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Highly Efficient Synthesis of 2,5-Dihydroxypyridine using *Pseudomonas* sp. ZZ-5 Nicotine Hydroxylase Immobilized on Immobead 150

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Received: 30 September 2018; Accepted: 13 November 2018; Published: 16 November 2018

Abstract: In this report, the use of immobilized nicotine hydroxylase from *Pseudomonas* sp. ZZ-5 ($HSPH_{ZZ}$) for the production of 2,5-dihydroxypyridine (2,5-DHP) from 6-hydroxy-3-succinoylpyridine (HSP) in the presence of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) is described. $HSPH_{ZZ}$ was covalently immobilized on Immobead 150 ($ImmHSPH_{ZZ}$). $ImmHSPH_{ZZ}$ (obtained with 5–30 mg of protein per gram of support) catalyzed the hydrolysis of HSP to 2,5-DHP. At a protein loading of 15 mg g^{-1}, $ImmHSPH_{ZZ}$ converted 93.6% of HSP to 2,5-DHP in 6 h. The activity of $ImmHSPH_{ZZ}$ was compared with that of free $HSPH_{ZZ}$ under various conditions, including pH, temperature, enzyme concentration, substrate concentration and stability over time, and kinetic parameters were measured. The results showed that $ImmHSPH_{ZZ}$ performed better over wider ranges of pH and temperature when compared with that of $HSPH_{ZZ}$. The optimal concentrations of $ImmHSPH_{ZZ}$ and substrate were 30 mg L^{-1} and 0.75 mM, respectively. Under optimal conditions, 94.5 mg L^{-1} of 2,5-DHP was produced after 30 min with 85.4% conversion. After 8 reaction cycles and 6 days of storage, 51.3% and 75.0% of the initial enzyme activity remained, respectively. The results provide a framework for development of commercially suitable immobilized enzymes that produce 2,5-DHP.

Keywords: 2,5-DHP; immobilization; $HSPH_{ZZ}$; $ImmHSPH_{ZZ}$; hydrolytic activity

1. Introduction

Nicotine is a major pyridine alkaloid found in tobacco that causes smoking addiction and several diseases such as pulmonary disease and cancer [1,2]. The manufacturing of tobacco products produces large amounts of toxic solid, liquid and airborne waste with high nicotine content [3,4]. Nicotine is very toxic to human health and the disposal of tobacco waste is a serious ecological problem [5,6]. As an environmentally friendly treatment, microbial degradation of nicotine is a promising approach because of its low cost and high efficiency. Several microorganisms including *Arthrobacter* species, *Pseudomonas* species, *Agrobacterium tumefaciens* S33, *Ochrobactrum intermedium*, *Aspergillus oryzae*,

Rhodococcus species, *Agrobacterium* species and *Sphingomonas* species have been reported to degrade nicotine [7–15]. Pyridine derivatives from nicotine are used extensively in functional materials and found in natural products that possess important biological activities. Recent studies have reported that several intermediates produced from nicotine degradation by microorganisms are important precursors (e.g., 2,5-dihydroxy-pyridine (2,5-DHP)) [5,16]. In addition, the drug 5-aminolevulinic acid, used in cancer therapy, has been produced from degradation of nicotine by microorganisms [17].

Immobilization of enzymes is an essential facet of modern biotechnology. Enzyme immobilization (especially on magnetic carriers) offers many advantages when compared with soluble enzyme preparations, including the ability to improve the catalytic properties of the enzyme, easy enzyme recovery and reuse, and reduced catalytic processing costs [18–20]. Various methods, including physical adsorption, covalent binding, cross-linking, entrapment and encapsulation, have been used for enzyme immobilization. Among them, the covalent binding method could make it difficult for the free enzyme to leach and exhibit high enzymatic activity due to the strong interaction between the enzyme and carrier function group [21–23].

In our previous work, the synthesis of 2,5-DHP from 6-hydroxy-3-succinoylpyridine (HSP) using HSP (6-hydroxy-3-succinoylpyridine) hydroxylase from *Pseudomonas* sp. ZZ-5 ($HSPH_{ZZ}$) was attempted, and the yield of 2,5-DHP reached 74.9% (*w*/*w*) in 40 min under optimal conditions [24]. However, recovery and reuse of $HSPH_{ZZ}$ could not be achieved. Therefore, to improve the catalytic properties and reduce the cost of the biocatalytic process, $HSPH_{ZZ}$ should be immobilized. Until now, there are no published reports on the synthesis of 2,5-DHP by immobilized HSP hydroxylase. In this context, the aim of this work was to optimize the synthesis of 2,5-DHP catalyzed by $HSPH_{ZZ}$ covalently immobilized on Immobead 150 ($ImmHSPH_{ZZ}$) by determining the optimal pH, temperature, and enzyme and substrate concentrations. Kinetic parameters K_m and k_{cat} were determined and reusability was also examined.

