SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
APPLIED MICROBIOLOGY
METABOLIC ENGINEERING

Received
16 January 2014

Accepted
3 June 2014

Published
23 June 2014

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Green strategy from waste to value-added-chemical production: efficient biosynthesis of 6-hydroxy-3-succinoyl-pyridine by an engineered biocatalyst

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Value-added intermediates produced by microorganisms during the catabolism of *N*-heterocycles are potential building blocks for agrochemical synthesis and pharmaceutical production. 6-Hydroxy-3-succinoyl-pyridine (HSP), an intermediate in nicotine degradation, is an important precursor for the synthesis of drugs and compounds with biological activities. In the present study, we show that an engineered biocatalyst, *Pseudomonas putida* P-HSP, efficiently produced HSP from the renewable raw material of tobacco-waste that contains a high concentration of nicotine. The genetically constructed strain P-HSP realized a high accumulation of HSP, and HSP production was 3.7-fold higher than the non-engineered strain S16. Under optimal conditions, HSP was produced at high concentrations of 6.8 g l^{-1} and 16.3 g l^{-1} from tobacco-waste and nicotine, respectively. This work demonstrates a green strategy to block the catabolic pathway of *N*-heterocycles, which is a promising approach for the mutasynthesis of valuable compounds.

Microbial degradation of *N*-heterocycles, such as nicotine, may produce numerous intermediates, and many of these intermediates are potential building blocks for agrochemical and pharmaceutical synthesis (Figure 1)[1,2]. Moreover, there is a rising interest in the microbial production of valuable chemicals from renewable raw materials. For example, tobacco production for cigarette and cigar manufacturing can generate approximately 75% residue (tobacco-waste), which is a renewable raw material that could potentially be used by bio-refineries to generate energy and other products[3–5]. Furthermore, tobacco-waste is considered to be an environmental pollutant because it contains a high concentration of nicotine, which is the main alkaloid in tobacco, constituting 0.6%–3% (w/w) of dry tobacco leaves[3,6]. Nicotine has not been seriously considered as a potential renewable resource except for its use as an insecticide. It may because nicotine is toxic, and the selective functionalization at two *N*-heterocyclic rings makes nicotine difficult to control by chemical means[7]. Microbial degradation of nicotine produces several metabolic intermediates, most of which are pyridine derivatives.

Pyridine derivatives are extensively seen in natural products, pharmaceuticals and functional materials. However, pyridine derivatives are mainly produced by organic synthesis, which is often accompanied by the formation of by-products, resulting in high costs[8]. Biocatalysis is a useful supplementary technology for the chemical industry in the reactions that are not easily conducted in the organic chemistry methods[1,7]. For example, the reactions involved in the formation of 2-substituted pyridines and their derivatives from pyridine *N*-oxides are useful synthetic methods; however, these methods require strict conditions for high product yields[9,10]. In microorganisms, the pyridine 2-hydroxylation step is specifically catalyzed by a molybdenum-containing protein, such as nicotinic acid dehydrogenase (EC1.17.1.5) in nicotinic degradation pathway, and nicotine hydroxylase (EC1.5.99.4) and Spm (Figure 2a) in nicotine degradation pathways[11,12]. This hydroxylation step can be introduced to replace the 2-substituted pyridine reaction in organic synthesis. Therefore, the bio-production of valuable intermediates from *N*-heterocycles has a promising potential for organic chemical synthesis.

