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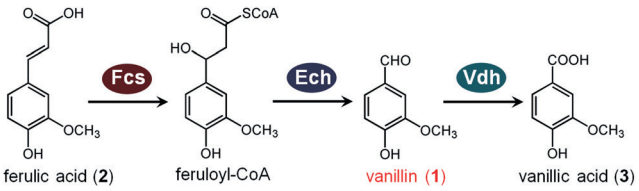
Temperature-Directed Biocatalysis for the Sustainable Production of Aromatic Aldehydes or Alcohols

Jun Ni[+], Yan-Yan Gao[+], Fei Tao, Hong-Yu Liu, and Ping Xu**

Abstract: *The biosynthesis of aromatic aldehydes and alcohols from renewable resources is currently receiving considerable attention because of an increase in demand, finite fossil resources, and growing environmental concerns. Here, a temperature-directed whole-cell catalyst was developed by using two novel enzymes from a thermophilic actinomycete. Ferulic acid, a model lignin derivative, was efficiently converted into vanillyl alcohol at a reaction temperature at 30 °C. However, when the temperature was increased to 50 °C, ferulic acid was mainly converted into vanillin with a productivity of 1.1 g L^{-1} h^{-1}. This is due to the fact that the redundant endogenous alcohol dehydrogenases (ADHs) are not active at this temperature while the functional enzymes from the thermophilic strain remain active. As the biocatalyst could convert many other renewable cinnamic acid derivatives into their corresponding aromatic aldehydes/alcohols, this novel strategy may be extended to generate a vast array of valuable aldehydes or alcohols.*

Recent years have witnessed a rising demand for bio-based polymers owing to the restricted availability of petrochemical resources and increasing environmental concerns.[1] In polymeric backbones, aromatic units offer rigidity, hydrophobicity, and fire resistance.[2] However, aromatic aldehyde monomers are predominantly produced by energy-intensive chemocatalysis of non-renewable petroleum feedstocks.[3] The use of microorganisms for converting renewable substances into aromatic monomers would provide a low-energy sustainable and green alternative. Unfortunately, aromatic aldehydes are rapidly converted into undesirable aromatic alcohols by numerous endogenous alcohol dehydrogenases (ADHs) with broad substrate specificity.[4] Recently, several attempts have been made to overcome this primary barrier in the biosynthesis of aromatic aldehydes.[5] For example, an *Escherichia coli* strain was generated by the deletion of six genes that contribute to benzaldehyde reduction, and used to increase the production of vanillin (**1**), benzaldehyde, and L-phenylacetylcarbinol.[5c] Nevertheless, microorganisms harbor many characterized and uncharacterized ADHs, and selecting the correct target genes for eliminating this activity is challenging.[5c,6]

Herein, we propose a novel strategy inspired by cell-free systems for the efficient biosynthesis of aromatic aldehydes, which does not require purified enzymes or knockout/knockdown of ADHs. The use of whole cells for biocatalytic reactions is an effective method for the production of value-added products.[7] We assumed that endogenous ADHs might lose their activity at high temperatures while the activities of functional enzymes from thermophilic strains can be retained in artificial whole-cell catalysis. To confirm the feasibility of this strategy, a model aromatic aldehyde, vanillin (**1**), was chosen as the target product.[8] Ferulic acid (FA, **2**), an easily available component of lignin, was used as the feedstock for the production of vanillin.[9] Normally, feruloyl-CoA synthetase (Fcs, encoded by *fcs*) and enoyl-CoA hydratase/aldolase (Ech, encoded by *ech*) are found in most FA-degrading strains. These strains convert **2** into **1** via a coenzyme A-dependent, non-β-oxidative pathway (Scheme 1). However, the instability and inefficiency of enzymes from mesophilic bacteria hamper their application.[10]



Scheme 1. Proposed route for the catabolism of ferulic acid into vanillin in *A. thermoflava*. Fcs = feruloyl-CoA synthetase, Ech = enoyl-CoA hydratase/aldolase, Vdh = vanillin dehydrogenase.