2. Results and Discussion

2.1. Immobilization of Purified $HSPH_{ZZ}$ on Immobead 150

The immobilization of $HSPH_{ZZ}$ was performed with Immobead 150, and the immobilized $HSPH_{ZZ}$ was termed $ImmHSPH_{ZZ}$. Protein loadings and immobilization times were 5–30 mg g^{-1} and 6 h, respectively. Table 1 shows that the immobilization efficiency of $HSPH_{ZZ}$ was 100% when the $HSPH_{ZZ}$ loading ranged from 5 mg g^{-1} to 20 mg g^{-1}. This result indicated that the support of Immobead 150 was not saturated at the high $HSPH_{ZZ}$ loading. The immobilization efficiency decreased to 45% when the $HSPH_{ZZ}$ loading increased to 30 mg g^{-1}. The retention of activity increased with increasing $HSPH_{ZZ}$ loading (5–15 mg g^{-1}). The highest retention of activity of $ImmHSPH_{ZZ}$ was achieved (95%) at a $HSPH_{ZZ}$ loading of 15 mg g^{-1}. The retention of activity decreased to 45% when $HSPH_{ZZ}$ loading was further increased from 20 to 30 mg g^{-1}. The preparation of $HSPH_{ZZ}$ immobilized on Immobead 150 was used to catalyze the hydrolysis of HSP to 2,5-DHP with $HSPH_{ZZ}$ loadings of 5, 10, 15 and 20 mg g^{-1} (Figure 1). The reaction products catalyzed by $ImmHSPH_{ZZ}$ were identified using liquid chromatography-mass spectrometry (LC-MS. The reaction time was 6 h, and the yields of 2,5-DHP were 85.1%, 92.7%, 93.6% and 82.6% at $HSPH_{ZZ}$ loadings of 5, 10, 15 and 20 mg g^{-1}, respectively. These results demonstrated that the immobilized $HSPH_{ZZ}$ showed high hydroxylase activity after covalent binding to Immobead 150.

2.2. Scanning Electron Microscopy (SEM) Analysis of $HSPH_{ZZ}$ and $ImmHSPH_{ZZ}$

The surface structure of $HSPH_{ZZ}$ before and after covalently immobilizing on Immobead 150 was observed using SEM (Figure 2). The hydroxylase bound to the surface of the beads and the surface morphology of $HSPH_{ZZ}$ immobilized to the beads differed when compared with that of the free enzyme. The $ImmHSPH_{ZZ}$ surface became spheroid in shape after immobilization, which may be caused by the formed covalent bond between an amino group located on the surface of the enzyme and

the support matrix oxirane ring of Immobead 150 [25]. Therefore, these results showed that nicotine hydroxylase $HSPH_{ZZ}$ was immobilized on Immobead 150.

Table 1. Effect of the protein to support ratio (protein loading) on the immobilization of purified HSPHZZ on Immobead 150.

-	Protein Loading (mg g-1)	Immobilization Efficiency (IE) (%)	Retention of Activity (R) (%)
Immobead 150	5	100	78
	10	100	85
-	15	100	95
-	20	100	75
-	25	90	51
-	30	67	45

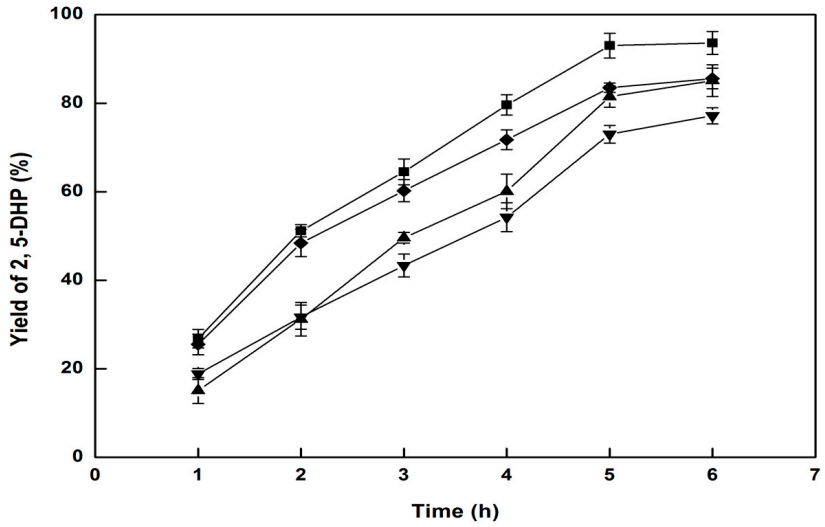


Figure 1. Synthesis of 2,5-dihydroxypyridine (2,5-DHP) by Immobead 150 ($ImmHSPH_{ZZ}$) at protein loading of (▲) 5, (♦) 10, (■) 15 and (▼) 20 mg g^{-1}. The reactions were performed in 20 mM Tris-HCl buffer (pH 9.0) containing 0.75 mM HSP, 30 mg/mL $ImmHSPH_{ZZ}$, 10 mM FAD and 0.5 mM NADH at 35 °C for 30 min.

