Chapter

Application of Biochar to Enzyme Carrier for Stress Tolerance of Enzymes

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Abstract

Biochar showed the high affinity to enzymes, and enzymes were sufficiently adsorbed on the surface of biochar. Enzymes were highly stabilized in water at high temperatures by adsorbing enzymes on biochar. The remaining activity of lysozyme adsorbed on adzuki bean charcoal showed around 50% after heat treatment at 90°C for 30 min though that of free lysozyme was almost lost. Likewise, the stability of enzymes was enhanced in organic solvents by adsorbing enzymes on biochar. The conformation of α-chymotrypsin adsorbed on bamboo charcoal was hardly influenced by organic solvents, while that of free α-chymotrypsin was strongly dependent of the kind of organic solvents. Moreover, the adsorption of α-chymotrypsin on bamboo charcoal improved the transesterification of *N*-acetyl-L-tyrosine ethyl ester with *n*-butanol in organic solvents. The transesterification rate of α-chymotrypsin adsorbed on bamboo charcoal was about 760 times higher than that of free α-chymotrypsin in *n*-butyl acetate.

Keywords: biochar, enzyme, enzyme carrier, adsorption, stress tolerance

1. Introduction

An enormous amount of greenhouse gas such as CO_2 has recently been emitted from industries and thereby has caused serious global warming problems [1, 2]. Accordingly, the application of biomass materials, which are carbon neutral, to energies and functional materials, is crucial to reduce greenhouse gas emissions [3, 4]. However, most of biomass materials such as forestry residues have hardly been utilized in the field of functional materials. Accordingly, the development in the high value-added application of biomass materials has been desired to provide the multiple effective utilization system of biomass materials.

Enzymes are biocatalysts, which exhibit their outstanding biological activity under mild conditions, and have widely been used in pharmacy, biotechnology, and chemical industry [5–7]. Typical applications of enzymes are biotransformation, biosensor, biofuel cell, and so on. Enzymes are generally stable in a cell. However, they are gradually denatured and inactivated under various physical and chemical stresses such as heat, organic solvents, and so on [8]. In order to enhance the stability of enzymes used in vitro, enzyme immobilization, where enzyme molecules are attached to solid carriers, has widely been used [9–11]. The main required features of enzyme carriers are chemical stability, thermal stability, insolubility under reaction

conditions, high affinity to enzymes, biocompatibility, the presence of reactive functional groups, availability, low price, regeneration, reusability, and so on. When enzymes are immobilized on carriers through adsorption, the catalytic activity, specificity, and stability of enzymes are influenced by the nature of carriers. Accordingly, the performance of enzymes can be enhanced by selecting an appropriate carrier.

A large quantity of the world's oldest biochar was excavated from the cave of Kara Iwatani of Hijikawacho, Ozu-shi, Ehime, Japan, with a beast bone and the human bone piece 300,000 years ago. Since the ancient period, the biochar has been used not only as a fuel but also as a soil conditioner to support human life for a long time in the world [12]. Consequently, as the biocompatibility of biochar can be expected, we have examined the application of biochar to enzyme carriers. As a result, we have found that enzymes are effectively adsorbed on biochar [13, 14], and biochar-adsorbed enzymes exhibit the high thermal stability in water [15–18]. Moreover, we have reported that the adsorption of enzymes on biochar sufficiently improves the enzyme activity in organic solvents [19–21].

In the chapter, the characterization of biochar, the adsorption of enzyme on biochar, the high temperature-tolerant property of biochar-adsorbed enzymes, and the organic solvent-tolerant property of biochar-adsorbed enzymes are discussed.

2. Heat tolerance of biochar-adsorbed enzyme

2.1 Preparation and characterization of biochar

Biochar has been prepared by pyrolyzing plant biomass waste such as bamboo waste at low temperatures under nitrogen atmosphere to produce functional groups, which were used as a binding site for the adsorption of enzymes (**Figure 1**) [13, 14]. The emission of carbon dioxide was reduced since plant biomass waste was not burned through the preparation of biochar. Moreover, the energy cost of the present preparation of biochar was suppressed, compared to that of the conventional preparation of charcoal. Consequently, the present preparation of biochar was a low-cost and environmentally benign process.

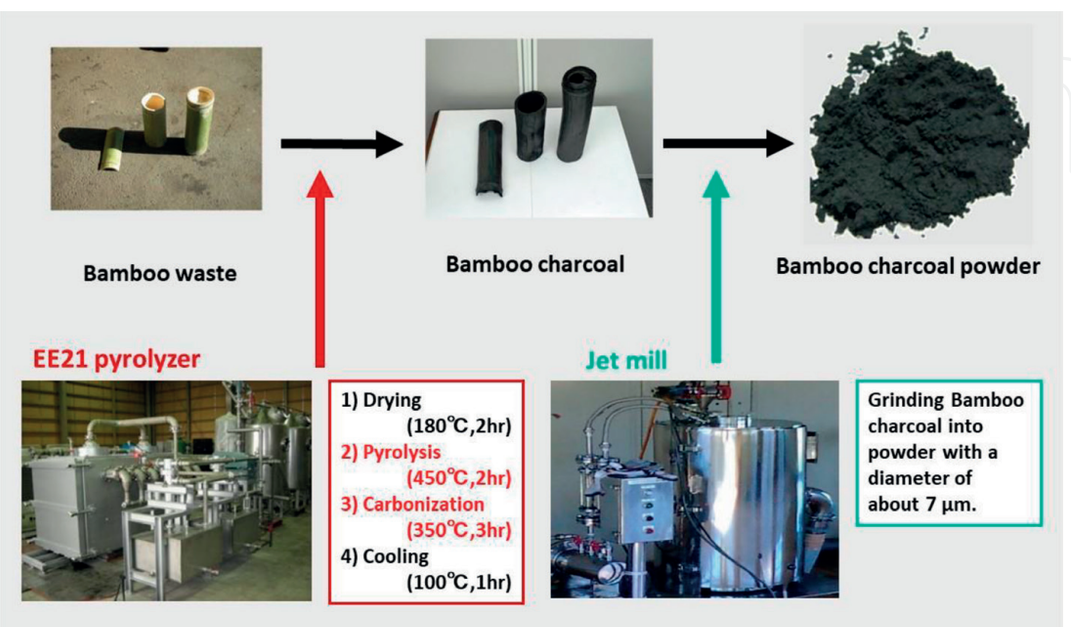

Figure 1.
Preparation process of biochar.

In order to observe the surface of biochar, we have examined SEM images [14]. As seen in **Figure 2**, the morphology of biochar was strongly dependent upon the kind of raw materials. The roughness of biochar 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. Moreover, Raman spectra of biochar showed that the structure of biochar was amorphous [13].

Table 1 shows the textural parameters of biochar obtained from low-temperature (−196°C) nitrogen adsorption isotherms, which allow the calculation of specific surface area, specific pore volume, and pore diameter peak [14]. In the table, the specific area of adzuki bean charcoal depicted the value obtained from the CO_2 isotherm. The specific surface area and specific pore volume of biochar showed much small, compared with that of conventional activated carbon. The carbonizing temperature affects the surface property of charcoal [22]. The specific pore volume increases with an increase in carbonizing temperature. Consequently, the pore of biochar was not formed enough at low temperatures.

Figure 3 shows CP/MAS ^{13}C-NMR spectra of biochar [13]. Aromatic carbon (140–141, 131 ppm) was mainly detected, and C=O (200 ppm), COOH, CHO (175–190 ppm), and aromatic oxygen (150–153, 145–146 ppm) were also detected. Moreover, in order to assess the chemical property of the surface of biochar, the measurement on X-ray photoelectron spectroscopy (XPS) was carried out [19]. **Figure 4** shows the elemental ratio of the surface of biochar detected by XPS. The main element was carbon atom, and oxygen and nitrogen atoms also existed on the surface of biochar to some extent. The ratios of oxygen and nitrogen atoms in adzuki bean charcoal were greater than those in any other charcoal. Narrow scan spectra of XPS showed C—C, C—H, C—O, O—C—O, C=O, COOH, and C—N, as seen in **Figure 5**. 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 [22, 23].