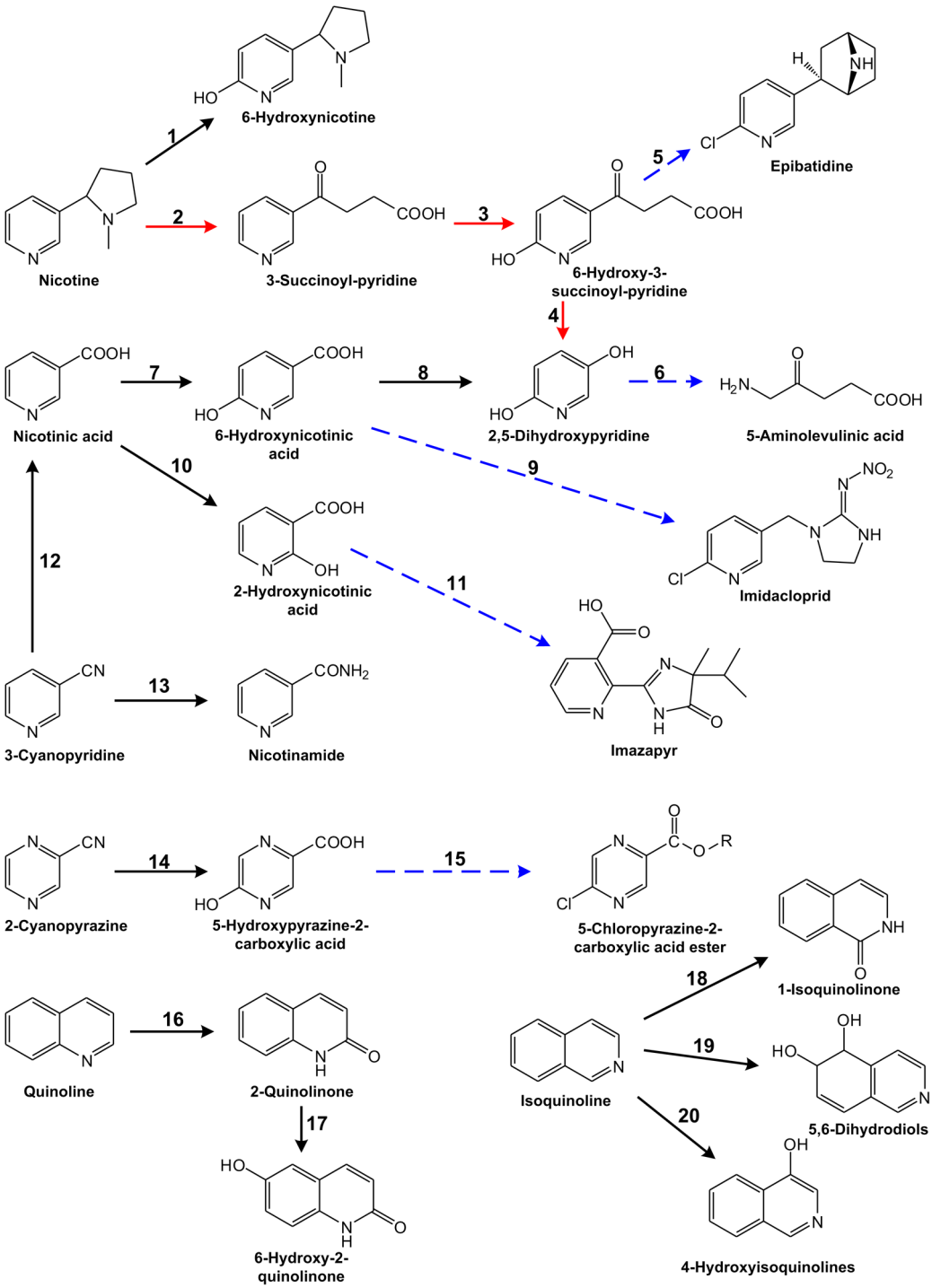

Figure 1 | Transformation of *N*-heterocycles and potential use in chemical synthesis. (1) *Arthrobacter nicotinovorans* can transform nicotine to 6-hydroxynicotine (precursor of insecticides)[16]. (2–6) *Pseudomonas putida* S16 transforms nicotine to 3-succinoylpyridine, 6-hydroxy-3-succinoylpyridine, and 2,5-dihydroxypyridine (precursor of 5-aminolevulinic acid) (red line)[12]. (7–11) Nicotinic acid can be transformed into 6-hydroxynicotinic acid (precursor of imidacloprid and nicotinoid insecticides) by *Serratia marcescens* IFO12648[36], 2,5-dihydroxypyridine by *Pseudomonas fluorescens* TN5[37], and 2-hydroxynicotinic acid by *Proteobacteria* sp.[38]. (12, 13) 3-Cyanopyidine can be transformed into nicotinic acid and nicotinamide (a vitamin used as a food supplement) by *Rhodococcus rhodochrous* J1[39,40]. (14, 15) *Agrobacterium* sp. DSM6336 has the capacity to transform 2-cyanopyrazine to 5-hydoxypyrazine-2-carboxylic acid[41], which can be used for the synthesis of 5-chloropyrazine-2-carboxylic acid esters. (16–20) Microbial degradation of quinoline and isoquinoline produces several intermediates, and these intermediates also have potential uses in pharmaceutical synthesis[30]. Solid-lined arrows: biological processes. Blue-dashed arrows: chemical processes.

6-Hydroxy-3-succinoyl-pyridine (HSP), a metabolic intermediate of nicotine, is a potential building block in the synthesis of drugs, insecticides, and other compounds that possess biological activities, such as the analgesic molecule epibatidine (Figure 1)[1]. HSP, which has a hydroxyl group and a side-chain, is a promising precursor for the synthesis of 2,5-di-substituted and 2,3,5-tri-substituted pyridine derivatives[13,14]. These 2,5-/2,3,5-substituted pyridine units are also present in several currently marketed drugs, including pioglitazone