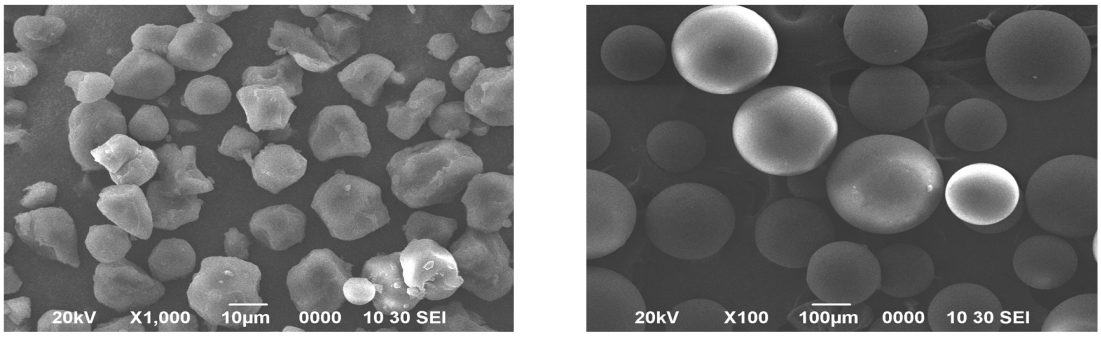


Figure 2. SEM micrographs of surface of $HSPH_{ZZ}$ (**a**) and $ImmHSPH_{ZZ}$ (**b**). Magnification: (**a**) ×1000; (**b**) ×100.

2.3. Effect of pH on the Activity of $HSPH_{ZZ}$ and $ImmHSPH_{ZZ}$

The effect of pH on the hydrolytic activity of $HSPH_{ZZ}$ and $ImmHSPH_{ZZ}$ was investigated at pH values between 5.0 and 10.0. As shown in Figure 3, free $HSPH_{ZZ}$ showed higher hydrolytic activities over the pH range of 8.0–9.0 with a maximum activity at pH 8.5. After immobilization on Immobead 150, the optimum pH range was between 7.5 and 9.5, and maximal activity was observed at pH 9.0, indicating that the immobilized $HSPH_{ZZ}$ exhibited higher tolerance to alkaline pHs. $ImmHSPH_{ZZ}$ maintained over 50% of its maximum activity at pH 7.0–10.0, whereas the free $HSPH_{ZZ}$ maintained over 50% of its maximum activity at pH 7.0–9.0. These observations showed that immobilization of HSPHzz improved enzymatic performance over a wider pH range. This improved pH-stability may be because of the covalent bond formed and possible secondary interactions between the enzyme and the functional groups of the carrier, which enhance the stability of the molecular structure of the immobilized enzyme at various pH values [26]. This behavior could be explained by influence of the partition effects on the enzymatic activities of the immobilized protein, which were from different concentrations of charged species in the micro-environment of the immobilized protein and reaction solution [27].

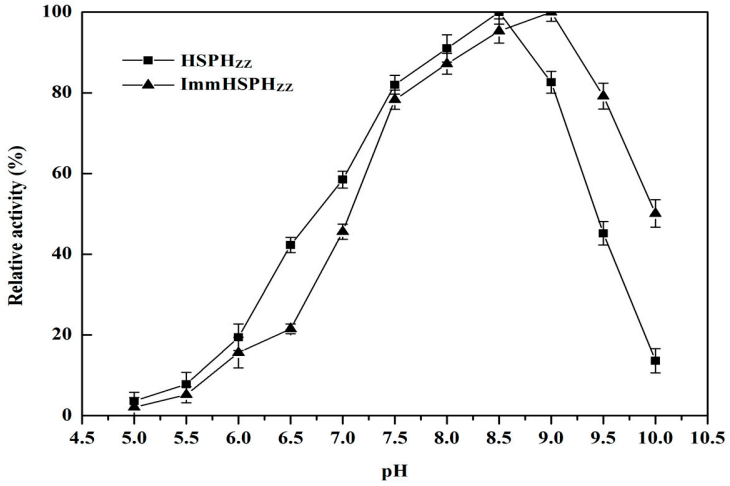
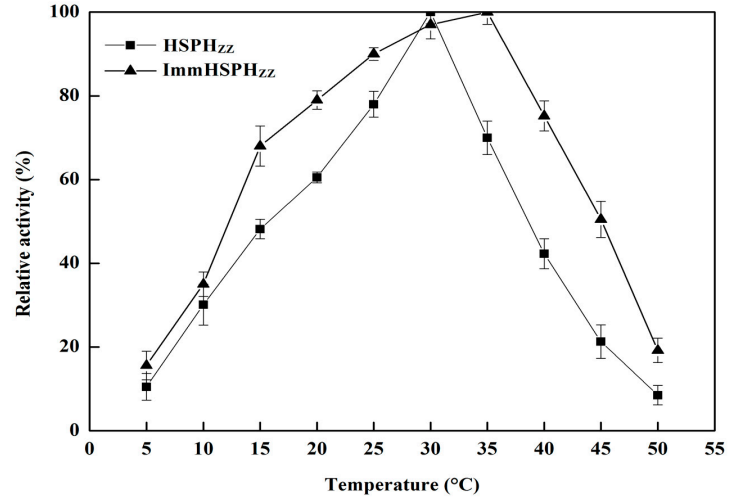


