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청정환경기술

Pigment Degradation by Lignin Peroxidase Covalently Immobilized on Magnetic Particles

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Abstract

Pigment red 53:1 is a dye used in various products as a component of the inks, suspected of being carcinogenic. Thus, the environmental and occupational issues related to it are important. The enzyme-based approach with reusability has advantages to consume less energy and generate less harsh side- products compared to the conventional strategies including separations, microbe, and electrochemical treatment. The degradation of Pigment red 53:1 by the lignin peroxidase immobilized on the surface of magnetic particles has been studied. The immobilization of the peroxidase was conducted on magnetic particle surface with the treatment of polyethyleneimine, glutaraldehyde, and the peroxidase, in sequence. The immobilization was confirmed using X-ray photon spectroscopy. The absorbance peak of the pigment was monitored at 495 nm of UV/Vis spectrum with respect to time to calculate the catalytic activities of the pigment for the immobilized lignin peroxidase. For the comparison, the absorbance of the lignin peroxidase free in solution was also monitored. The catalytic rate constant values for the free lignin peroxidases and the immobilized those were 0.51 and 0.34 min^{-1}, respectively. The reusable activity for the immobilized lignin peroxidase was kept to 92% after 10 cycles. The stabilities for heat and storage were also investigated for both cases.

Keywords : Pigment, Degradation, Lignin peroxidase, Immobilization, Magnetic particles

1. Introduction

The dye and pigment industries manufacture around 1 million tonnes of product annually, but an approximate 10% of the colorants are lost during either the production stage or the application process [1]. Not all of the dye and pigment currently used could be degraded and/or removed with physical and chemical processes, and sometimes the degradation products are more toxic [2]. Conventional strategies for the physical treatment of wastewater contaminated with dyes or pigments are based on separation processes such as coagulation or adsorption onto activated carbon. Such procedures, however, only serve to transfer the contaminant from wastewater to the solid state without effectively destroying the pollutant [3]. Biological treatment has been suggested to enhance the degradation of textile dyes [4-6]. The utilization of microbes requires supplies of nutrients and excretion of wastes for their survival. Therefore, as a biological treatment, the enzymes immobilized on support matrices have been intensively studied. For example, laccase has been known to degrade broad range of dyes [7]. Several processes using laccases as well as immobilized laccases have been developed for the treatment of dyes [8-11].

Pigment red 53:1, 5-chloro-2-((2-hydroxy-1-naphthyl)azo)-4-methylbenzenesulphonic acid, barium salt, has been widely used as a dye and suspect of carcinogen. This pigment has been studied for the degradation by Fenton, photo-Fenton and UV/H_2O_2 systems. It was found that the efficiencies of the Fenton reactions increased with temperature, but the formation of solid agglomerates was observed when the reactions were carried out above 50 ℃ indicating a coagulant action of Fe^{+2} or Fe^{+3} [12]. Still, the dye has not been investigated for the enzymatic degradation. Generally, lignin peroxidase showed a significant activity in the oxidation of selected azo, quinoline and fluorone dyes as the primary oxidant under mild experimental conditions [13].

The immobilization of enzymes has advantage to separate the enzymes from the other substances and reuse them. It has been

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published that the immobilized laccase showed the catalytic activity better than the free one [14]. Since magnetic particles can be easily and rapidly separated from the reaction medium in an external magnetic field without being subjected to heavy mechanical stress compared with filtration or centrifugation and has been widely investigated [15]. Therefore, in this study, we have assessed the potential of the lignin peroxidase immobilized on the magnetic particles to continuously degrade Pigment red 53:1. Since it has a high molar absorption coefficient (λ_{max} = 495 nm in visible region), the decrease in Pigment red 53:1 concentration during degradation processes can be monitored with respect to time. Furthermore, the reusability and the activity change induced by the thermal effect and the storage have been studied. Such an understanding is necessary for the design and application of Pigment red 53:1 treatment processes.