First, we attempted to identify efficient and thermostable enzymes for converting **2** into **1**. A thermophilic actinomycete, *Amycolatopsis thermoflava* N1165, was found to rapidly degrade more than 35 mM FA (**2**) at 50 °C and produce approximately 61.3 mg L^{-1} of vanillin (**1**; Figure 1 A). To the best of our knowledge, this is the first report of microbial production of **1** at 50 °C. In addition, a small amount of vanillic acid (**3**) was also detected, which indicated that **1** could be degraded further. According to phylogenetic analysis, *A. thermoflava* N1165 is closely related to *Amycolatopsis* sp. ATCC39116 (see the Supporting Information, Figure S1). Hence, we speculated that similar to *Amycolatopsis* sp. ATCC39116, the catabolism of **2** in *A. thermoflava* N1165 was also catalyzed by Fcs and Ech (Scheme 1).[11] Furthermore, the enzymes involved in the degradation of **2** in the thermophilic bacterium might be more stable.

[*] Dr. J. Ni,[+] Y. Y. Gao,[+] Dr. F. Tao, H. Y. Liu, Prof. P. Xu
State Key Laboratory of Microbial Metabolism and School of Life Sciences & Biotechnology
Shanghai Jiao Tong University
Shanghai 200240 (P. R. China)
E-mail: taofei@sjtu.edu.cn
pingxu@sjtu.edu.cn

[+] These authors contributed equally to this work.

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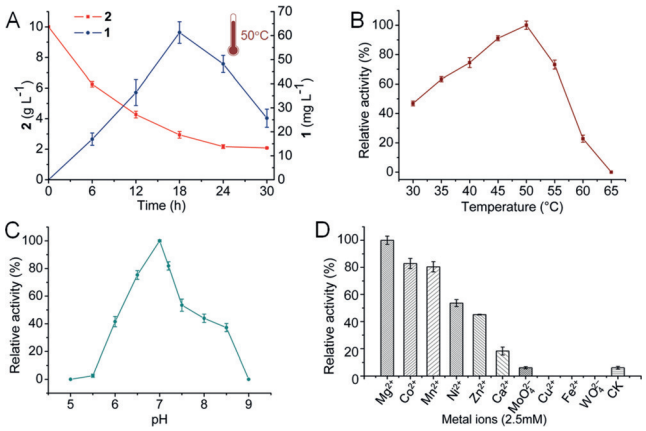

Figure 1. Activity assay for *At*Fcs. A) Time course of the degradation of **2** and the production of vanillin in *A. thermoflava*. B) Temperature dependence of the activity of *At*Fcs with the whole-cell catalyst. Influence of C) the pH and D) metal ions on the enzymatic activity of purified *At*Fcs.

In *A. thermoflava* N1165, two predicted proteins (WP_027935342 and WP_027935341) of 491 and 287 amino acids, respectively, showed considerable identity with previously characterized Fcs and Ech from the vanillin producer *Streptomyces* sp. V-1.[12a] To further investigate the relationship of *At*Fcs and *At*Ech with the Fcs and Ech enzymes from various other strains, multiple sequence alignment (MSA) was performed, and two phylogenetic trees were constructed (Figure S2). Next, *At*Fcs and *At*Ech were purified (Figure S3). The concentrations of purified *At*Fcs and *At*Ech were 0.94 mg mL^{-1} and 7.25 mg mL^{-1}, respectively.