Figure 3. Temperature optima of $HSPH_{ZZ}$ and $ImmHSPH_{ZZ}$. The buffers (20 mM) used: sodium acetate (pH 5.5–6.0), sodium phosphate (pH 6.5–7.5), Tris-HCl (pH 8.0–9.0) and *N*-cyclohexyl-3-aminopropanesulfonic acid (pH 9.5–10.0).

2.4. Effect of Temperature on the Activity of $HSPH_{ZZ}$ and $ImmHSPH_{ZZ}$

The effect of temperature on the hydrolytic activity of $HSPH_{ZZ}$ and $ImmHSPH_{ZZ}$ was evaluated. Immobilization of $HSPH_{ZZ}$ strongly increased its thermostability when compared with that of the free $HSPH_{ZZ}$ (Figure 4). Maximal activity of $ImmHSPH_{ZZ}$ was observed at ~35 °C and more than half the maximal activity was observed at 15–45 °C. In contrast, the maximal activity of $HSPH_{ZZ}$ was ~30 °C, and more than half the maximal activity was observed at 20–35 °C. Thermal stabilities of $HSPH_{ZZ}$ and $ImmHSPH_{ZZ}$ were investigated at 40 °C with increasing incubation periods up to 6 days. After incubation for 6 days, the enzymatic activity of $ImmHSPH_{ZZ}$ was only partly reduced (~73%), whereas the activity of $HSPH_{ZZ}$ had reduced to 13%. Thus, temperature has a lower impact on the activity profile of $ImmHSPH_{ZZ}$ when compared with that of $HSPH_{ZZ}$. This result demonstrated that the immobilized $HSPH_{ZZ}$ showed higher thermostability than the free enzyme [25,26]. The thermostability of the immobilized $HSPH_{ZZ}$ is in accordance with that of other previously reported immobilized enzymes. This improved thermostability may be because of the formation of the covalent linkage between $HSPH_{ZZ}$ and Immobead 150, which changes the conformation of the enzyme as a result of the temperature changes [27].



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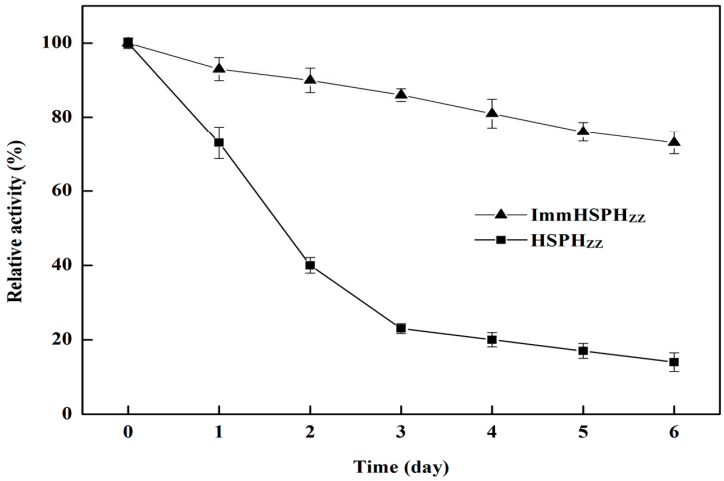

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Figure 4. Temperature optima (**a**) and thermostability (**b**) of $HSPH_{ZZ}$ and $ImmHSPH_{ZZ}$. (**a**) Temperature optimums of $HSPH_{ZZ}$ and $ImmHSPH_{ZZ}$ were determined with HSP as substrate in 20 mM Tris-HCl buffer (pH 9.0) at temperatures ranging from 5 to 50 °C. (**b**) Thermostability of $HSPH_{ZZ}$ and $ImmHSPH_{ZZ}$. The residual enzyme activity was measured after incubation of the enzyme at 40 °C.