2. Material and methods

2.1. Chemicals

Pigments Red 53:1 and magnetic particles (3 μm diameter, ProMag®HP) were obtained from TCI Japan (Tokyo, Japan) and Bangs Lab. (Fishers, IN, USA), respectively. Lignin Peroxidase, Polyethylenimine (PEI, M.W. 750k), Glutaraldehyde, and Bradford reagent were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Enzyme Immobilization

Figure 1 schematically illustrates the chemistries used to graft enzymes to magnetic particles. A 150 μL aliquot of stock particle solution, containing 2.7×10^8 particles, was washed in 50 mM of carbonate buffer, pH 8.2, three times. The particles were coated with 3% (w/v) PEI in 2 mL of 50 mM carbonate buffer, pH 8.2, for 1 h, magnetically separated from the PEI solution, and thoroughly washed with carbonate buffer. Particle aggregates were resuspended with a microtip ultrasonic disrupter (Branson Ultrasonics Corporation, Danbury, CT). The particles were thoroughly washed with 20 mM Na_3PO_4, pH 7.5 and functionalized with 2 mL glutaraldehyde by reacting 2.5% (v/v) glutaraldehyde, 20 mM Na_3PO_4, pH 7.5, with the PEI-coated particles for 45 min. The particles were immersed in 50 mL of 100 mM Na_3PO_4, (pH 7) containing 300 U of lignin peroxidase for 3 h. The immobilization of the enzymes was confirmed with X-ray photoelectron spectroscopy (XPS). The spectra of XPS were obtained using a spectrometer equipped with a concentric hemispherical analyzer (PHI 5800, Physical Electronics, Inc., Chanhassen, MN, USA). For XPS characterization, a silicon wafer (Sehyung Wafer Tech., Seoul, S. Korea) was utilized as a substrate where the magnetic particles were physically adsorbed. After the adsorption, the chemical treatments described above were performed. Using the Bradford reagent, the concentrations of the injected and unbound enzymes were calculated. Therefore, the concentration of the immobilized enzyme was estimated and about 1.1 μM and 8.2 ng-protein/mg-particle. Unless otherwise noted, all reactions were carried out at room temperature and the water was prepared with a Milli-Q purification system (Millipore, Bedford, MA).

2.3. Activity Measurement

The catalytic activity of the lignin peroxidase was studied by monitoring the decolorization of the Pigment red 53:1 solution (10 mg^{-1} and pH 5.8). The 5 mL pigment solution and the 50 μL enzyme solution (300 mU) were transferred to seven different sterilized-tubes for the free and immobilized enzyme, respectively. Each tube was shaken slowly (1 turn-over per 1 minute) and moved to a UV/Vis spectrometer (UV-1800, Shimadzu, Tokyo, Japan) one by one every 2 hr. Right before the movement, the particles were collected by magnetic decantation for reusability. The concentration of pigment in the solution was evaluated by measuring the absorbance of the solution at the λ_{max} (495 nm) of the pigment in the visible region. All of these procedures were repeated three times.

3. Results and Discussion

3.1. XPS Characterization

The results of XPS for each layer were summarized in Table 1. The analysis was performed at each step of the surface treatments. Table 1 contains significant amounts of silicon, oxygen, nitrogen, and carbon. Since each element has its own binding energy, the peak distribution for the energy represents the relative amount of the element. Before any treatment, the surface of the silicon wafer consisted of silicon and oxygen. After the

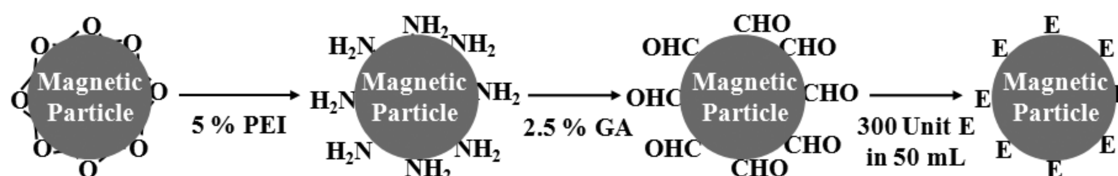

Figure 1. Chemical scheme used to immobilize enzyme on magnetic particles, GA and E indicate glutaraldehyde and enzyme.