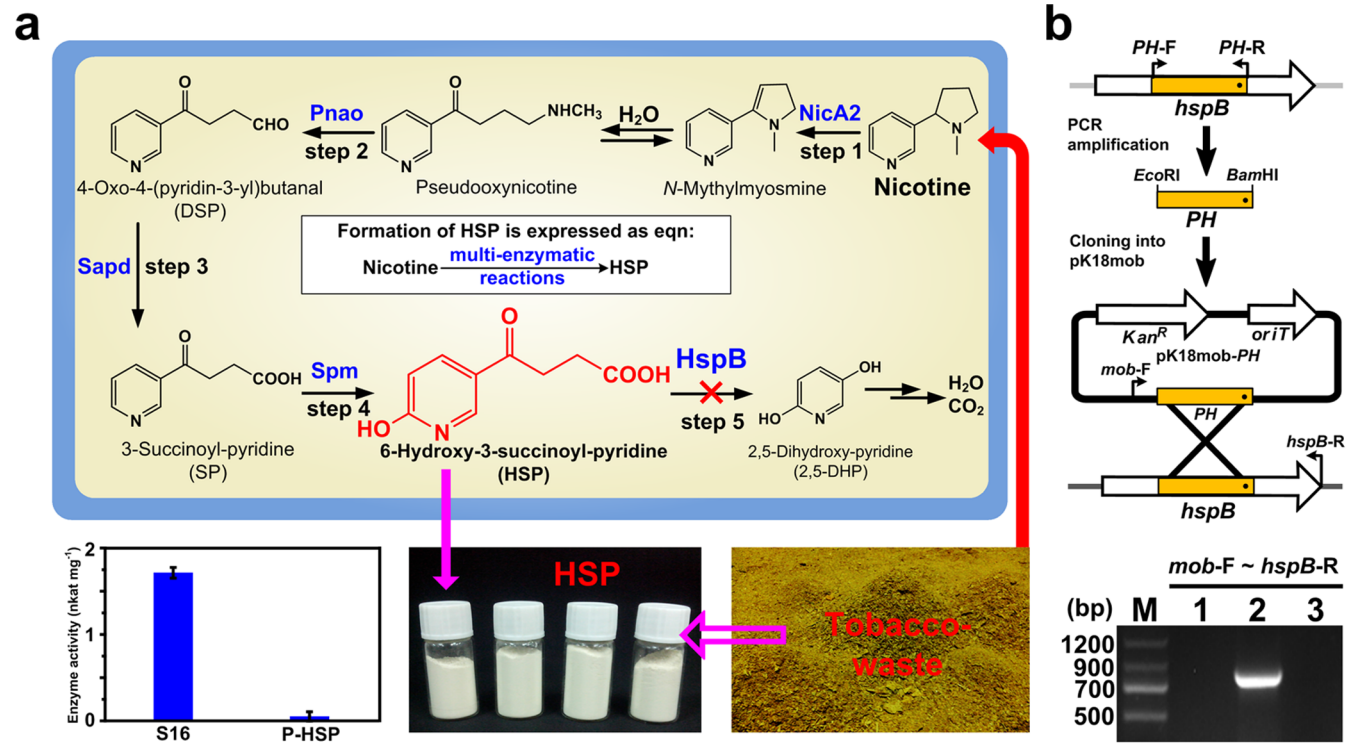

Figure 2 | Characteristics of *P. putida* P-HSP. (a) Metabolic pathway of nicotine in *P. putida* P-HSP was locally blocked (a, top). Enzymatic steps 1–5 are catalyzed by NicA2, nicotine oxido-reductase; Pnao, pseudooxynicotine amine oxidase; Sapd, DSP dehydrogenase; Spm, SP monoxygenase and HspB, respectively. The HSP monooxygenase activities of strain *P. putida* S16 and P-HSP are shown in (a, bottom panels). The photographs (a, bottom panels) were taken in the lab by the first author Yu. (b) Construction and verification of engineered strain P-HSP.

(Actos, for diabetes), eszopiclone (Lunesta, for insomnia), and the recently approved crizotinib (Xalkori, for cancer)[15]. Additionally, HSP is involved in several nicotine degradation pathways, and HSP production may facilitate investigation of these pathways[16–18]. No procedure for organic chemistry-mediated synthesis of HSP has been reported; however, biotransformation of HSP from nicotine is a practical commercial method[1].

Compared with purified enzymes, whole-cell catalysts are readily available and relatively inexpensive, and they are particularly useful for multi-enzymatic reactions[19,20]. Because most enzymes that catalyze the conversion of nicotine to HSP have not yet been purified and studied *in vitro*[16,17], whole-cell catalysis is a better option for HSP production[21,22]. In previous studies, HSP concentration produced by whole cells of a wild strain of *Pseudomonas putida* S16 was rather low at a concentration of 1.45 g l^{-1}, with a yield of 43.5%[22]. Although the growth and catalytic conditions could be optimized, it was very difficult to achieve high product yield and productivity. Therefore, microorganism engineering may be an attractive strategy for a high accumulation of HSP.

In this study, we constructed a whole-cell catalyst, *P. putida* P-HSP, by blocking the catabolic pathway of nicotine (Figure 2). The catalyst efficiently accumulated HSP, and high yield and productivity were obtained in batch and fed-batch biotransformations using tobacco-waste and nicotine as substrates, under optimized production conditions.

Results

Construction of engineered *P. putida* P-HSP. HSP, converted from 3-succinoyl-pyridine, is transformed into 2,5-dihydroxy-pyridine (2,5-DHP) by the enzyme HspB in *P. putida* S16[23]. HspB is essential for the conversion of HSP in strain S16; therefore, it is estimated that the inactivation of *hspB* would cause HSP accumulation (Figure 2a). The *hspB* gene in strain S16 was disrupted by homologous recombination as described in the Methods section (Figure 2b) to produce *P. putida* P-HSP (*P. putida* S16 *hspB*::pK18mob). HspB activity was not detected in *P. putida* P-HSP (Figure 2a).

