Hishinuma K, Kurihara S, Nishigami J, Takemoto T, Endo N, Kato S, "Adsorption of α-chymotrypsin on plant biomass charcoal", J. Surface Eng. Materials Adv. Technol., Vol. 3, 2013, pp. 269-274.
[20] Noritomi H, Kai R, Iwai D, Tanaka H, Kamiya R, Tanaka M, Muneki K, Kato S, "Increase in thermal stability of proteins adsorbed on biomass charcoal powder prepared from plant biomass wastes", J. Biomedical Sci. Eng., Vol. 4, 2011, pp. 692-698.
[21] Noritomi H, Ishiyama R, Kai R, Iwai D, Tanaka M, Kato S, "Immobilization of lysozyme on biomass charcoal powder derived from plant biomass wastes", J. Biomaterials Nanobiotechnol., Vol 3, 2012, pp. 446-451.
[22] Noritomi H, Kurihara S, Endo N, Kato S, "Heat-resistant properties of α-chymotrypsin adsorbed onto biomass charcoal powder", J. Biomaterials Nanobiotechnol., Vol. 5, 2014, pp. 179-185.
[23] Noritomi H, Kurihara S, Endo N, Kato S, Uchiyama K, "Effect of adsorption condition on thermal stability of proteins adsorbed onto biomass charcoal powder", International J. GEOMATE, Vol. 11, 2016, pp. 2123-2128.
[24] Kumar A, Venkatesu P, "Overview of the stability of α-chymotrypsin in different solvent media", Chemical Reviews, Vol. 112, 2012, pp. 4283-4307.
[25] Asada T, Ishihara S, Yamane T, Toba A, Yamada A, Oikawa K, "Science of bamboo charcoal: study on carbonizing temperature of bamboo charcoal and removal capability of harmful gases," Journal of Health Science, Vol. 48, 2002, pp. 473-479.
[26] Nishimiya K, Hata T, Imamura Y, Ishihara S, "Analysis of chemical structure of wood charcoal by X-ray photoelectron spectroscopy," Journal of Wood Science, Vol. 44, 1998, pp. 56-61.
[27] Reichardt C, Solvents and Solvent Effects in Organic Chemistry 2nd ed. Wiley-VCH, 1988, p. 408.
[28] Fersht A, Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding, W. H. Freeman and Company, 1999, pp. 216-244.
[29] Fogler HS, Elements of Chemical Reaction Engineering 2nd ed. Prentice Hall PTR, 1992, pp. 607-628.
[30] Surewicz WK, Mantsch HH, "New insight into protein secondary structure from resolution-enhanced infrared spectra", Biochim. Biophys. Acta, Vol. 952, 1988, pp. 115-130.