Figure 6 shows the relationship of the ζ potential of biochar with the solution pH [15]. The ζ potential of adzuki bean charcoal drastically decreased with

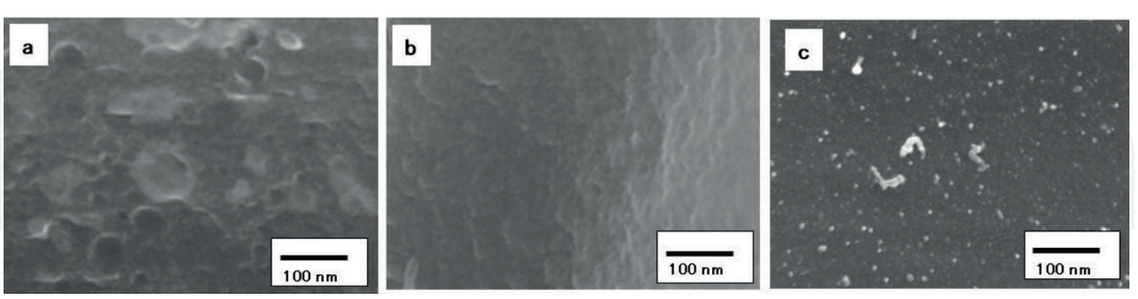

Figure 2.
SEM images of (a) adzuki bean charcoal, (b) bamboo charcoal, and (c) wood charcoal.

Biochar	Specific surface area [m^2/g]	Pore volume [cm^3/g]	Pore diameter peak [nm]
Adzuki bean charcoal	204[a]	—	—
Bamboo charcoal	294	0.041	Less than 2.6
Wood charcoal	117	0.025	Less than 2.6

[a]*Specific area of adzuki bean charcoal was obtained from the CO_2 isotherm.*

Table 1.
Structural characteristics of biochar.

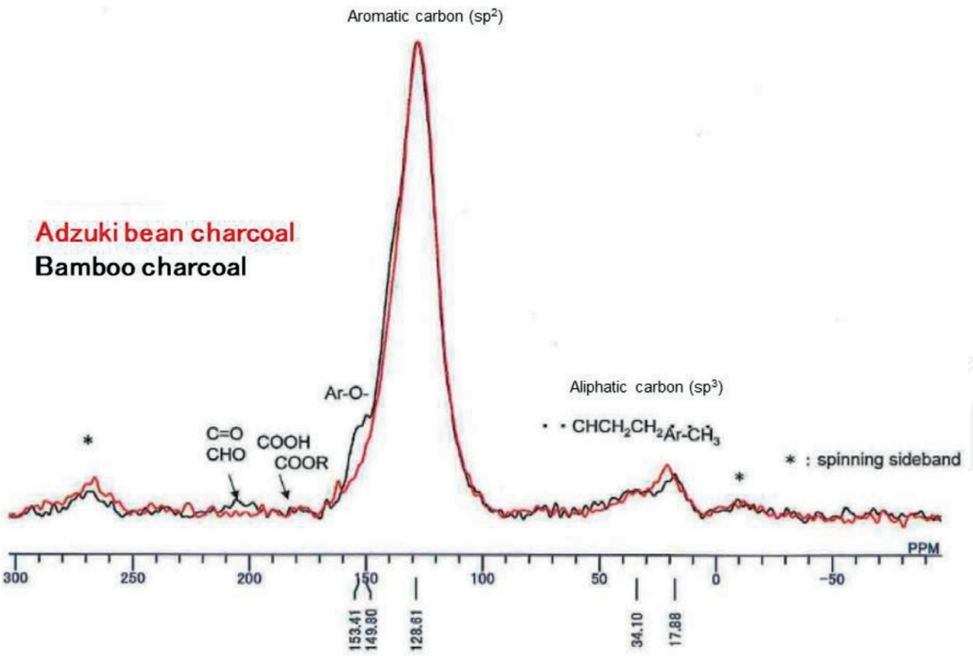

Figure 3.
CP/MAS ^{13}C-NMR spectra of biochar.

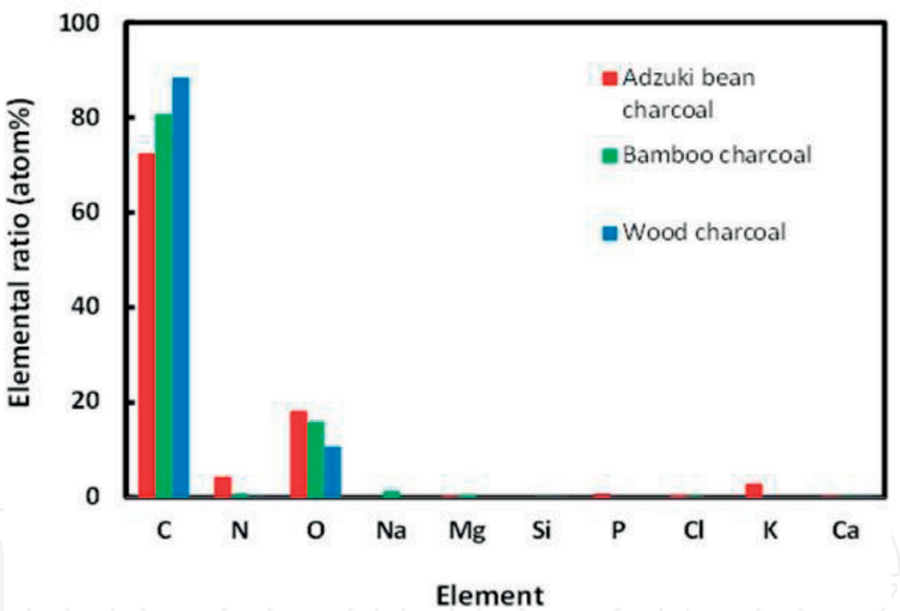

Figure 4.
The elemental ratio of the surface of biochar detected by XPS.

increasing the pH value, exhibiting a negative value above pH 4, drops till pH 7, and was almost constant in the alkaline region. The pH dependence of the ζ potential of bamboo charcoal exhibited the same tendency to the case of adzuki bean charcoal.

2.2 Adsorption of enzymes on biochar

Figure 7 shows the time course of the amount of lysozyme adsorbed on adzuki bean charcoal at pH 7 and 25°C when hen egg white lysozyme was employed as a model enzyme [16]. The amount of lysozyme adsorbed on adzuki bean charcoal increased with an increase in adsorption time, reached a plateau around 24 h, and

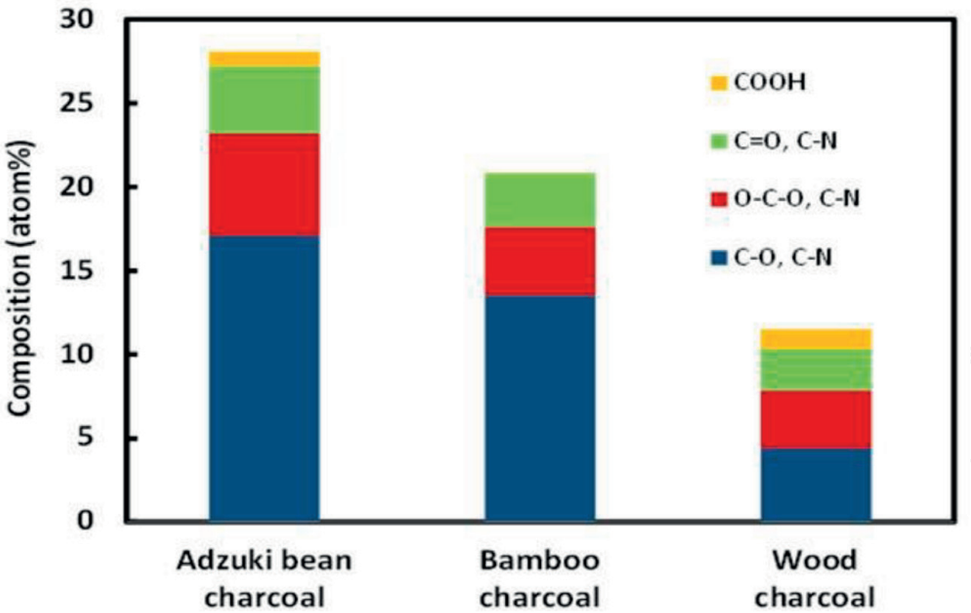

Figure 5.
The chemical bond ratio of biochar obtained from narrow scan spectra of XPS.