HSP transformation capacity was regained in an *hspB*-complemented strain (*P. putida*S16 *hspB*::pK18mob (pME-*hspB*)) by transforming a plasmid containing *hspB* into strain P-HSP. Both engineered and unmodified strains were grown in LB media with 1 g l^{-1} nicotine for 24 hours. The culture color of wild strain S16 was not changed; however, the culture color of the *hspB*-complemented strain turned to saddle brown (the color of 2,5-DHP) (Figure 3a). The insertion of pK18mob in *hspB* blocks the transcription of the entire *nic2* cluster[17], which also blocks the expression of 2,5-DHP dioxygenase gene. Therefore, 2,5-DHP transformed from HSP in the *hspB*-complemented strain cannot be further converted because 2,5-DHP dioxygenase was not expressed. The resting cell experiment results (Figure 3b, c) confirm that the *hspB*-complemented strain regains the potential to convert HSP.

Reaction catalyzed by *P. putida* P-HSP. To determine the HSP production capacity of *P. putida* P-HSP, biotransformation was carried out using 3.4 g dry cell weight (DCW) l^{-1} whole cells of *P. putida* P-HSP as the biocatalyst, 3.0 g l^{-1} nicotine as the substrate, and double-distilled H_2O (ddH_2O) (final pH 10.0) as the reaction buffer at 30°C with shaking at 120 rpm. After 5 h of reaction, the sample was centrifuged, and the supernatant was analyzed by HPLC. A single peak with a retention time of 12.93 min was observed, which corresponds to the peak and spectrum of HSP (Figure 4a). The compound was analyzed by LC-MS ([M-H]$^-$, m/z 194.0456) (Figure 4b) and NMR (^{1}H NMR (DMSO, 400 MHz): δ 12.12, 8.25, 7.86, 6.38, 3.05, 2.5; ^{13}C NMR (DMSO, 100 MHz): δ 194.57, 174.28, 162.93, 141.27, 138.67, 120.00, 116.61, 32.34, 28.25) (Figure 4c, d). The signals are consistent with the molecular weight and published

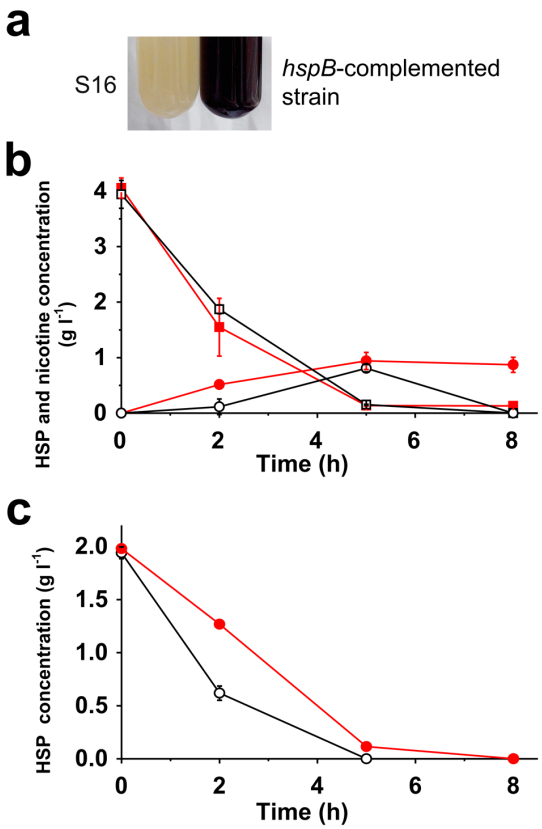

Figure 3 | Degradation of nicotine and HSP by *P. putida* S16 and the *hspB*-complemented strain (*P. putida* S16 *hspB*::pK18mob (pME-*hspB*)). (a) Both strains were grown in LB media with the addition of 1 g l^{-1} nicotine for 24 h. (b, c) Resting cells of *P. putida* S16 (black line) and *hspB*-complemented strain (red line) were used for nicotine and HSP degradation at 30°C, 3.4 g DCW l^{-1}, in 0.05 mol l^{-1} sodium phosphate buffer (pH 7.0). Each value is the mean of three parallel replicates ± SD. Symbols: HSP, ●; nicotine, ■.

NMR data of HSP. These results confirm that HSP was the only product generated from nicotine by whole cells of *P. putida* P-HSP.

Transformation by whole cells of *P. putida* P-HSP. The reaction was carried out under various conditions. The reaction buffer (ddH_2O) was adjusted with HCl to different pH values. The pH value (Figure 4e) and substrate concentration (Figure 4g) significantly affected HSP production. The optimal pH value was determined to be 9.0, and the optimal nicotine concentration was 6.0 g l^{-1}. There was no significant change in HSP production at different reaction temperatures (Figure 4f), which is good for industrial production. From 1.7 g DCW l^{-1} to 3.4 g DCW l^{-1}, a significant increase in HSP production was observed with an increase in the concentration of *P. putida* engineered cells. However, there was a minimal increase in HSP production with *P. putida*-engineered cell concentrations of over 3.4 g DCW l^{-1} (Figure 4h). Thus, the optimal concentration of *P. putida* engineered cells was determined to be 3.4 g DCW l^{-1}. Taking into account the conversion rate and biomass, *P. putida* P-HSP was most productive in the late-exponential phase of cell growth.