Table 1. XPS results for each step chemically treated on magnetic particles

	Silicon oxide	PEI treatment	Glutaraldehyde treatment	Enzyme immobilization
C 1s	0.1%	19.0%	23.7%	25.8%
N 1s	0.1%	7.0%	6.1%	7.6%
O 1s	67.6%	50.0%	48.9%	46.7%
Si 2p	32.2%	24.0%	21.3%	19.7%
S 2p				0.2%

adsorption of PEI, the compositions of the carbon and the nitrogen increased tremendously. The increase indicated that PEI adsorbed successfully on the wafer. The results of the adsorption were consistent with that of the previous research [16]. The changes in the carbon and the nitrogen, led by the glutaraldehyde surface reaction and the peroxidase immobilization as next steps, were expected, respectively.

3.2. Pigment Degradation Kinetics

The degradation was monitored by measuring the absorbance intensities (Figure 2), and it was presented with respect to time for the immobilized and free lignin peroxidase (Figure 3). The change of the intensity was more rapid for the free lignin peroxidase. The rate difference for the peroxidases seems due to the accessibility of the substrates to the enzymes. However, the intensity, reached little change after 12 h, was almost identical for both cases. Therefore, the enzyme immobilized on the surface still retains the activity for the degradation. The degradation kinetics of the enzyme was analyzed with the Michaelis-Menten equation, as shown below.

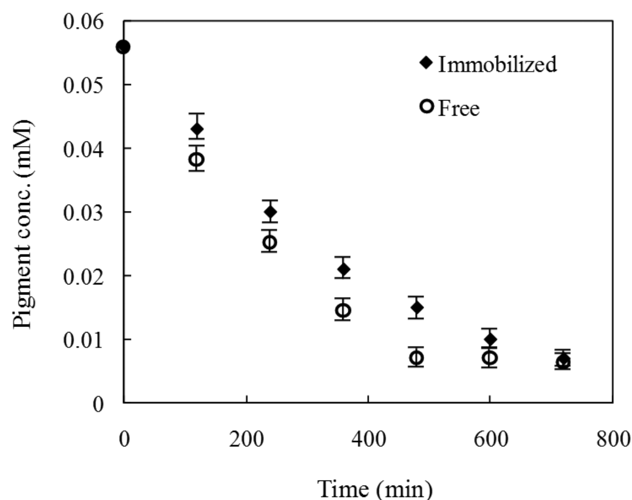

Figure 3. Pigment degradation with respect to time for the immobilized and free lignin peroxidase.

$$-\frac{d[Pigment]}{dt}=\frac{k_{cat}[Pigment][E]}{k_m+[Pigment]}$$

where [*Pigment*] is the concentration of the Pigment red 53:1, k_{cat} is the catalytic constant, k_m is the Michaelis-Menten constant, and [*E*] is the concentration of the lignin peroxidase [17].

For the calculation of the constants, the concentration of the pigment was estimated from the absorbance intensity using the calibration curve. The curve was made from the measurement of the intensity for the pigment solution of the determined concentration. Since the intensity of the reacted-pigment solution was measured with respect to time, the rate of change in the pigment concentration was also obtained. Therefore, the Lineweaver-

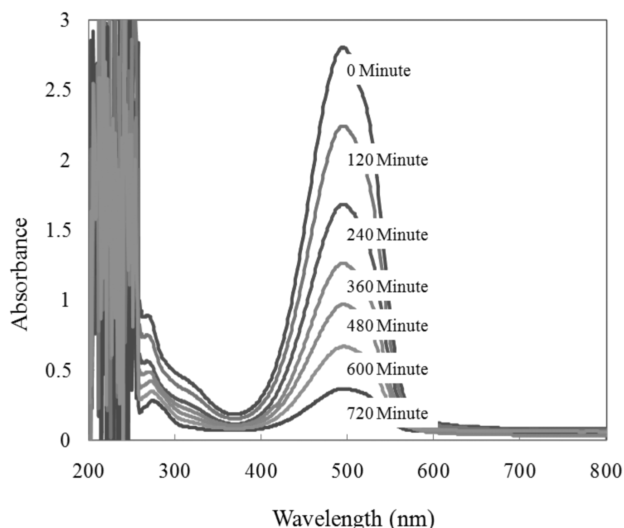

Figure 2. Absorbance intensity change for the immobilized lignin peroxidase.