Purified *At*Fcs was further characterized in terms of its enzymatic properties (Figure 1). The optimum temperature for *At*Fcs is 25 °C (Figure S4) while the enzyme activity of the whole cells expressing *At*Fcs was highest at 50 °C (Figure 1 B). These observations are in agreement with previous studies, which had reported that the activities of several purified enzymes were significantly more sensitive to heat than their activities in whole cells.[13] The optimum pH value for *At*Fcs is 7 (Figure 1 C), and six ions showed varying degrees of positive effects on *At*Fcs activity (Figure 1 D). The K_m, V_{max}, k_{cat}, and catalytic efficiency (k_{cat}/K_m) values for *At*Fcs were determined to be 0.62 mM, 171.4 U mg^{-1}, 148.6 s^{-1}, and 239.7 mM^{-1} s^{-1}, respectively (Figure S5). An enzyme assay for *At*Ech was not performed because of unavailability the substrate HMPHP-CoA.[12a] Alternatively, the catalytic activity of purified *At*Ech was determined in a mixed enzymatic reaction (Figures S6). Purified *At*Ech was catalytically active at 50 °C, and was involved in the biosynthesis of vanillin in *A. thermoflava* N1165.

Next, pETDuet-*fcs-ech* was constructed for co-expressing the two codon-optimized genes encoding *At*Ech and *At*Fcs and transferred to *E. coli*, generating the strain VA1 (Figure S7). The recombinant strain VA1 was used as the whole-cell biocatalyst. About 5 mM FA (**2**) were converted into only 0.025 mM vanillin (**1**) after 24 h at 30 °C (Figure 2 A). Notably, a considerable amount of vanillyl alcohol (**4**) accumulated, which accounted for 99.2 % of the final products. This result demonstrated that endogenous ADHs in *E. coli* could convert

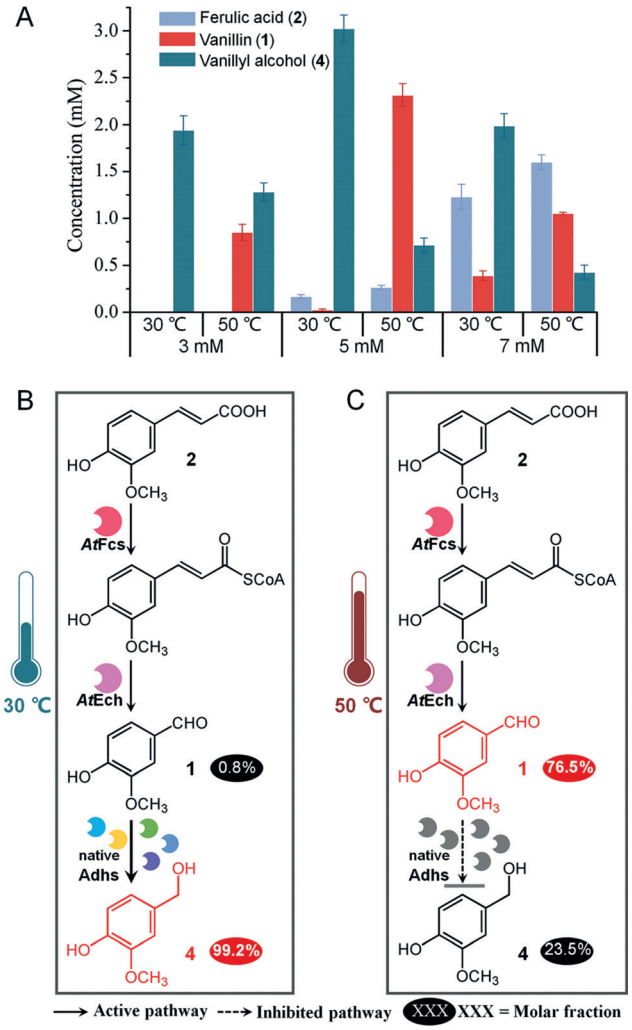

Figure 2. Temperature-directed formation of vanillin or vanillyl alcohol. A) Biotransformation of **2** into **1** and **4** using whole cells of the recombinant strain VA1 at different temperatures. B) Proposed temperature-regulated mechanism.