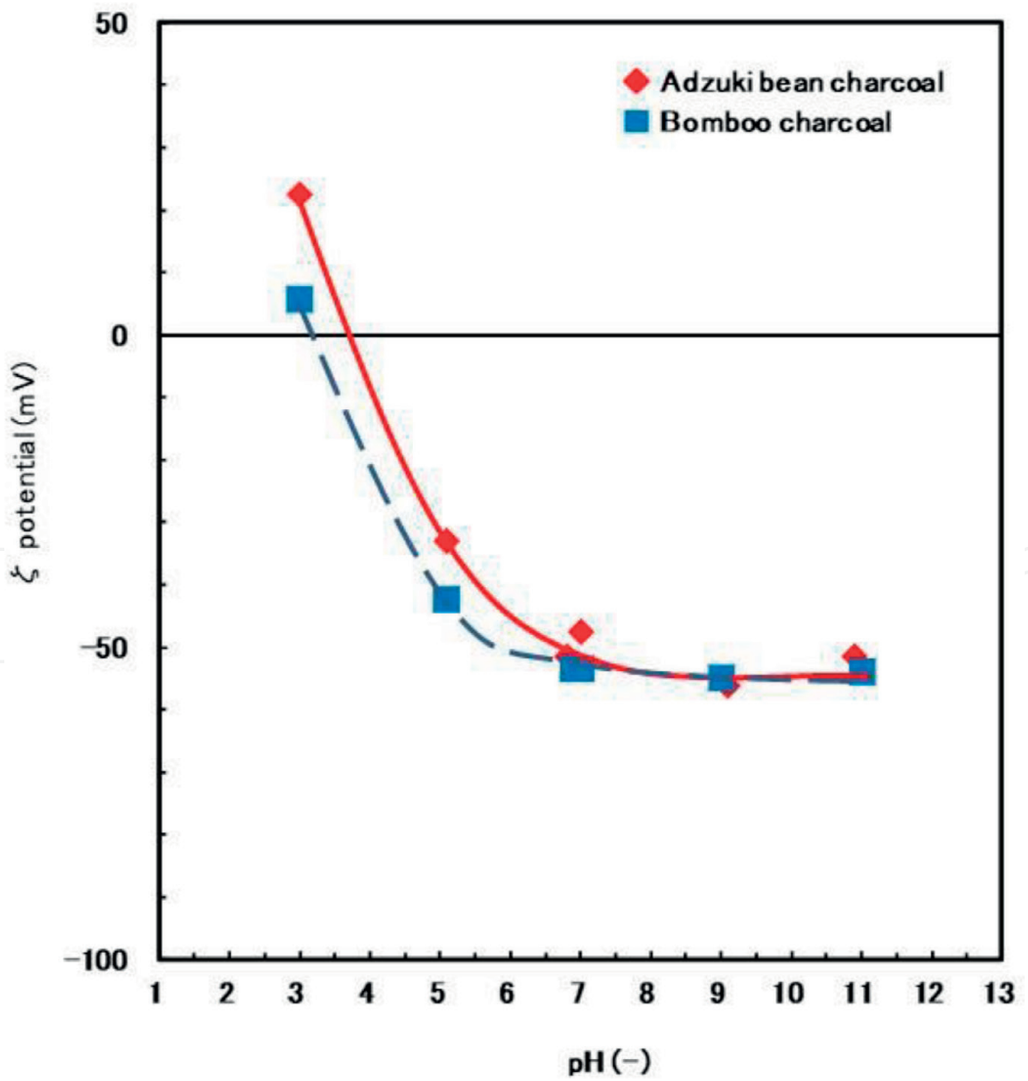

Figure 6.
Effect of solution pH on ζ potential of biochar.

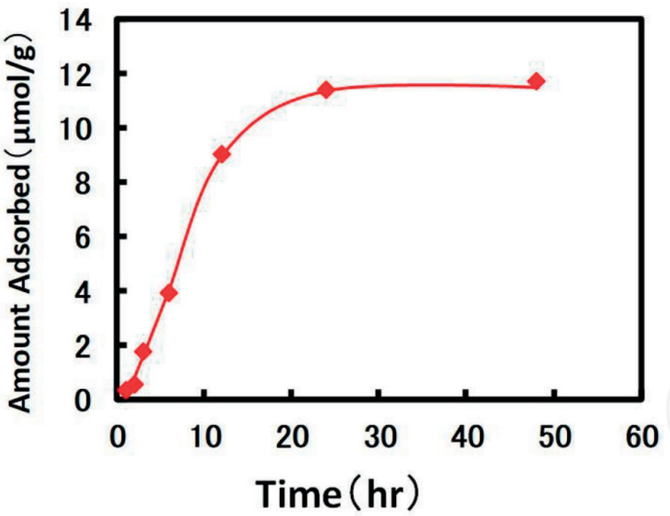

Figure 7.
Time dependence of amount of lysozyme adsorbed onto adzuki bean charcoal.

Biochar	Amount of enzymes adsorbed (μmol/g)	
	Lysozyme	α-Chymotrypsin
Adzuki bean charcoal	11	17
Bamboo charcoal	9	9.8
Wood charcoal	12	21

Table 2.
Amount of enzymes adsorbed on biochar.

was 11 μmol/g (0.16 g/g). As overall concentration of adzuki bean charcoal was 3 g/L in an aqueous solution, overall lysozyme concentration in the aqueous solution corresponded to 33 μM (0.48 mg/mL). From this result, the adsorption of lysozyme on adzuki bean charcoal was carried out for 24 h.

As seen in **Table 2**, the amount of enzymes adsorbed on biochar was strongly dependent on the kinds of enzymes and/or biochars [14, 15]. The amount of lysozyme adsorbed on biochar was almost the same among three different charcoals although the specific surface area of adzuki bean charcoal or bamboo charcoal was more than twice larger than wood charcoal, as seen in **Table 1**.

Figure 8 shows the adsorption isotherms of lysozyme on biochar. These isotherms gradually increased [13]. The amount adsorbed on adzuki bean charcoal exhibited large, compared to the amount adsorbed on bamboo charcoal. The curves in the figure were the fitting lines with Freundlich adsorption isotherm equation (Eq. 1).

$$W = K_F C^{1/n} \quad (1)$$

Here, W and C are the amount adsorbed and the concentration of lysozyme, respectively. K_F and n are experimental constants [24]. The curves of adzuki bean charcoal and bamboo charcoal had the correlation constants (r^2) of 0.974 and 0.998, respectively. On the other hand, the data were fitted by Langmuir adsorption isotherm equation; the curves of adzuki bean charcoal and bamboo charcoal depicted the correlation constants (r^2) of 0.721 and 0.694, respectively.

Figure 9 shows the relationship of the amount of lysozyme adsorbed on biochar with the pH value of aqueous solutions at 25°C [14]. The curve of the amount

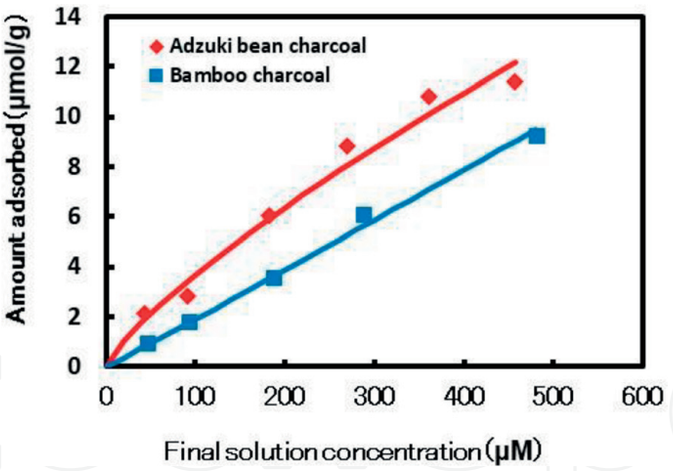

Figure 8.
Adsorption isotherms of lysozyme onto biochar; adsorption was carried out by incubating buffer solution (pH 7) containing a certain amount of lysozyme and 3 g/L biochar at 120 rpm and 25°C for 24 h.