HSP production by batch and fed-batch transformation. Large-scale biotransformation was performed at pH 9.0, 30°C, an initial nicotine concentration of 6.0 g l^{-1}, and 3.4 g DCW l^{-1} of whole cells of *P. putida* P-HSP. Time courses of HSP production reactions in a cell-free system and with resting cells of wild strain S16, engineered strain of P-HSP and heat-killed strain of P-HSP were analyzed and are described in Figure 5. Figure 5a shows that HSP production with engineered strain (concentration, 6.40 ± 0.46 g l^{-1}; yield, 99.4%) is much higher than that with unmodified strain (concentration, 1.73 ± 0.19 g l^{-1}; yield, 38.9%) after 5 h biotransformation. It appears that the engineered strain is 3.7 times more efficient at HSP producing compared to the unmodified strain. Moreover, with the engineered strain, HSP concentration didn't decrease after it reached the maximum value; however, HSP concentration decreased in the reaction system of the unmodified strain from 7 h to 24 h. The results indicate that the engineered strain successfully blocked the pathway realizing the accumulation of HSP. Nicotine concentrations were basically unchanged in the control samples of the cell-free system and heat-killed resting cells of *P. putida* P-HSP.

HSP rapidly accumulated when nicotine or crude tobacco-waste extract was used as a substrate under batch and fed-batch transformations. For batch transformation (Figure 5b, c), the same catalyst was used four times for 4.5 h, 4.5 h, 6.0 h, and 20 h, respectively. HSP concentrations with the productive yield for the reactions were 6.81 ± 0.38 g l^{-1} (98%), 4.83 ± 0.070 g l^{-1} (100%), 2.98 ± 0.045 g l^{-1} (92%), and 1.46 ± 0.24 g l^{-1} (34%), respectively. Fed-batch biotransformation was conducted with intermittent substrate feeding to avoid substrate inhibition. The time course of nicotine feeding and HSP production are shown in Figure 6. The maximum HSP concentration obtained from nicotine was 16.3 ± 1.5 g l^{-1} at 23 h, with a productivity of 0.71 g l^{-1} h^{-1} and yield of 75% (Figure 6a). During the initial stage of biotransformation, stoichiometric formation of HSP from tobacco-waste and nicotine was observed. However, the yield decreased during the later stage of biotransformation, which suggests that the activity of resting cells and enzymes were affected by nicotine and high HSP concentration, respectively.

When crude tobacco-waste extract was used as the substrate, P-HSP cells produced 6.84 ± 0.36 g l^{-1} HSP at 27 h, with a productivity of 0.25 g l^{-1} h^{-1} and a yield of 36% (Figure 6b). The HSP concentration obtained using crude tobacco-waste extract was lower than that obtained using pure nicotine. This may be due to the fact that crude tobacco-waste extract has complex components, of which many are secondary metabolites of tobacco that could inhibit enzyme activity. However, preparation of crude tobacco-waste extract by steam distillation from tobacco-waste is a simple and green process that avoids the use of organic solvents for nicotine extraction.

Discussion

N-Heterocycles are common structural motifs in natural molecules and in man-made chemical active substances, some of which are environmental pollutants, such as nicotine from tobacco, pyrrole/pyridine/pyrrolidine/pyrazine derivatives that are used as drugs, herbicides, insecticides, paints, and some carcinogenic azaarenes[7,24–26]. Microbial degradation is one of the best strategies to remove these pollutants when they have contaminated soil and water. Many microorganisms possessing different *N*-heterocycle degradation pathways have previously been isolated[27,28]. Meanwhile, microbial catabolism of *N*-heterocycles produces a lot of intermediates, which provide a huge pool of building blocks for the synthesis of agrochemicals and pharmaceuticals[2,29,30]. In some cases, the synthesis and modification of these intermediates are difficult via organic chemistry methods. Biocatalysis, born in the early 1900s and flourished with the concept of "green chemistry" in the 1990s, was introduced as a supplementary technology. Its highly chemo-, regio- and stereoselective bioconversions simplify certain manufacturing processes, making them economically attractive and environmentally acceptable[1]. Some biochemical pathways, which have the potential for biotransformation of *N*-heterocycles into value-added chemicals, are listed in Figure 1[28,31]. Microbial catabolism has great potential for the production of *N*-heterocycle intermediates, which could be used as an important supplementary technology in the chemical industry.