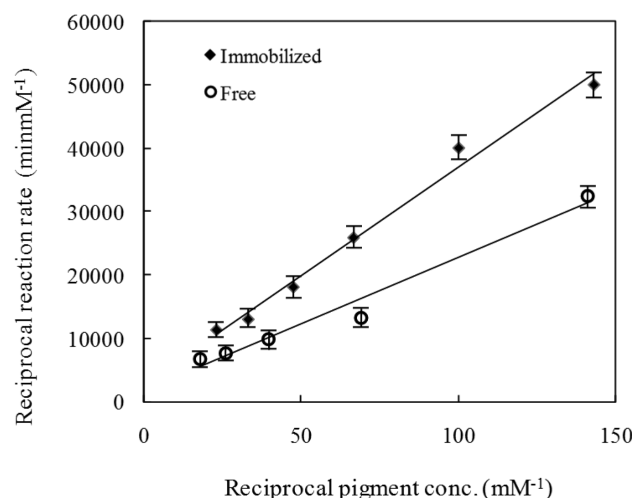

Figure 4. Lineweaver-Burk plot for Pigment red 53:1 degradation, ◆ for immobilized and ○ for free. Figure caption should be provided and placed at the below.

Burk plot was available and shown in Figure 4. The double reciprocal plots were constructed with linear relations, y = 343.7x + 2625 (R^2 = 0.987) and y = 209.5x + 1789 (R^2 = 0.972) for the immobilized and the free, respectively. The parameters, k_{cat} and k_m, found from the plots were 0.51 min^{-1} and 0.11 mM for the free enzyme. For the immobilized enzyme, k_{cat} and k_m were 0.34 min^{-1} and 0.14 mM. The lower k_{cat} and higher k_m indicate the less catalytic ability and affinity of the immobilized enzyme towards substrate compared to the free enzyme. The reduction in the affinity may be due to the lower accessibility of the substrate to the active site of the immobilized enzyme. The immobilized enzyme has 67% of the k_{cat} value of free one, which suggests that the immobilization system provides efficient catalytic ability.

3.3. Lignin Peroxidase Activity

Stabilities of thermal and storage, and reusability of the immobilized enzyme were evaluated. The activity of the immobilized enzyme was shown with respect to the number that the enzyme was used (Figure 5). The activity was monitored up to the 10th cycle of use, and the activity at the last time was around 92% on average, compared to that at the first time the immobilized-enzyme was used. This activity measurement was performed three times, and the results of the measurements were 94% one time and 91% two times. At 20, 25, 30, 35, and 40 ℃, the activity was monitored. The activity was the highest at 25 ℃, and the relative activities at the temperatures were calculated (Figure 6). The activities at 20 ℃ were little different for the free and immobilized enzymes. However, above 25 ℃, the behavior of the activity was changed. Similar results have been reported for the enzyme, which was immobilized on meso-porous particles [18]. This result seems to indicate that the structure of the enzyme was transformed with the increase from the temperature. Furthermore, it was observed that the immobilized enzyme showed less decrease in the activity compared to the free one, as previously investigated with the immobilization on the block co-polymer and xerogel [19,20]. At 40 ℃, 10% was reduced for the immobilized enzyme and 20% was for the free one. The difference between these activities appears to suggest that the structure change of the immobilized one was less than that of the free. Likewise, the activity change for the storage was less for the immobilized one than for the free one (Figure 7). The results of the storage were obtained from the measurements performed every fifth day for 30 d. These results also seem to suggest that the immobilization may interfere in the structure change.

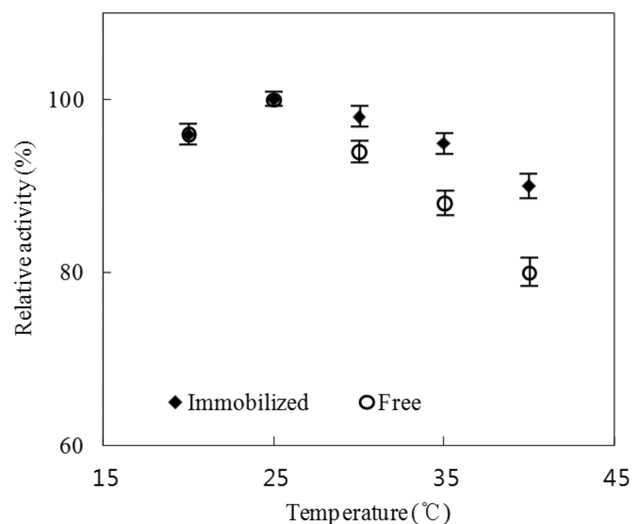

Figure 6. Stability change with respect to thermal effect.