2.5. Effect of Enzymatic Concentration on the Activity of $ImmHSPH_{ZZ}$

Different enzymatic concentrations (10–50 mg mL^{-1}) were studied to determine the optimal conditions for synthesis of 2,5-DHP. Interestingly, a lower yield of 2,5-DHP was obtained at higher concentrations of $ImmHSPH_{ZZ}$ (40–50 mg mL^{-1}; Figure 5). The highest 2,5-DHP yield (95.6%) was obtained at an enzyme concentration of 30 mg mL^{-1}. In general, the product yield increased as the concentration of the enzyme increased. However, the presence of excess catalyst (>30 mg mL^{-1}) can result in enzyme agglomeration and diffusion problems, which can reduce reaction efficiency [28,29]. An $ImmHSPH_{ZZ}$ loading of 30 mg mL^{-1} offered the efficient hydrolysis of HSP and synthesis of 2,5-DHP.

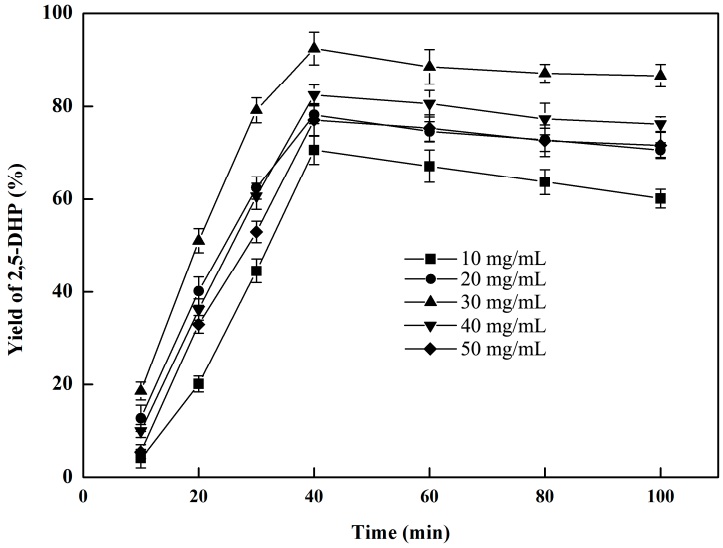


Figure 5. Effect of enzyme concentration of ImmHSPH$_{ZZ}$ on the production of 2,5-DHP.

2.6. Effect of HSP Concentration on the Activity of ImmHSPH$_{ZZ}$

The yields of 2,5-DHP produced by ImmHSPH$_{ZZ}$ at different HSP concentrations (0.25–2.0 mM) are shown in Figure 6. The reaction rate increased with increasing HSP concentrations between 0.25 and 0.75 mM. The highest yield of 2,5-DHP was achieved at a HSP concentration of 0.75 mM. The yield of 2,5-DHP decreased when the HSP concentration was further increased from 1.0 to 2.0 mM. This may be because higher amounts of HSP limit substrate mass transfer to the active center of ImmHSPH$_{ZZ}$ [30,31]. The Michaelis-Menten equation was used for kinetic analysis of ImmHSPH$_{ZZ}$. The kinetic parameters of ImmHSPH$_{ZZ}$ for HSP were calculated under optimal conditions and at the NADH concentration of 1.0 mM. As shown in Table 2, the k_{cat}/K_m value of ImmHSPH$_{ZZ}$ (24.0 S^{-1} mM^{-1}) toward HSP is higher than that of HSPH$_{ZZ}$ (10.6 S^{-1} mM^{-1}). Moreover, the k_{cat}/K_m value of ImmHSPH$_{ZZ}$ (23.6 S^{-1} mM^{-1}) toward NADH is higher than that of HSPH$_{ZZ}$ (8.3 S^{-1} mM^{-1}). Kinetic parameters (K_m, k_{cat} and K_{cat}/K_m) for immobilized enzymes often differ when compared with the corresponding parameters obtained for the free enzyme [32,33]. These results demonstrated that ImmHSPH$_{ZZ}$ exhibited higher substrate affinity and catalytic efficiency when compared with that of HSPH$_{ZZ}$.