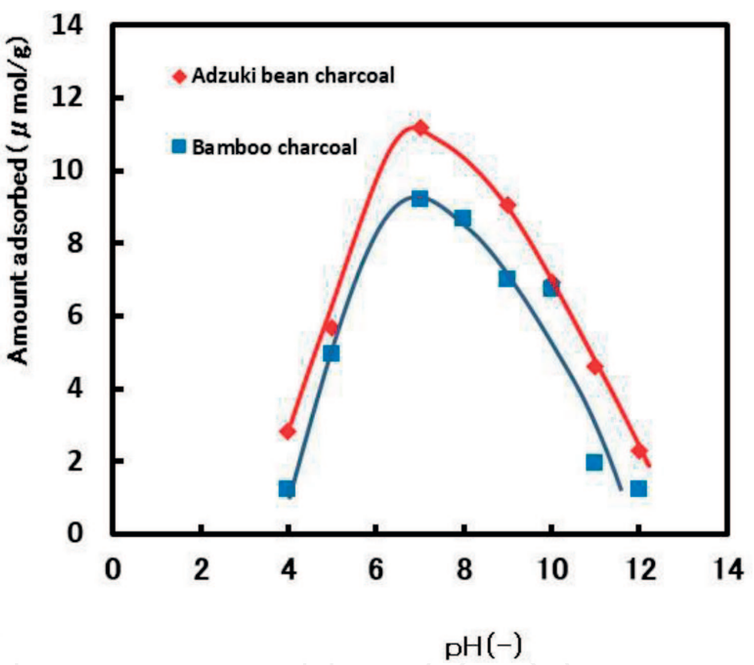

Figure 9.
Effect of pH on the amount of lysozyme adsorbed onto biochar; adsorption was carried out by incubating buffer solution (appropriate pH) containing 500 μM lysozyme and 3 g/L biochar at 120 rpm and 25°C for 24 h.

adsorbed on adzuki bean charcoal had the optimum value around neutral pH, similar to the case of bamboo charcoal. The net charge of protein molecules alters with the pH of aqueous solutions. Since the isoelectric point (pI) of lysozyme is 11, the net charge of lysozyme becomes more positive below pH 11. Concerning the ζ potential of biochar, the ζ potential of adzuki bean charcoal drastically decreased with increasing the pH value, exhibited a negative value above pH 4, dropped till pH 7, and was almost constant in the alkaline region, as shown in **Figure 6**. The pH dependence of the ζ potential of bamboo charcoal exhibited the same tendency to the case of adzuki bean charcoal. When the pH value was around the pI of lysozyme or the pH where the ζ potential of biochar approached 0 volts, a dramatic decrease in the amount of lysozyme adsorbed on biochar was observed. On the other hand, in the vicinity of neutral pH where lysozyme and the surface of biochar were charged positively and negatively, respectively, the high amount of adsorption

tended to be obtained. Consequently, these results indicate that the electrostatic interaction between the positively charged lysozyme and the negatively charged surface of biochar mainly contributes to the adsorption.

2.3 Heat stress tolerance of enzymes adsorbed on biochar

Modest heating causes enzymes dissolved in an aqueous solution to be denatured and inactivated by unfolding of enzyme molecules due to the disruption of weak interactions such as ionic bonds, hydrogen bonds, and hydrophobic interactions, which are prime determinants of enzyme tertiary structures [25]. In order to assess the heat stress tolerance of enzymes adsorbed on biochar, an aqueous solution containing lysozyme adsorbed on adzuki bean charcoal was incubated at high temperatures [15]. **Figure 10** shows photographs of aqueous solutions containing free lysozyme, the mixture of lysozyme and adzuki bean charcoal, and lysozyme adsorbed on adzuki bean charcoal before and after heat treatment was carried out at 90°C for 30 min, while under such heat conditions, raw eggs become hard-boiled eggs. The solution of free lysozyme immediately became turbid since thermally denatured enzymes were drastically aggregated by heat, as shown in **Figure 10(d)**. The enzyme aggregation is precipitated above 10 μM lysozyme [26]. The formation of enzyme aggregation was enhanced since the present concentration of lysozyme was 33 μM. Adzuki bean charcoal was easily dispersed in an aqueous solution due to the good wettability to water as seen in **Figure 10(b)**. Likewise, the mixture of lysozyme and adzuki bean charcoal was immediately precipitated by heat treatment due to the aggregation of denatured enzymes, as shown in **Figure 10(e)**. On the other hand, lysozyme adsorbed on adzuki bean charcoal was easily dispersed in an aqueous solution, as seen in **Figure 10(c)**. After the heat treatment, lysozyme adsorbed on adzuki bean charcoal was sufficiently dispersed in the solution, and the white enzyme aggregation and the cohesion among adzuki bean charcoals adsorbing lysozyme were not observed in the solution, as shown in **Figure 10(f)**. When enzymes dissolved in an aqueous solution are placed at high temperatures, most of enzymes are instantaneously unfolded by the disruption of weak interactions consisting of ionic bonds, hydrogen bonds, and hydrophobic interactions of enzymes [25, 27]. Additionally, unfolded enzymes are aggregated with each other, and the chemical deterioration reactions occur in unfolded enzymes. In particular, enzyme aggregation easily occurs upon the exposure of the hydrophobic surfaces of an enzyme, and this phenomenon becomes the major problem because of the fast

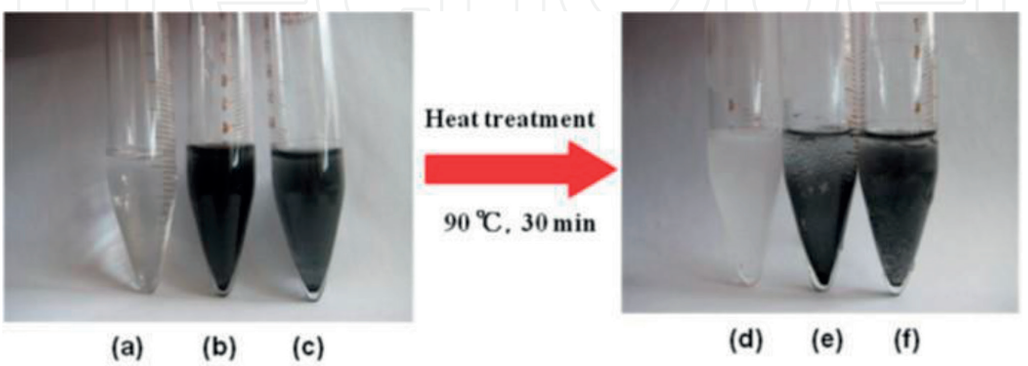

Figure 10.
Photographs of lysozyme solutions before and after heat treatment at 90°C for 30 min: (a) an aqueous solution containing free lysozyme before heat treatment, (b) an aqueous solution containing free lysozyme solution and adzuki bean charcoal before heat treatment, (c) an aqueous solution containing lysozyme adsorbed on adzuki bean charcoal before heat treatment, (d) an aqueous solution containing free lysozyme after heat treatment, (e) an aqueous solution containing free lysozyme solution and adzuki bean charcoal after heat treatment, and (f) an aqueous solution containing lysozyme adsorbed on adzuki bean charcoal after heat treatment.

irreversible inactivation. The adsorption of lysozyme on adzuki bean charcoal could inhibit the formation of enzyme aggregation.

When the remaining activity is defined as the ratio of the activity of lysozyme after heat treatment to that before heat treatment, time courses of remaining activities of free lysozyme and lysozyme adsorbed on adzuki bean charcoal through the heat treatment at pH 7.0 and 90°C are shown in **Figure 11** [16]. The remaining activities of free lysozyme and lysozyme adsorbed on adzuki bean charcoal decreased with an increase in time. As shown in **Figure 11**, the remaining activities of free and adsorbed lysozyme exhibited the correlation of first-order kinetics with heat treatment time. **Table 3** shows inactivation rate constants and half-lives of free and adsorbed lysozymes obtained from the curve fitting in **Figure 11**. The half-life of adsorbed lysozyme was seven times greater than that of free lysozyme. The remaining activity of free lysozyme was almost lost after heat treatment for 30 min, and the remaining activity in the mixture of lysozyme and adzuki bean charcoal exhibited 2%, while the remaining activity of lysozyme adsorbed on adzuki bean charcoal showed around 50%. The robust thermal stability of adsorbed lysozyme may be attributable to the suitable interaction of lysozyme with the surface of adzuki bean charcoal.

To extend our study, the remaining activities of lysozyme adsorbed on biochar obtained from several kinds of plant biomass wastes have been measured after heat treatment at 90°C for 30 min. Lysozyme adsorbed on bamboo charcoal or wood charcoal exhibited the high thermal stability, similar to the case of lysozyme-adsorbed adzuki bean charcoal, as shown in **Figure 12**. On the other hand, the

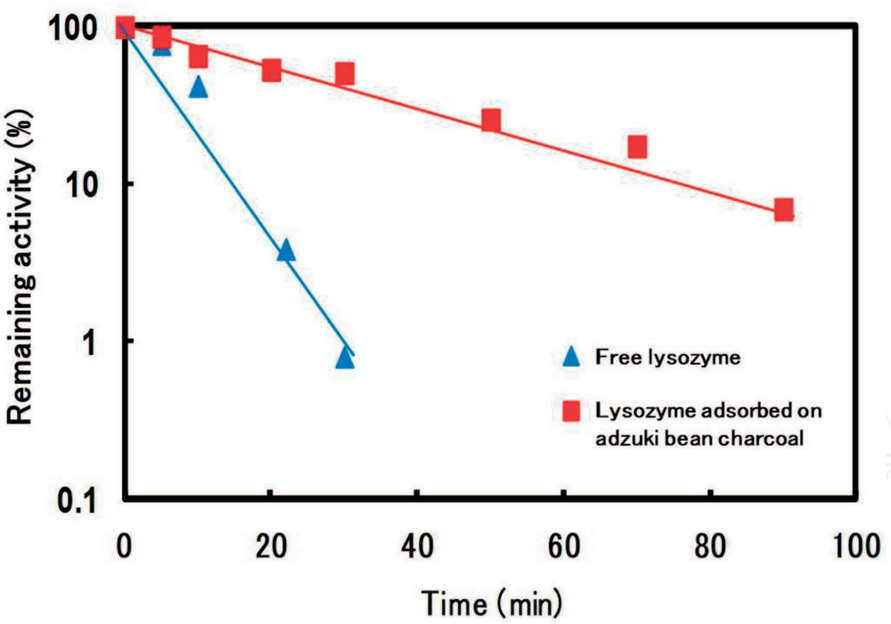

Figure 11.
Time course of remaining activity of free lysozyme and lysozyme adsorbed on adzuki bean charcoal through the heat treatment at pH 7.0 and 90°C.