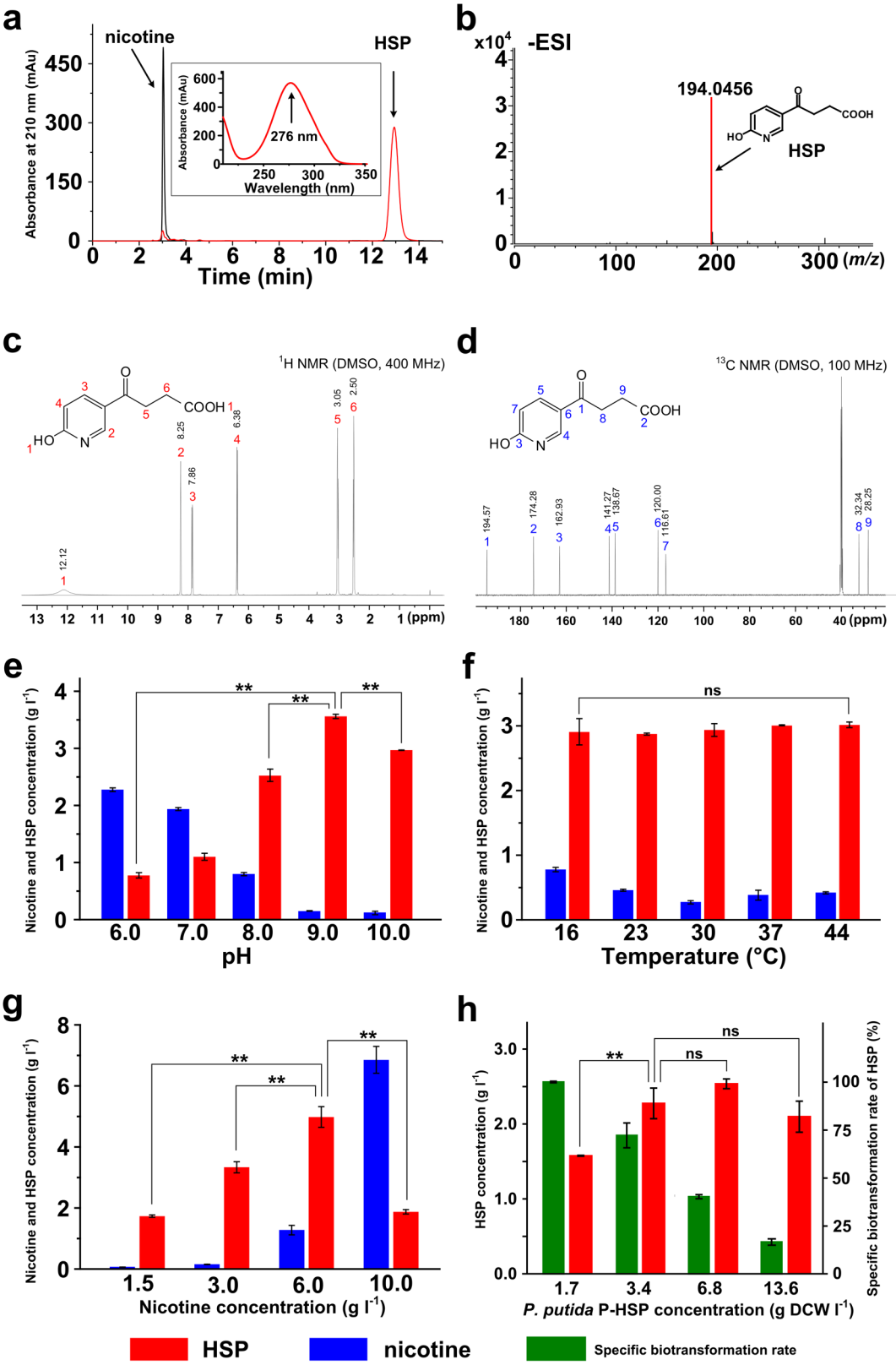

Figure 4 | Feasibility of HSP production and optimization of biotransformation conditions. (a) HPLC analysis of the reaction sample at 0 min (black line) and 5 h (red line) for the catalysis of HSP from nicotine by whole cells of *P. putida* P-HSP. The inset shows the spectrum of the HPLC signal at 12.93 min. (b) LC-MS analysis of the sample after a 5-h reaction. (c, d) ^{1}H and ^{13}C NMR profiles of the product. Effect of pH (e), temperature (f), and nicotine concentration (g) on HSP production. (h) Effect of biomass concentration on the specific biotranformation rate of HSP. Each value is the mean of three parallel replicates ± SD. *t*-test was used to calculate statistical significance. Symbols: ns, no significance ($p > 0.05$); **, $p <= 0.01$.