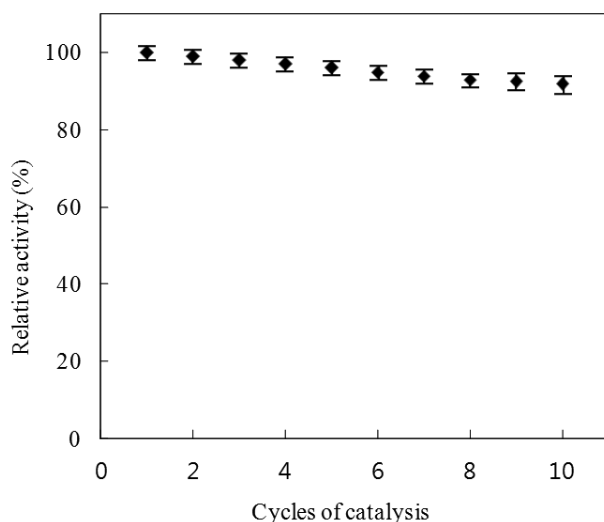

Figure 5. Relative activity change with respect to the number of use.

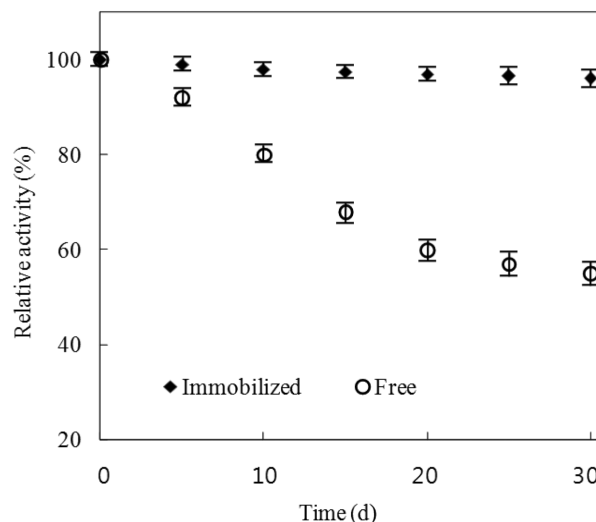

Figure 7. Stability change with respect to storage.

4. Conclusions

In this study, the Pigment red 53:1 degradation kinetics, the stabilities of thermal and storage, and the reusability of the lignin peroxidase, immobilized on the magnetic particles through covalent bindings, were evaluated. For the immobilization, the surface of the particles was treated with polyethyleneimine, glutaraldehyde, and the peroxidase, in sequence. The decolorization was monitored with UV/Vis spectrometer, and the catalytic activity was estimated into 0.51 and 0.34 min^{-1} for the free and immobilized enzyme, respectively. At the 10th repeat of the use, the activity was retained about 92% compared to the first use. For the stability of the thermal and storage, the immobilized enzyme showed less decrease in the activity than the free one. As the results of this research, the lignin peroxidase immobilized on the magnetic particles has feasibility for the degradation process of the Pigment red 53:1.