Samples	Rate constant (min^{-1})	Half life (min)
Free lysozyme	0.168	4
Lysozyme adsorbed on adzuki bean charcoal	0.027	28

Table 3.
Rate constants and half lives of inactivation of lysozyme at 90°C.

mixture of lysozyme and bamboo charcoal or wood charcoal showed several percent of remaining activity.

The solution pH generally affects the activity and stability of enzymes in aqueous solutions [5]. **Figure 13** shows the remaining activity of lysozyme adsorbed on bamboo charcoal against the solution pH of adsorption medium. The remaining activity of bamboo charcoal-adsorbed lysozyme was markedly influenced by the pH of adsorption medium and showed the maximum value at pH 5.

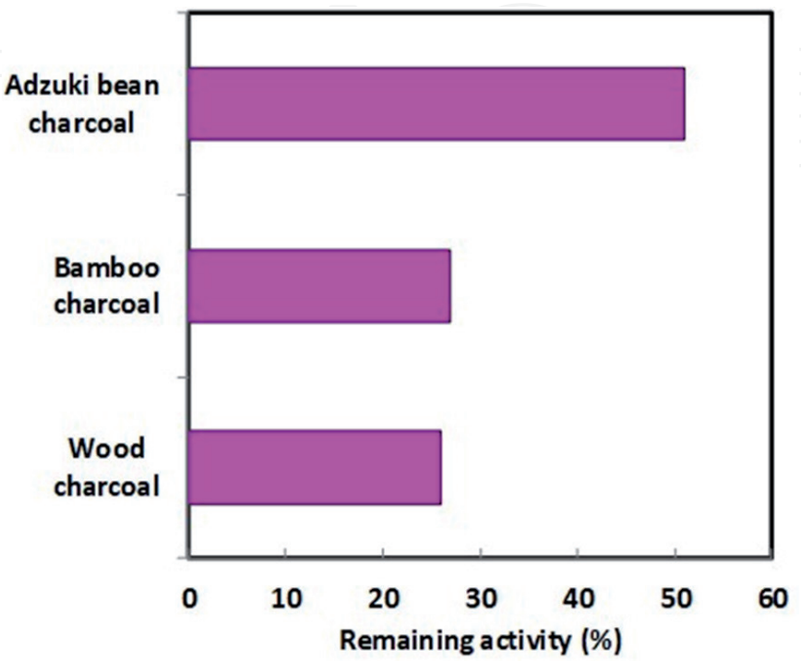

Figure 12.
Effect of kind of biochar on remaining activity of lysozyme adsorbed on biochar after heat treatment at 90°C for 30 min.

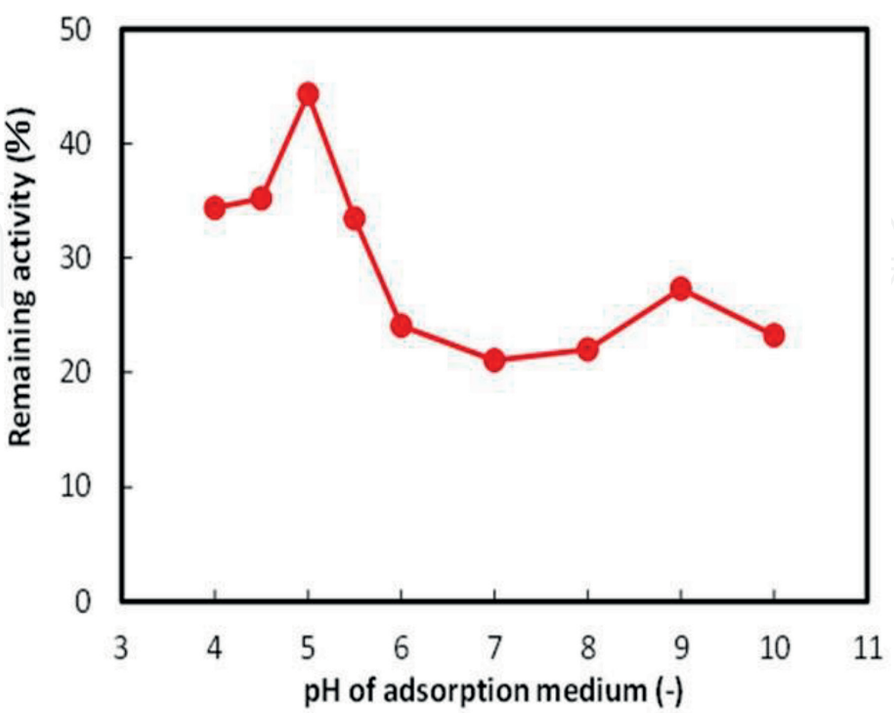

Figure 13.
Effect of pH of adsorption medium on the remaining activity of lysozyme adsorbed on bamboo charcoal after the heat treatment at 90°C for 30 min.

Three-dimensional structures of enzymes consist of secondary structures such as α-helix and β-sheet [28]. To elucidate the influence of adsorption on the structure of lysozyme, bamboo charcoal-adsorbed lysozyme has been measured by Fourier-transform infrared (FTIR) spectroscopy. **Figure 14** shows the FTIR spectra of native lysozyme and lysozyme adsorbed on bamboo at different pH. The most sensitive spectral region to enzyme secondary structural components is amide I (1700–1600 cm^{-1}), which is due almost entirely to the C═O stretch vibrations of peptide linkages [28]. The spectral pattern of bamboo charcoal-adsorbed lysozyme was influenced by the pH of adsorption medium. To evaluate the change in the secondary structure of bamboo-adsorbed lysozyme, the ratio of the absorbance at 1681 cm^{-1} to the absorbance at 1647 cm^{-1} (ABS_{1681}/ABS_{1647}) has been assessed since the band located at ca. 1681 cm^{-1} is assigned to intramolecular β-sheet and the band located at ca. 1647 cm^{-1} is assigned to α-helix. The ABS_{1681}/ABS_{1647} ratio at pH 5 (0.86), where the remaining activity showed the maximum value, was similar to that of native lysozyme (0.88). Likewise, the ABS_{1681}/ABS_{1647} ratio at pH 4 (0.92) was near that of native lysozyme. On the other hand, the ABS_{1681}/ABS_{1647} ratios at pH 7 (0.69) and 9 (0.61) were different from that of native lysozyme. The effect of solution pH of adsorption medium on the thermal stability of bamboo charcoal-adsorbed lysozyme has been summarized as follows. The structure of bamboo charcoal-adsorbed lysozyme was nearly the native structure of lysozyme when lysozyme was adsorbed on bamboo charcoal at pH 4, but the electrostatic interaction between lysozyme and bamboo charcoal could not sufficiently contribute to the thermal stability. The electrostatic interaction between lysozyme and bamboo charcoal could strongly retain the structure of lysozyme at high temperatures when lysozyme was adsorbed on bamboo charcoal at pH 5, where the native structure of lysozyme was maintained. The structure of lysozyme was partially destroyed since the electrostatic interaction was too strong to maintain the native structure of lysozyme, and the thermal stability of bamboo charcoal-adsorbed lysozyme dropped when lysozyme was adsorbed on bamboo charcoal at pH 7 and 9. Therefore, these results indicate that biochar-adsorbed enzymes exhibit the excellent thermal stability when the