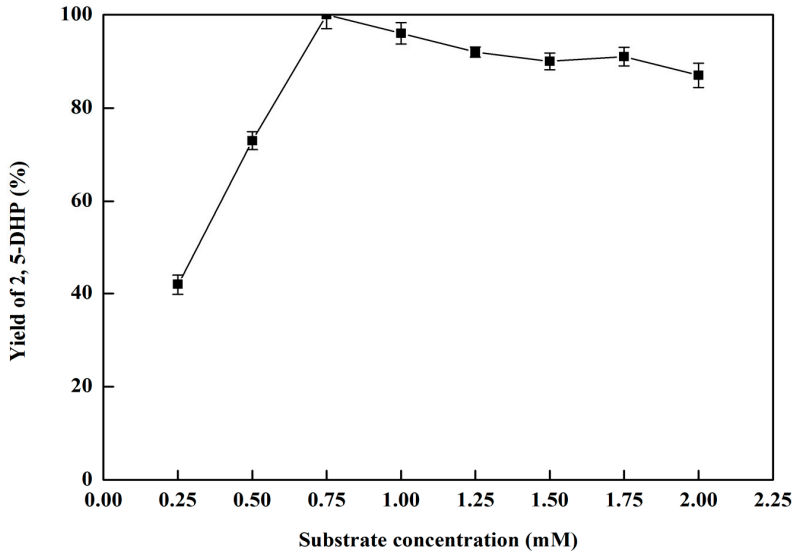


Figure 6. Effect of substrate concentration on the production of 2,5-DHP for HSPH$_{ZZ}$ and ImmHSPH$_{ZZ}$.

Table 2. Kinetic parameters of HSPHZZ and ImmHSPHZZ for the substrate of HSP or DADH.

Protein	Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
$HSPH_{ZZ}$	HSP	1.9 ± 0.3	0.18 ± 0.02	10.6 ± 2.1
-	NADH	1.5 ± 0.4	0.18 ± 0.04	8.3 ± 1.9
$ImmHSPH_{ZZ}$	HSP	4.8 ± 0.4	0.20 ± 0.02	24.0 ± 2.1
-	NADH	5.2 ± 0.2	0.22 ± 0.05	23.6 ± 1.9

2.7. 2,5-DHP Production from HSP by ImmHSPH$_{ZZ}$ under Optimum Conditions

The production of 2,5-DHP from HSP by $ImmHSPH_{ZZ}$ was measured under optimal conditions (at 35 °C and pH 9.0 in 20 mM Tris-HCl buffer) with $ImmHSPH_{ZZ}$ at a concentration of 30 mg mL^{-1}. The immobilized enzyme produced 2,5-DHP (97.2 mg L^{-1}) at a conversion of 85.4% (*w*/*w*) after a reaction time of 30 min (Figure 7). In our previous work, the yield of 2,5-DHP using free $HSPH_{ZZ}$ reached 74.9% in 40 min under optimal conditions [24]. Therefore, the yield of 2,5-DHP catalyzed by $ImmHSPH_{ZZ}$ is higher when compared with that of $HSPH_{ZZ}$, and 2,5-DHP was produced in a shorter reaction time (30 min) when HSP was catalyzed by $ImmHSPH_{ZZ}$. The production of 2,5-DHP from HSP by $ImmHSPH_{ZZ}$ was also higher than that of the nicotine HSP hydroxylase from *A. tumefaciens* S33 (a conversion of 69.7% in 50 min) [10]. These results demonstrated that enzymatic transformation of HSP to 2,5-DHP by $ImmHSPH_{ZZ}$ was superior to free $HSPH_{ZZ}$ and likely to be suitable for commercial applications.

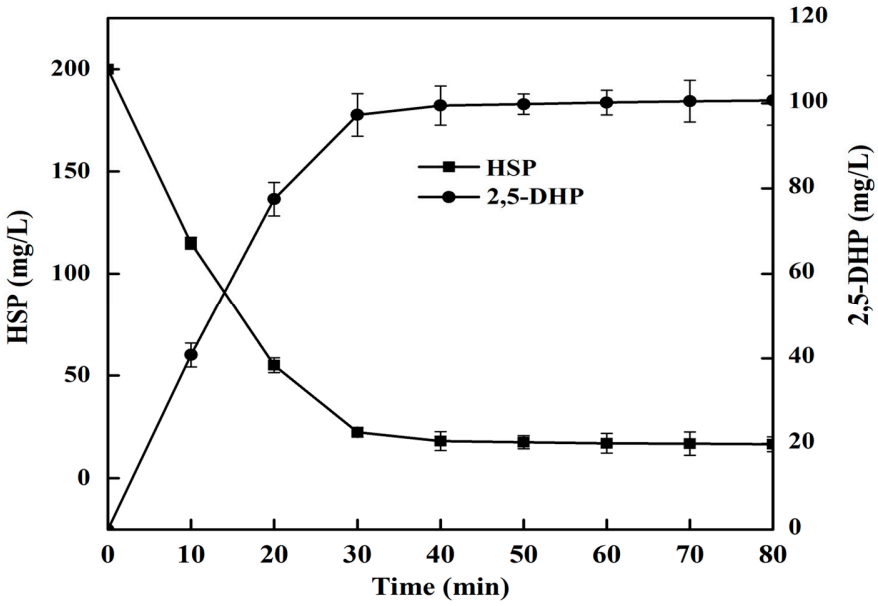
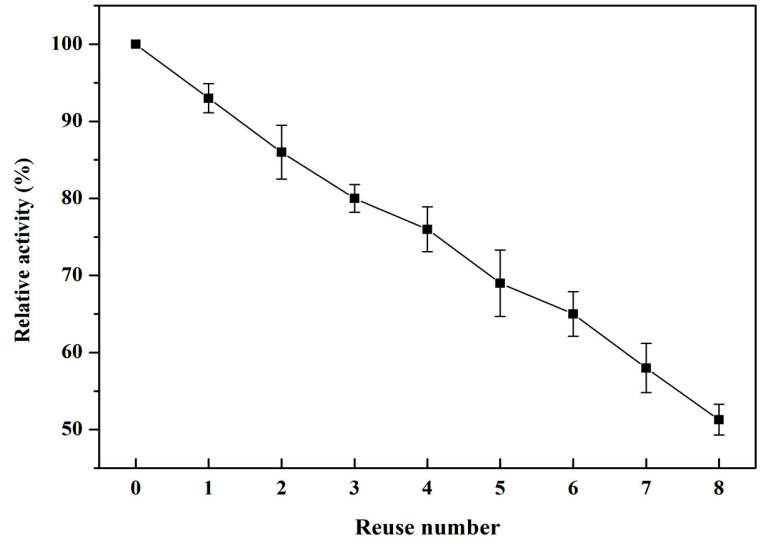