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References

1. Couto, S. R., Dominguez, A., and Sanromán, A., "Photocatalytic Degradation of Dyes in Aqueous Solution Operating in a Fluidized Bed Reactor," *Chemosphere*, **46**(1), 83-86 (2002).
2. Spadaro, J. T., Lorne, I., and Renganathan, V., "Hydroxyl Radical Mediated Degradation of Azo Dyes: Evidence for Benzene Generation," *Environ. Sci. Technol.*, **28**, 1389-1393 (1994).
3. Shu, H.-Y., and Huang, C.-R., "Degradation of Commercial Azo Dyes in Water Using Ozonation and UV Enhanced Ozonation Process," *Chemosphere*, **31**(8), 3813-3825 (1995).
4. Bortone, G., "Effects of an Anaerobic Zone in a Textile Wastewater Treatment Plant," *Water Sci. Technol.*, **32**, 133-140 (1995).
5. Haug, W., Schmidt, A., Nortemann, B., Hempel, D. C., Stolz, A., and Knackmuss, H. J., "Mineralization of the Sulfonated Azo Dye Mordant Yellow 3 by a 6-aminonaphthalene-2-sulfonate Degrading Bacterial Consortium," *Appl. Environ. Microbiol.*, **57**, 3144-3149 (1991).
6. O'Neill, C., Lopez, A., Esteves, S., Hawkes, F., Hawkes, D. L., and Wilcox, S., "Azo-dye Degradation in an Anaerobic-Aerobic Treatment System Operating on Simulated Textile Effluent," *Appl. Biochem. Biotechnol.*, **53**, 249-254 (2000).
7. Thurston, C. F. "The Structure and Function of Fungal Laccases," *Microbiol.*, **140**, 19-26 (1994).
8. Böhmer, S., Messner, K., and Srebotnik, E., "Oxidation of Phenanthrene by a Fungal Laccase in the Presence of 1-hydroxybenzotriazole and Unsaturated Lipids," *Biochem. Biophys. Res. Commun.*, **244**, 233-238 (1998).
9. Call, H. P., and Mucke, I., "Process Development and Mechanisms in the Mediated Bleaching of Pulps by Laccase," *Abstr. Pap. Am. Chem. Soc.*, **211**, 147-CELL (1996).
10. D'Annibale, A., Rita Stazi, S., Vinciguerra, V., and Giovannozzisermanni, G, "Oxirane-immobilized Lentinula Edodes Laccase: Stability and Phenolics Removal Efficiency in Olive Mill Wastewater," *J. Biotechnol.*, **77**:265-273 (2000).
11. Davis, S., and Burns, R. G., "Covalent Immobilization of Laccase on Activated Carbon for Phenolic Effluent Treatment," *Appl. Microbiol. Biotechnol.*, **37**, 474-479 (1992)
12. Ilha, C. E. G., dos Santos, A. J. M. G., and SouzaDe, J. R., "Degradation of Monoazo Pigments Red 53:1 and Red 48:2 by Fenton, Photo-Fenton and UV/Peroxide Reactions," *Clean*, **37**(10), 799-805 (2009).
13. Saladino, R., Guazzaroni, M., Crestini, C., and Crucianelli, M., "Dye Degradation by Layer-by-Layer Immobilized Peroxidase/Redox Mediator Systems," *Chem. Cat. Chem.*, **5**(6), 1407-1415 (2013).
14. Zhang, J. N., Song, M. Y., Wang, X. Y., Wu, J. R., Yang, Z. P., Cao, J. H., Chen, Y., and Wei, Q. F., "Preparation of a Cellulose Acetate/Organic Montmorillonite Composite Porous Ultrafine Fiber Membrane for Enzyme Immobilizatione," *J. Appl. Polym. Sci.*, **133**(33), 43818-43821 (2016).
15. Xia, T. T., Liu, C. Z., Hu, J. H., and Guo, C., " Improved Performance of Immobilized Laccase on Amine-Functioned Magnetic Fe_3O_4 Nanoparticles Modified with Polyethylenimine," *Chem. Eng. J.*, **295**, 201-206 (2016).
16. Park, J.-W., "Phosphatidic Acid Production by PLD Covalently Immobilized on Porous Membrane," *Clean Technol.*, **21(4), 224-228 (2015).**
17. Lee, J. M., Biochemical Engineering, Prentice Hall, Englewood Cliffs, NJ, USA, 1992, 21-31
18. Hu, Z., Xu, L., and Wen, X., "Mesoporous Silicas Synthesis and Application for Lignin Peroxidase Immobilization by Covalent Binding Method," *J. Environ. Sci.*, **25**(1), 181-187 (2013).
19. Tay, T., Köse, E., Kecili, R., and Say, R., "Design and Preparation of Nano Lignin Peroxidase by Protein Block Copolymerization Approach," *Polym.*, **8**(6), 223 (2016).
20. Asgher, M., Iqbal, H. M. N., and Irshad, M., "Characterization of Purified and Xerogel Immobilized Novel Lignin Peroxidase Produced from Trametes Versicolor IBL04 using Solid State Medium of Corncobs," *BMC Biotechnol.*, **12**, 46 (2012).