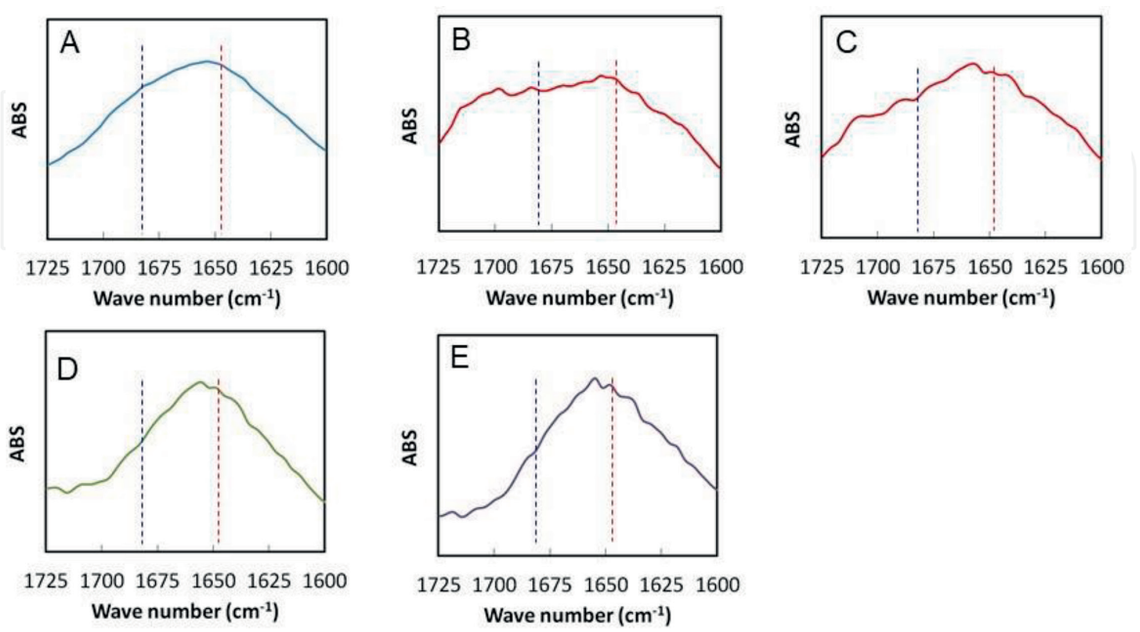

Figure 14.
(A) FTIR spectrum of native lysozyme. (B) FTIR spectrum of lysozyme adsorbed on bamboo charcoal at pH 4. (C) FTIR spectrum of lysozyme adsorbed on bamboo charcoal at pH 5. (D) FTIR spectrum of lysozyme adsorbed on bamboo charcoal at pH 7. (E) FTIR spectrum of lysozyme adsorbed on bamboo charcoal at pH 9.

native structure of enzymes is kept after the adsorption and the adsorption force is strong enough to retain the structure of enzymes against the heat stress.

2.4 Organic solvent stress tolerance of enzymes adsorbed on biochar

Biotransformation catalyzed by an enzyme in nonaqueous media has been applied to numerous synthetic processes because of the following benefits [29]: (1) The solubility of nonpolar reactants and products is improved. (2) Synthetic reactions can take place by the 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 can easily be recycled by the filtration or the centrifugation. (6) The product can easily be recovered by the evaporation when the volatile organic solvent is used as a reaction medium. (7) The contamination such as the growth of microorganisms can be inhibited by using organic solvents. However, the enzyme tends to show the low activity in organic solvents, compared to that in water since an organic solvent in general works as a denaturant of enzymes [30].

Figure 15 shows the scheme of reaction catalyzed by α-chymotrypsin (α-CT) [30, 31]. When *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) is used as a substrate, the enzymatic reaction proceeds by the formation of enzyme intermediates between the active site of enzymes and the substrate. In water α-CT catalyzes the hydrolysis reaction of *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) with water to give *N*-acetyl-L-tyrosine (*N*-Ac-Tyr-OH). On the other hand, in organic solvents α-CT mainly catalyzes the transesterification reaction of *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) with another substrate, *n*-butanol (BuOH), to produce *N*-acetyl-L-tyrosine butyl ester (*N*-Ac-Tyr-OBu). Thus α-CT, which is hydrolase, can beneficially catalyze the synthetic reaction in organic solvents although expensive energy substances such as adenosine triphosphate (ATP) are needed for conventional synthetic reactions by enzymes to take place in water [24].

In order to evaluate the catalytic activity of enzymes adsorbed on biochar in an organic solvent, the transesterification catalyzed by α-CT adsorbed on the different kind of biochar in acetonitrile has been examined [19]. **Figure 16** 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 biochar-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 biochar-adsorbed α-CT were much higher than those catalyzed by free α-CT. α-CT adsorbed on bamboo charcoal was the most effective of all, with respect

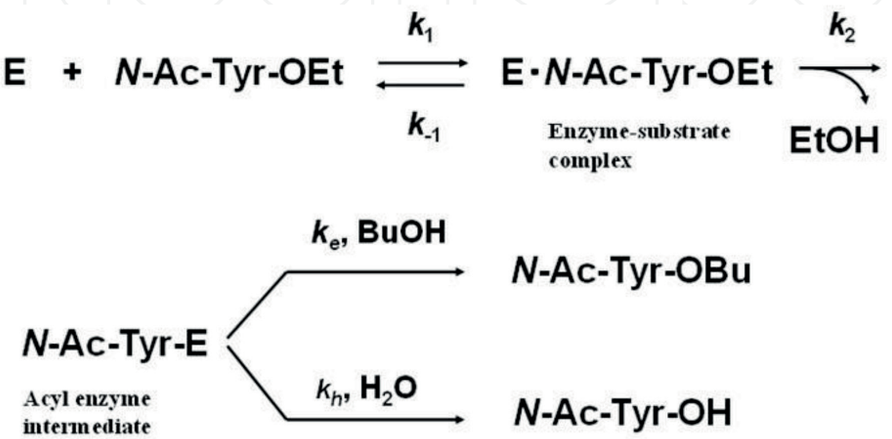

Figure 15.
α-CT-catalyzed transesterification of N-acetyl-L-tyrosine ethyl ester (N-Ac-Tyr-OEt) with n-butanol (BuOH) to N-acetyl-L-tyrosine butyl ester (N-Ac-Tyr-OBu) and competing hydrolysis (N-Ac-Tyr-OH).

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 nonaqueous enzymology, the initial rate of *N*-Ac-Tyr-OBu catalyzed by α-CT adsorbed on bamboo charcoal was about 50 times higher than that catalyzed by free one. Enzymes are aggregated in an organic solvent, and most of them cannot 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 on biochar are directly in contact with the bulk organic phase since they are located on the surface of biochar. Accordingly, biochar-adsorbed enzymes can effectively proceed with the reaction, compared to free enzymes, since mass transfer of substrates and products is rapidly facilitated [32].

In order to elucidate the influence of a kind of biochar on the secondary structure of α-CT, the FTIR spectra of free and biochar-adsorbed α-CT were measured. **Table 4** 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 biochar. As mentioned above, 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 as shown in **Figure 16**. 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 [33]. 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.

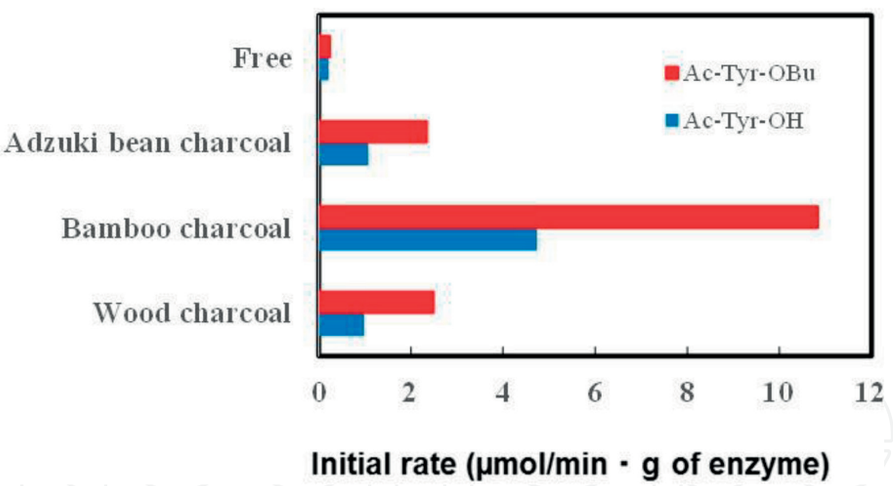

Figure 16.
Dependence of kind of biochar on biochar-adsorbed α-CT-catalyzed transesterification. Free or biochar-adsorbed α-CT was placed in acetonitrile containing 5% (v/v) water, 10 mM N-Ac-Tyr-OEt, 1000 mM BuOH, and 1 mM acetanilide, and the resulting mixture was shaken at 120 rpm and 25°C.

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

Table 4.
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.

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 on biochar.