Figure 7. Time course of 2,5-DHP (**circles**) production from HSP (**boxes**) for $ImmHSPH_{ZZ}$ under the optimum condition.

2.8. Stability of Storage and Reusability

The stability of storage and reusability is an important requirement for industrial enzyme applications. Immobilization of the enzyme may improve resistance to reaction conditions and conformational changes, which reduces the likelihood of denaturation [34,35]. The storage stability and operational stability of $ImmHSPH_{ZZ}$ towards HSP were evaluated (Figure 8). The results showed that the immobilized enzyme kept 51.3% of its activity up until eight cycles. These results may be explained that $HSPH_{ZZ}$ release from the support during recycle use due to weak interaction between Immobead 150 and the enzyme. To investigate the storage stability of $ImmHSPH_{ZZ}$, the activity of $HSPH_{ZZ}$ after incubating the enzyme at 4 °C for different time intervals was measured. After 6 days, the relative activity of $ImmHSPH_{ZZ}$ remained greater than 75.0% of the initial activity, whereas that

of $HSPH_{ZZ}$ was only ~9% of the initial activity. These results indicated that the storage stability of $ImmHSPH_{ZZ}$ was clearly superior when compared with that of $HSPH_{ZZ}$.



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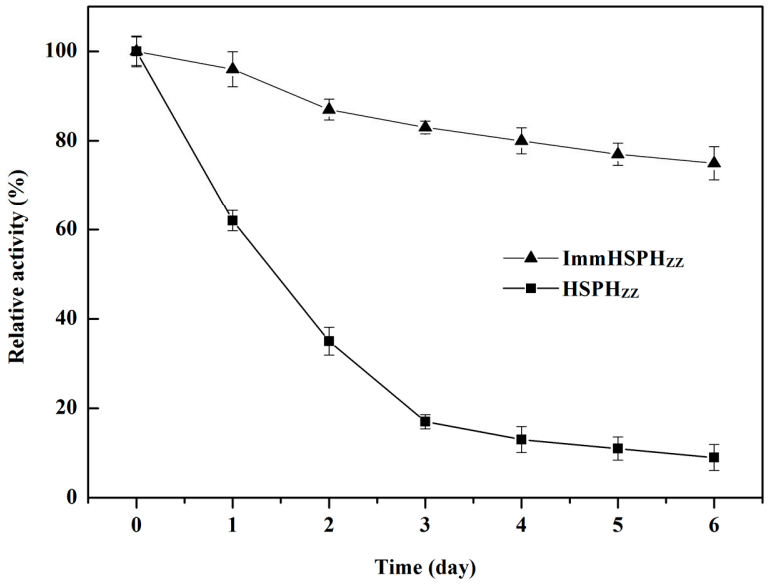

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Figure 8. Stability of reusability (**a**) and storage (**b**) of immobilized $ImmHSPH_{ZZ}$.

3. Materials and Methods

3.1. Materials

2,5-DHP was purchased from SynChem OHG (Altenburg, Germany). The Immobead 150 support and succinic acid were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). HSP was obtained from Professor Hongzhi Tang at Shanghai Jiao Tong University. All other chemicals used were analytical grade quality and obtained from commercial sources in China.