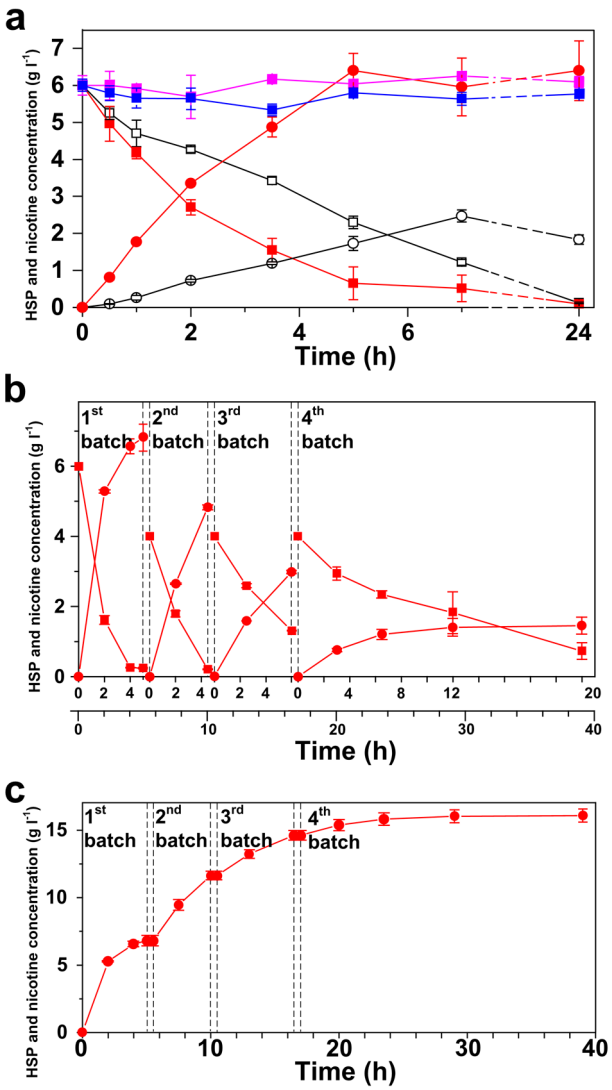

Figure 5 | Time course of batch biotransformation by *P. putida* S16 and *P. putida* P-HSP at pH 9.0, 30°C, and 3.4 g DCW l^{-1}. (a) *P. putida* S16 (black line) and *P. putida* P-HSP (red line) were used for HSP production under the same conditions. Cell-free system (blue line) and heat-killed cells (pink line) were used as controls. (b) For batch biotransformation, the catalysts were used four times after collection by centrifugation. (c) Total HSP production by four reactions. Each value is the mean of three parallel replicates ± SD. Symbols: HSP, ●; nicotine, ■.

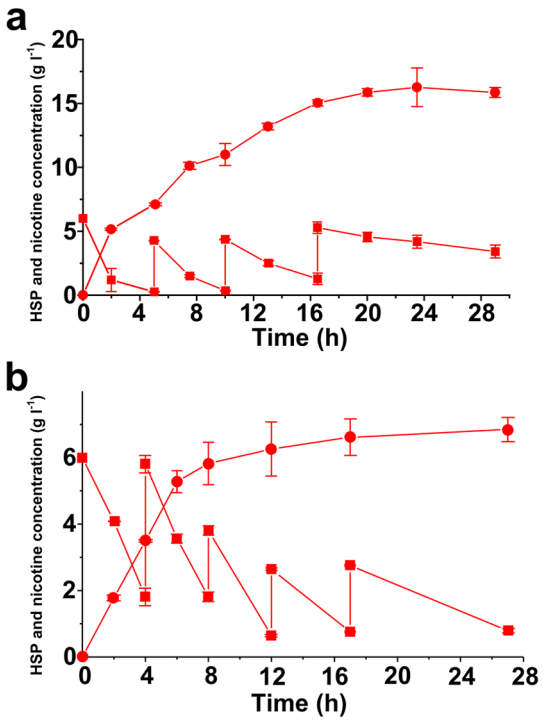

Figure 6 | Time course of fed-batch biotransformation by *P. putida* P-HSP at pH 9.0, 30°C, and 3.4 g DCW l^{-1}. Nicotine (a) and crude tobacco-waste extract (b) were used as substrates by *P. putida* P-HSP under optimal conditions. Each value is the mean of three parallel replicates ± SD. Symbols: HSP, ●; nicotine, ■.

Whole-cell catalysis is an important tool for biocatalysis, especially for multi-enzymatic reactions, although it is hindered by some shortcomings. Wang et al. developed a promising method for the production of HSP using whole cells of strain S16[22]. However, protein expression is sometimes inconsistent in the wild strains[32]; therefore, it is difficult to maintain the catalytic stability of native producers. Consequently, biotransformation conditions require extensive optimization to promote HSP accumulation and prevent its transformation to 2,5-DHP. However, it is notable that some HSP had been degraded before it reached the highest concentration when using wild strain S16 as the catalyst[22]. Metabolic engineering, which can enhance the accumulation of target compounds by blocking the catabolic pathways, is therefore a good strategy to overcome the shortcomings of whole-cell biocatalysis[33]. The results of these experiments demonstrated that engineered strain P-HSP realized a high accumulation of HSP. In fact, the metabolic engineering of strain S16 presents two advantages: (1) the engineered strain has high efficiency and yield, and (2) the catalytic process is easy to control. Moreover, mutasynthesis was first used in the production of intermediates in nicotine degradation, and it can be also used for the production of other intermediates, such as 3-succinoyl-pyridine and 2,5-DHP (Figure 1)[12].

In summary, our results demonstrate the feasibility for efficient production of a value-added chemical, HSP, with high product yield and productivity from renewable waste by blocking the catabolic pathway in a metabolically-engineered microorganism. The green strategy introduced in this study may provide a technically and economically interesting route for value-added-chemical production from industrial waste or renewable sources.

Methods

Chemicals. L-(-)-Nicotine (99% purity) was purchased from Fluka Chemie GmbH (Buchs, Switzerland), and HSP was prepared by our lab as previously described[22]; both were used as standards. Crude tobacco-waste extract (~8.7% (w/v) nicotine) was obtained by steam distillation according to the method described by Nanohar and Sridharan[5]. Nicotine (~90%) was further purified by extraction with chloroform. All other reagents and solvents used in this study were of analytical grade and commercially available.

Strains and cultivation conditions. *P. putida* strain S16 from our own collection was cultivated as previously described[34] and recently deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (accession no. DSM 28022). The *P. putida* P-HSP strain was aerobically cultivated at 30°C in Luria-Bertani (LB) medium containing filtration-sterilized kanamycin (50 ng l^{-1}) and nicotine (1 g l^{-1}). Engineered strain P-HSP was deposited at the China Center for Type Culture Collection under accession no. CCTCC M 2014135.