Figure 17 shows time course of remaining activities of free α-CT and bamboo charcoal-adsorbed α-CT through the heat treatment at 50°C [20]. The state of free α-CT in acetonitrile, where α-CT was dispersed as the solid state, was unchanged during the heat treatment, although enzymes dissolved in an aqueous solution immediately form the aggregation of thermally denatured enzymes [15]. Likewise, the enzyme aggregation and the cohesion among bamboo charcoal-adsorbed α-CT were not observed in acetonitrile during the heat treatment. However, the remaining activities of free α-CT and BCP-adsorbed α-CT gradually dropped with an increase in heat time. The relation between the remaining activity of free α-CT and heat time could be correlated by first-order kinetics, while the relation between bamboo charcoal-adsorbed α-CT and heat time could be correlated by second-order kinetics. When the curve fitting was carried out in the figure, the half-life of inactivation of free α-CT was 33 min, and the half-life of inactivation of bamboo charcoal-adsorbed α-CT was 125 min. Therefore, the half-life of bamboo charcoal-adsorbed α-CT showed 3.8-fold, compared with that of α-CT. On the other hand, the half-life of inactivation of bamboo charcoal-adsorbed α-CT is 15 min in aqueous solutions at 45°C [17]. Consequently, the thermal stability of bamboo charcoal-adsorbed α-CT in acetonitrile was greater than that in water. As a result, the electrostatic interaction between α-CT and bamboo charcoal, which mainly contributes to the adsorption of α-CT on bamboo charcoal, is strengthened in acetonitrile as the dielectric constant of acetonitrile is much smaller than that of water [34].

In moist air, the catalytic activity of solid enzymes is markedly dependent on the thermodynamic water activity (a_w), which is defined as the ratio of the water partial pressure to the vapor pressure of pure water [35]. Similarly, the catalytic activity of biochar-adsorbed enzymes might be influenced in hydrophilic organic solvents containing low water content. **Figure 18** shows the relationship of the initial transesterification rate (V_e) and the initial hydrolysis rate (V_h) with the water activity

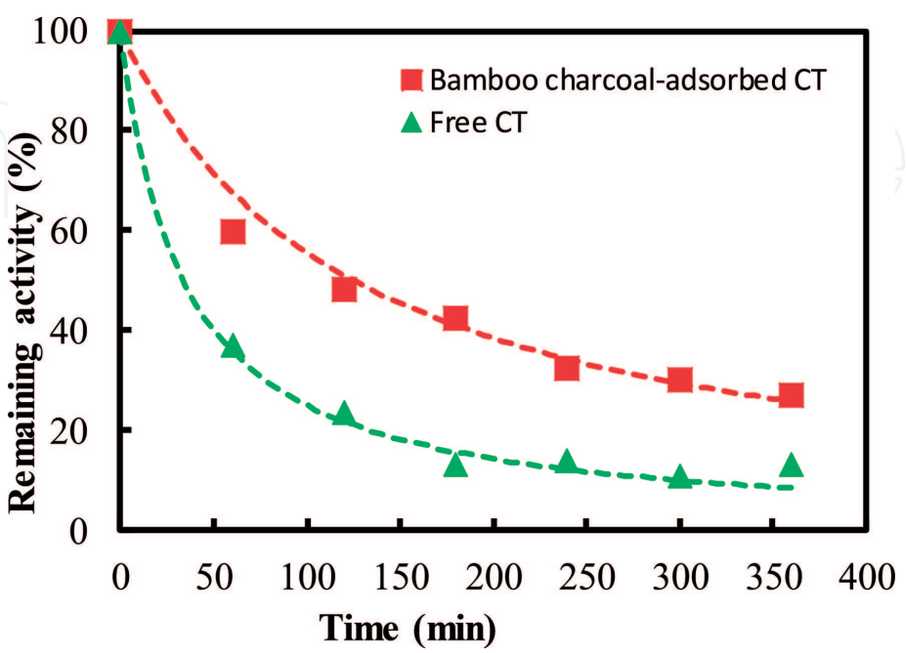

Figure 17.
Time course of remaining activities of free α-CT and bamboo charcoal-adsorbed α-CT through the heat treatment at 50°C.

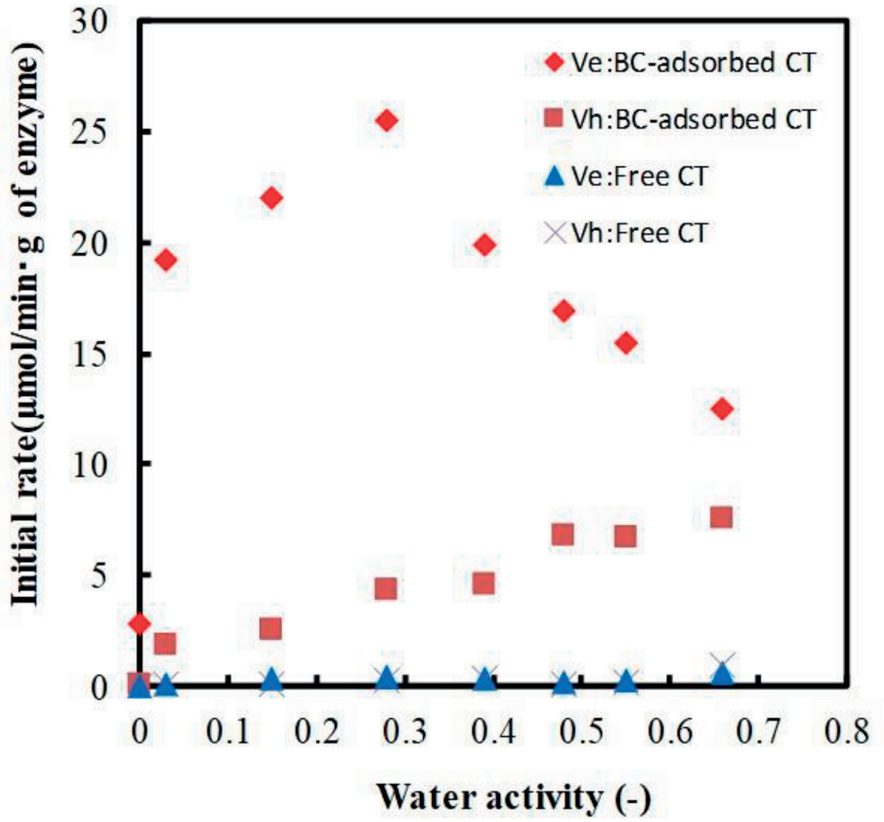

Figure 18.
Effect of water activity on the transesterification rate (V_e) and hydrolysis rate (V_h) of free α-CT and bamboo charcoal (BC)-adsorbed α-CT in acetonitrile.

in acetonitrile at 25°C [20]. Low water activity inhibited the inherent enzymatic hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) with water, whereas the enzymatic transesterification of *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) with *n*-butanol (BuOH) was enhanced. The water activity strongly affected the initial transesterification rates catalyzed by bamboo charcoal-adsorbed α-CT and free α-CT, and the correlation between these parameters showed a bell-shaped curve. The initial transesterification catalyzed by bamboo charcoal-adsorbed α-CT exhibited about 60-fold, compared to that catalyzed by free α-CT when the maximum initial transesterification rates of free α-CT and bamboo charcoal-adsorbed α-CT were shown. On the other hand, the initial hydrolysis rates catalyzed by free α-CT and bamboo charcoal-adsorbed α-CT gradually increased with increasing the water activity. The relationship of the catalytic activity of enzymes with the water activity in organic solvents tends to depict a bell-shaped curve. The balance between the kinetic rigidity of enzyme structures and their thermodynamic stability results in the optimum water activity [29, 36]. The kinetic rigidity drops with an increase in water activity, while the native structure of enzymes is gradually influenced by the thermodynamic stability. Consequently, the catalytic activity of enzymes increases with increasing the flexibility in the rigid structure of enzymes and then decreases with increasing the disturbance of enzyme structures. On the other hand, the hydrolysis is promoted with an increase in the water activity since the high water activity results in the high overall water concentration. Concerning the reaction selectivity, which was defined as the ratio of the initial transesterification rate (V_e) to the initial hydrolysis rate (V_h), bamboo charcoal-adsorbed α-CT was much better than free α-CT.

Table 5 shows the absorbance ratio at 1650 and 1630 cm^{-1} (ABS_{1650}/ABS_{1630}) of bamboo charcoal-adsorbed α-CT [20]. The higher the absorbance ratio, the higher the secondary structure. The water activity did not affect the absorbance ratio

Water activity (−)	ABS_{1650}/ABS_{1630} (−)
0.03	1.3
0.28	1.3
0.55	1.3
0.73	1.3

Table 5.
Ratio of the absorbance at 1650 cm^{-1} to the absorbance at 1630 cm^{-1} (ABS_{1650}/ABS_{1630}) of bamboo charcoal-adsorbed α-CT provided by the FTIR measurement.