3.2. Multipoint Immobilization of HSPH$_{ZZ}$ on Immobead 150

The support of Immobead 150 (0.2 g) was added to a HSPH$_{ZZ}$ solution (5 mL) in 20 mM sodium bicarbonate buffer (pH 8.5), with HSPH$_{ZZ}$ loadings ranging from 5 mg g^{-1} to 30 mg g^{-1}. The mixture was incubated at 4 °C for 6 h in an orbital shaker operating at 150× rpm. To check for covalent binding, the mixture was washed with 1 M NaCl and ethylene glycol (30%, *v*/*v*), centrifuged, vacuum freeze-dried and stored at 10 °C. The immobilization efficiency (IE, %) and retention of activity (R, %) were calculated according to Madalozzo et al. [36].

3.3. Characterization of HSPH$_{ZZ}$ and ImmHSPH$_{ZZ}$

The activity of HSPH$_{zz}$ and ImmHSPH$_{ZZ}$ hydroxylase was determined by the catalysis of HSP to 2,5-DHP, as described previously [24]. The production of 2,5-DHP was measured by an electron spray ionization (ESI) source on an AB Sciex Triple Quad 5500 mass spectrometer (AB Sciex, Framingham, MA, USA) with an Agilent 1290 infinity liquid chromatography (LC) system for ultra high performance liquid chromatography (UHPLC). The mobile phase was methanol/acetic acid (*v*/*v*, 25:75) at a flow rate of 0.5 mL min^{-1}. One unit of HSPH$_{ZZ}$ and ImmHSPH$_{ZZ}$ were defined as the amount of enzyme required to synthesize 1 mol 2,5-DHP per min under the standard conditions.

The activities of HSPH$_{ZZ}$ and ImmHSPH$_{ZZ}$ were determined as a function of pH (5.0–10.0), temperature (5–50 °C), enzymatic concentration (10–50 mg mL^{-1}) and substrate (HSP) concentration (0.25–2.0 mM). The enzymatic activities of the free and immobilized enzyme were determined using HSP at concentrations ranging from 0.01 to 1 mM. The corresponding K_m and k_{cat} values were calculated using Hanes–Wolff plots and the Michaelis–Menten equation.

3.4. Stability of ImmHSPH$_{ZZ}$

The reusability of ImmHSPH$_{ZZ}$ was examined by the hydrolytic activity assay under optimal conditions. At the end of each cycle, the separation of ImmHSPH$_{ZZ}$ from the reaction mixture was achieved by filtration and washed with 1 mL of 20 mM bicarbonate buffer (pH 8.5). Recovered ImmHSPH$_{ZZ}$ was then dried at room temperature for 24 h before use in the next cycle.

Relative activity was calculated by defining the first reaction as 100%.

3.5. SEM Assay of ImmHSPH$_{ZZ}$

The ImmHSPH$_{ZZ}$ was sputtered with gold and the structures were observed using a Quanta-200 scanning electron microscope (FEI, Amsterdam, Holland).

3.6. Statistical Analysis

Triplicate experiments were performed for each parameter investigated and the mean and standard deviation values were reported.

4. Conclusions

In this study, nicotine hydroxylase from *Pseudomonas* sp. ZZ-5 was immobilized on Immobead 150 using covalent binding methods to optimize enzymatic production of 2,5-DHP from HSP. This preparation was characterized by SEM techniques to characterize the morphology of the immobilized enzyme on the support and the enzyme-support interaction. ImmHSPH$_{ZZ}$ displayed higher hydrolysis activity and catalytic performance than free HSPH$_{ZZ}$, and exhibited better thermal stability, storage stability, and reusability when compared with the free enzyme. Under optimal conditions, ImmHSPH$_{ZZ}$ produced 94.5 mg L^{-1} of 2,5-DHP from 200 mg L^{-1} of HSP after 30 min with 85.4% conversion. These results demonstrated that immobilized HSPH$_{ZZ}$ is a prospective system for the enzymatic production of 2,5-DHP in biotechnology applications.

Author Contributions: C.D. designed and performed the experiments, analyzed the data and prepared the manuscript. Y.Z., H.T., Z.L., J.L., Z.Z., and S.L. performed the experiments and assisted in data analysis. D.M. and T.W. performed experiments, analyzed the data and assisted in manuscript preparation. All authors read and approved the final manuscript.

Funding: This research was funded by grants from the National Natural Science Foundation of China (21406210), Henan Province Foreign Cooperation Projects (152106000058), Program for Science & Technology Innovation Talents in the Universities of Henan Province (18HASTIT040) and Training Plan for Young Backbone Teachers in the Universities of Henan Province (2014GJS-082).

Acknowledgments: Authors greatly acknowledge the financial support from the National Natural Science Foundation of China, Henan provincial science and technology department and Henan Provincial Department of Education.

Conflicts of Interest: The authors declare no conflicts of interest.

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