Construction of engineered catalyst and complemented strain. A partial sequence of *hspB* (fragment *PH*) was amplified by PCR with the primers *PH*-F (5′-ccgGAATTCggggacaaatgtggtggtg-3′) and *PH*-R (5′-cgcGGATCCcaagaactacccgaacaga-3′) (Figure 2b). Fragment *PH* and plasmid pK18mob were digested with the restriction enzymes *Eco*RI and *Bam*HI and ligated to generate pK18mob-*PH* (Figure 2b). To construct the *hspB* deletion strain (*P. putida* P-HSP), plasmid pK18mob-*PH* was transferred into strain S16, and triparental filter mating was performed as previously described[23]. Integrants were isolated on M9 minimal medium plates containing 50 ng l^{-1} kanamycin and verified by analysis of

PCR products using the primers *mob*-F (5′-cggctcgtataatgtgtgga-3′) and *hspB*-R (5′-ctacagaaaggtttccatagt-3′) (Figure 2b).

To construct the *hspB*-complemented strain, *hspB*, including the promoter region, was amplified from the genomic DNA of strain S16 using the primers C-F (5′-ccgGAATTCgcaccgatgactacatcagttttga-3′) and C-R (5′-ccgCTCGAGctacagaaaggtttccatagtctct-3′). The 1,332-bp fragment was purified and inserted into the *Eco*RI and *Xho*I sites of the *E. coli-P. putida* shuttle vector pME6032[35], generating recombinant plasmid pME-*hspB*. Plasmid pME-*hspB* was then transformed into strain *P. putida* P-HSP (*P. putida* S16 *hspB*::pK18mob) to produce the *hspB*-complemented strain (*P. putida* S16 *hspB*::pK18mob (pME-*hspB*)).

Catalyst preparation and transformation optimization. For whole-cell preparation, 1% (v/v) pre-cultures of *P. putida* P-HSP were added to 1 l of fresh LB medium in a 2.5-l conical flask (containing 1 g l^{-1} nicotine and 50 ng l^{-1} kanamycin) and incubated at 30°C with shaking at 200 rpm for 10 h until growth reached the late-exponential phase. Cells were collected by centrifugation at 8,000 rpm for 5 min and then washed twice with 0.9% NaCl. Biotransformation reactions were performed in 250-ml conical flasks containing 50 ml of the reaction mixtures with shaking at 120 rpm. The reaction conditions were optimized by testing the following parameters: pH, 6.0–10.0; temperatures, 16°C–44°C; nicotine concentrations, 1.5 g l^{-1}–10 g l^{-1}; and cell concentrations, 1.7 g DCW l^{-1}–13.6 g DCW l^{-1}.

Batch and fed-batch transformation. Large-scale production of HSP was carried out in a 2.5-l conical flask containing 500 ml of the reaction mixture under optimal conditions. The resting cells of wild *P. putida* S16 were prepared in the same way as *P. putida* P-HSP, and both strains were used for the biotransformation of nicotine. A cell-free system and heat-killed cells were used as control under the same conditions. For batch transformation, the biocatalyst was collected by centrifugation after the reaction, and reused in the next batch. For fed-batch transformation in the conical flask, crude tobacco-waste extract and nicotine were directly fed at the desired time. Each sample was analyzed in triplicate, and the mean value was used for further calculations. In the multi-enzyme steps reaction that forms HSP, one molecule of nicotine produces one molecule of HSP (Figure 2a). The yield was defined as the ratio between the molar concentrations of the consumed nicotine and the produced HSP (mol/mol).

Analytical methods. The activity of HSP hydroxylase (HspB) was assayed according to previous reports[23]. The OD_{620nm} was determined using a UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). The concentrations of nicotine and HSP were measured by high-performance liquid chromatography (HPLC) (Agilent, Santa Clara, CA, USA) using an Eclipse XDB-C18 column (5 μm 4.6 × 150 mm, Keystone Scientific, Bellefonte, PA, USA). Briefly, the sample was analyzed with a mobile phase of 12% (v/v) methanol and 88% (v/v) 1 mM H_2SO_4 at a flow rate of 0.5 ml min^{-1}. Liquid chromatography-mass spectrometry (LC-MS) was performed on an Agilent 6230 TOF-MS equipped with electrospray ionization sources. DCW was calculated from the optical density ($OD_{620\ nm}$) with a linear correlation factor (1 OD_{620nm} = 0.56 g DCW l^{-1}).

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Acknowledgments

The work was supported in part by the National Basic Research Program of China (grant no. 2011CBA00800), and by the Chinese National Natural Science Foundation (grant nos. 31230002, 31270154, and 31121064). We also acknowledge the "Shanghai Rising-Star Program" (grant no. 13QA1401700).

Author contributions

P.X., H.Y. and H.T. conceived and designed the project. P.X. and H.T. contributed reagents and materials. H.Y. analyzed data. H.Y. and P.X. wrote the manuscript. All authors have read and approved.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Yu, H., Tang, H. & Xu, P. Green strategy from waste to value-added-chemical production: efficient biosynthesis of 6-hydroxy-3-succinoyl-pyridine by an engineered biocatalyst. *Sci. Rep.* **4**, 5397; DOI:10.1038/srep05397 (2014).