1 into the corresponding alcohol (Figure 2 B). Surprisingly, when the reaction temperature was increased to 50 °C, the same amount of **2** was converted into 2.32 mM of **1**, and only 0.71 mM of **4** were detected. We surmised that the high temperature may reduce the activities of endogenous ADHs in the recombinant *E. coli*, whereas the *At*Fcs and *At*Ech from the thermophilic microorganism retained their activities (Figure 2 C). Thus **1** formed a sizeable amount of the final products. While the whole-cell biocatalyst almost entirely lost its ability to convert **1** into **4** at 60 °C (Figure S8), the production of **1** considerably decreased at 60 °C using the current system, which indicated that the activities of the exogenous enzymes were also affected by very high temperatures (Table S1). In addition, increasing or decreasing the substrate concentration yielded the same results, and some amount of **2** was not converted when the initial concentration was increased (Figure 2 A).

The k_{cat}/K_m value of *At*Fcs was much higher than that reported previously for Fcs,[12] which indicated that *At*Fcs possessed better catalytic efficiency. As shown in Figure 3,

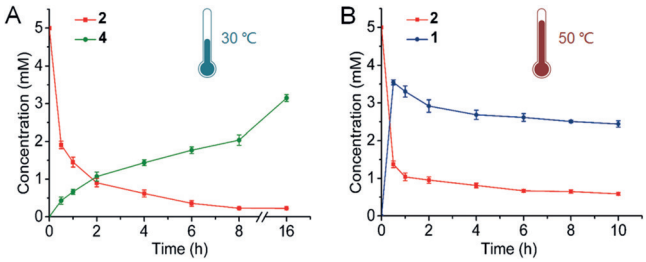

Figure 3. Time course of the production of **1** and **4** with the temperature-directed biocatalyst.

VA1 cells rapidly degraded 5 mM **2** and produced up to 3.15 mM **4** after 16 h at 30 °C. Notably, **4** was produced from **2** using the low-cost method. Meanwhile, 3.55 mM of **1** were formed from the same amount of **2** in 30 min at 50 °C by VA1-mediated whole-cell biocatalysis, and the corresponding productivity was 1.1 $g L^{-1} h^{-1}$ for **1**. To the best of our knowledge, this productivity far exceeds all previously reported values for microbial systems (Table S2). In addition to the excellent catalytic efficiency of *At*Fcs, the high reaction temperature may also contribute towards the high productivity. Currently, **1** and **4** are commonly used as building blocks for aromatic polymers (e.g., in the synthesis of thermosetting resins and thermoplastics).[14] In summary, the *At*Fcs- and *At*Ech-based thermostable whole-cell system can efficiently produce the aromatic monomers **4** or **1** from the sustainable feedstock **2** by altering the reaction temperature.

Aside from **2**, several other renewable cinnamic acid derivatives can also be easily obtained from lignin.[15] Therefore, the substrate specificity of purified *At*Fcs for several cinnamic acid derivatives was also investigated. *At*Fcs catalyzed the bioconversion of cinnamic acid (**5**), 3-hydroxycinnamic acid (**6**), *para*-coumaric acid (**7**), caffeic acid (**8**), and 4-methoxycinnamic acid (**9**; Figure 4). Thus *At*Fcs may potentially be used for the biotransformation of various cinnamic acid derivatives because of its broad substrate spectrum and relatively high activity.

Furthermore, we determined the conversion capacity of the temperature-directed whole-cell biocatalyst VA1 for **5**, **6**, **7**, **8**, and **9**. Similar to **2**, these cinnamic acid derivatives were mainly converted into their corresponding aromatic alcohols, namely benzyl alcohol (**10**), 3-hydroxybenzyl alcohol (**11**), 4-hydroxybenzyl alcohol (**12**), 3,4-dihydroxybenzyl alcohol (**13**), and 4-methoxybenzyl alcohol (**14**), within 24 h at 30 °C (Figure 5). Notably, 4-hydroxybenzaldehyde (**17**), 3,4-dihydroxybenzaldehyde (**18**), and *para*-anisaldehyde (**19**) were not present in the final products. This demonstrated that the endogenous ADHs in *E. coli* can convert aromatic aldehydes into the corresponding alcohols. The corresponding aromatic aldehydes of these cinnamic acid derivatives, namely benzaldehyde (**15**), 3-hydroxybenzaldehyde (**16**), **17**, **18**, and **19**, formed a major proportion of the final products when the reaction temperature was increased to 50 °C. The highest productivities achieved for these aromatic aldehydes/alcohols using the whole-cell biocatalyst VA1 are listed in Table 1. These aromatic aldehydes/alcohols are widely used in industry. For example, *para*-anisaldehyde (**19**) is a valuable material with applications in perfume making and pharmaceuticals.[16]