(ABS_{1650}/ABS_{1630}) of bamboo charcoal-adsorbed α-CT, indicating that the secondary structure of bamboo charcoal-adsorbed α-CT does not depend on the water activity. Accordingly, the adsorption firmly makes the conformation of bamboo charcoal-adsorbed α-CT maintained. The absorbance ratio (ABS_{1650}/ABS_{1630}) of bamboo charcoal-adsorbed α-CT is higher than that of free α-CT, as seen in **Table 4**. The results illustrate that the water activity effectively affects the catalytic activity of bamboo charcoal-adsorbed α-CT having a native structure, compared to that of free α-CT.

The catalysis of free α-CT and bamboo charcoal-adsorbed α-CT was markedly dependent upon the nature of organic solvents as shown in **Figure 19**. The catalytic activity of bamboo charcoal-adsorbed α-CT was much superior to that of free α-CT in organic solvents. The initial transesterification rate of free α-CT in *n*-octane exhibited 813-fold, compared to that in *n*-butyl acetate, while the initial transesterification rate of bamboo charcoal-adsorbed α-CT in *n*-octane was 1.3 times greater than that in *n*-butyl acetate.

There have been some reports that the native conformation of enzymes may be altered when enzymes are immersed in organic solvents [37, 38]. **Table 6** 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 bamboo charcoal-adsorbed α-CT after they were immersed in organic solvents for 24 h. The absorbance ratio (ABS_{1650}/ABS_{1630}) of bamboo charcoal-adsorbed α-CT after the solvent immersion was similar to that before the solvent immersion. On the other hand, the absorbance ratio (ABS_{1650}/ABS_{1630}) of free α-CT was altered by the immersion in octane. Those results indicate that the conformation of bamboo charcoal-adsorbed α-CT is hardly influenced by the nature of solvents, compared to the case of free α-CT.

Figure 20 shows the relation between the catalytic activity and the hydrophobicity defined as log P where P is a partition coefficient for a given solvent between *n*-octanol and water [39]. The initial transesterification rate of free α-CT and bamboo charcoal-adsorbed α-CT increased with an increase in the hydrophobicity of organic solvents. Likewise, the initial hydrolysis rates of free α-CT and bamboo charcoal-adsorbed α-CT tended to increase with increasing the hydrophobicity of organic solvents. The hydrophobicity contributes to the partition of water between enzyme molecules and the bulk organic phase in reaction systems [29, 40]. When a certain amount of water is added into organic solvents, the amount of water associated with enzyme molecules increases in an increase in the hydrophobicity of organic solvents. Thereby, the flexibility of enzyme molecules, which is essential for catalytic activity, is enhanced by the hydration of enzyme molecules, and then the catalytic activity increases. On the other hand, hydrolysis reaction is promoted by the increase of water molecules around enzymes. As shown in **Figure 20**, the initial transesterification rate of bamboo charcoal-adsorbed α-CT was much superior to that of free α-CT at the low hydrophobicity. For instance, the initial transesterification rate of bamboo charcoal-adsorbed α-CT was about 760 times higher than that

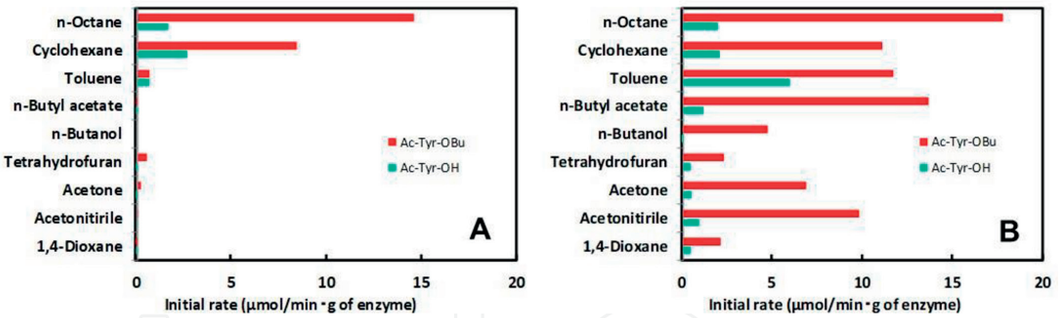

Figure 19.
Solvent dependence o of transesterification catalyzed by free α-CT (A) and BCP-adsorbed α-CT (B).

Solvent	ABS_{1650}/ABS_{1630} (−)	
	Free α-CT	**Bamboo charcoal-adsorbed α-CT**
None	1.14	1.47
Acetonitrile	1.15	1.51
n-Octane	1.20	1.53

Table 6.
Ratio of the absorbance at 1650 cm^{-1} to the absorbance at 1630 cm^{-1} (ABS_{1650}/ABS_{1630}) of free α-CT and bamboo charcoal-adsorbed α-CT provided by the FTIR measurement after the solvent immersion.

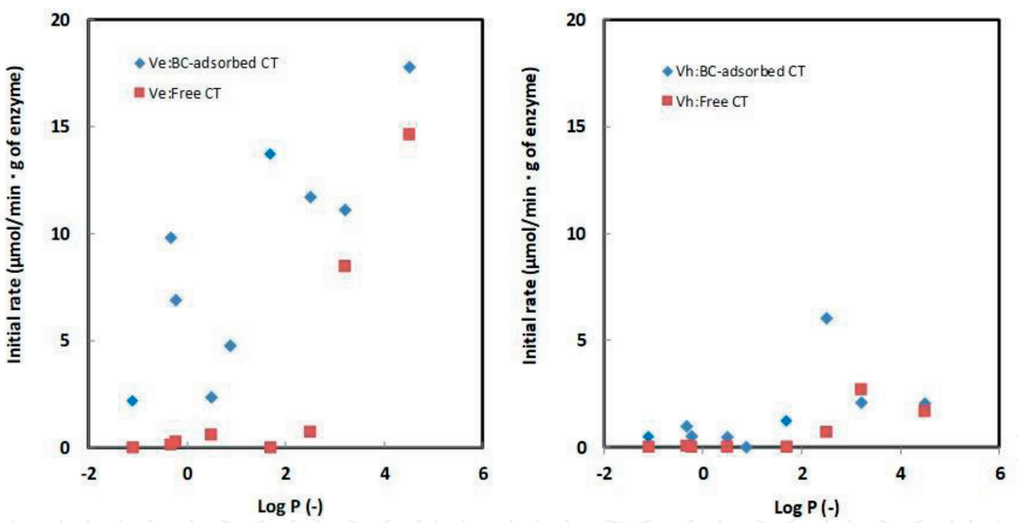

Figure 20.
Relationship of log P of solvents with transesterification rate (A) or hydrolysis rate (B) of free α-CT and bamboo charcoal (BC)-adsorbed α-CT.

of free α-CT in *n*-butyl acetate (log P = 1.7). Moreover, bamboo charcoal-adsorbed α-CT depicted the high initial transesterification rate in acetone and acetonitrile, compared to the case of free α-CT. An enzymatic reaction in hydrophilic solvents has the advantage of the solubility of a variety of substrates including drug derivatives, which are poorly soluble in hydrophobic solvents [41]. As discussed above, since the native conformation of bamboo charcoal-adsorbed α-CT was maintained in hydrophilic organic solvents, bamboo charcoal-adsorbed α-CT could exhibit the high catalytic activity, compared to the case of free α-CT. On the other hand, no correlation between the catalytic activity and the other parameter (e.g., dielectric constant, hydrogen bonding parameter, solubility parameter, and viscosity) was shown.

3. Conclusions

This chapter has introduced the study on the application of the biochar to enzyme carriers to develop the high value-added application of biomass materials. Biochar was thermal stable, chemical stable, insoluble under reaction conditions, available, low cost, regeneration, and reusable. Moreover, as biochar had functional groups for the interaction of enzymes and a high affinity to enzymes, enzymes were firmly adsorbed on biochar. On the other hand, the original weakness of enzymes due to the heat and organic solvent stresses could be much improved by adsorbing enzymes onto the biochar. Moreover, enzymes are strictly influenced not only by heat and organic solvents but also by ultraviolet, X-ray, sound wave, shake, freeze, pressure, shearing force, extreme ionic strength, urea, surfactant metal ion, reductant, and so on. It would be expected that suitable carriers having the high tolerance against those stresses are developed for an enzyme carrier by selecting a kind of biochar as well because there are a great variety of biomass in the earth.

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