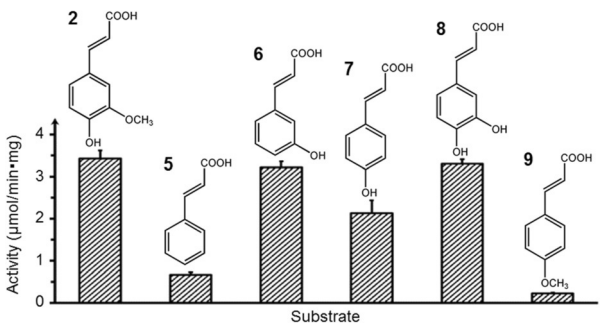

Figure 4. The substrate specificity of *At*Fcs.

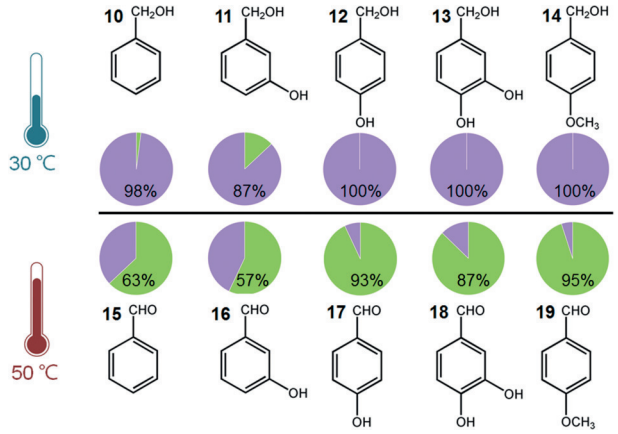

Figure 5. The temperature-directed whole-cell system for the formation of aromatic aldehydes or alcohols.

In conclusion, we have constructed a novel temperature-directed whole-cell catalyst for the production of various valuable aromatic aldehydes or alcohols from renewable compounds by using novel enzymes from a thermophilic actinomycete. In the past, endogenous ADHs have prevented the biosynthesis of several (aromatic) aldehydes.[17] For example, a previous study focused on improving the production of isobutyraldehyde by the deletion of eight ADH-encoding genes in *E. coli*.[17b] Our novel strategy rendered the redundant ADHs inactive while retaining the activities of functional enzymes from thermophilic strains at high temperatures. Searching for enzymes with higher thermal stability and activity and further elevating the reaction temperature to completely deactivate the ADHs in the mesophilic host may improve the practicability of this strategy in future. Recently, a growing number of aldehyde biosynthesis enzymes have been characterized in detail, and various methods have been developed to engineer thermostable enzymes.[18] Thus this

Table 1: The highest productivities for the aromatic aldehydes/alcohols.

Product	10/15	11/16	12/17	13/18	14/19
Productivity [$g L^{-1} h^{-1}$]	0.104[a] 0.085[b]	0.112[a] 0.147[b]	0.066[a] 0.898[b]	0.241[a] 0.505[b]	0.036[a] 0.109[b]

[a] Productivity of aromatic alcohols with whole-cell biocatalyst VA1 at 30 °C. [b] Productivity of aromatic aldehydes with whole-cell biocatalyst VA1 at 50 °C.

novel strategy may also be used to efficiently generate an enormous array of other aldehydes. Moreover, it provides a green process for generating valuable chemicals with microbes via aldehyde intermediates.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: alcohols · aldehyde · biocatalysis · enzymes · lignin · vanillin

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