Vanillin dehydrogenase (VDH) is a crucial enzyme involved in the degradation of lignin-derived aromatic compounds. Herein, the VDH from Corynebacterium glutamicum was characterized. The relative molecular mass (Mr) determined by SDS-PAGE was ~51 kDa, whereas the apparent native Mr values revealed by gel filtration chromatography were 49.5, 92.3, 159.0 and 199.2 kDa, indicating the presence of dimeric, trimeric and tetrameric forms. Moreover, the enzyme showed its highest level of activity toward vanillin at pH 7.0 and 30°C, and interestingly, it could utilize NAD\(^+\) and NADP\(^+\) as coenzymes with similar efficiency and showed no obvious difference toward NAD\(^+\) and NADP\(^+\). In addition to vanillin, this enzyme exhibited catalytic activity toward a broad range of substrates, including p-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, o-phthaldialdehyde, cinnamaldehyde, syringaldehyde and benzaldehyde. Conserved catalytic residues or putative cofactor interactive sites were identified based on sequence alignment and comparison with previous studies, and the function of selected residues were verified by site-directed mutagenesis analysis. Finally, the vdh deletion mutant partially lost its ability to grow on vanillin, indicating the presence of alternative VDH(s) in Corynebacterium glutamicum. Taken together, this study contributes to understanding the VDH diversity from bacteria and the aromatic metabolism pathways in C. glutamicum.

Corynebacterium glutamicum, a fast growing Gram-positive soil bacterium, is one of the most important microorganisms in industrial biotechnology. Since its discovery, C. glutamicum has been widely used for industrial production of amino acids, vitamins, nucleotides and various other bio-based chemicals\(^1\). As a soil bacterium, recent studies have demonstrated that C. glutamicum is able to utilize a large variety of lignin derived aromatic compounds (e.g. vanillin, ferulate, p-coumarate, cinnamate, etc.) for growth\(^2,3\). The outstanding capability of C. glutamicum in assimilation of aromatic compounds provides it with a distinct advantage in using lignocellulosic hydrolysates as sustainable and inexpensive feedstocks in industrial fermentation, thanks to its capability to detoxify and assimilate great amounts of lignin derived aromatic inhibitors in lignocellulosic hydrolysates as an alternative source to sugars for carbon and energy.

The main lignin-derived aromatic inhibitors in lignocellulosic hydrolysates are ferulate, vanillin, p-coumarate, 4-hydroxybenzoic acid (4-HBA), and vanillic acid, and most of which can be assimilated into TCA cycle intermediates by C. glutamicum\(^4,5\). Catabolism of ferulate follows a CoA-dependent non-β-oxidative pathway that contains feruloyl-CoA synthetase (Fcs) and enoyl-CoA hydratase/aldolase (Ech), yielding vanillin\(^6\). Vanillin is further converted into protocatechuic acid catalyzed by an aldolase (VanAB)\(^7,8\). Although some peripheral pathways converting various phenylpropanoids (such as vanillin, vanillate, cinnamate) to protocatechuic acid have been suggested in C. glutamicum, and the genes vanAB encoding vanillate dehydrogenase that catalyzes the conversion of vanillate to protocatechuic acid have been functionally identified\(^5,6\), the upstream vanillin dehydrogenase gene (vdh) has not been experimentally investigated.

The vanillin dehydrogenase is a critical enzyme for the degradation of lignin derived phenylpropanoids (such as vanillin, vanillate, cinnamate, and cinnamate) and studies on vanillin dehydrogenase gene (vdh) in Gram-negative bacteria have been well documented. For instance, vdh has been characterized in Pseudomonas fluorescens\(^9\), Pseudomonas putida\(^10,11\), Pseudomonas sp. strain HR199\(^12\), and Sphingomonas paucimobilis SYK-
The vanillin dehydrogenase activity of VDH ATCC13032, a putative vanillin dehydrogenase in *C. glutamicum* and investigated the enzyme activity, substrate specificity and roles in catabolism of aromatic compounds in *C. glutamicum*, thus contributing to a deeper understanding of the aromatic metabolism pathways in *C. glutamicum*.

**Results**

Identification of *vdh* gene from *C. glutamicum* genome and phylogenetic analysis. Based on BLAST Search and genome sequence analysis, the gene coding for a putative vanillin dehydrogenase (ncgl2578, named as *vdh*ATCC13032 in this study) was identified, which composed of 1,455 bp and encoded a protein of 484 amino acids with a theoretical molecular mass of 51.5 kDa. The *vdh*ATCC13032 shares 35%, 41% and 59% amino acid sequence identity with the *vdh* genes from *Pseudomonas aeruginosa* DK2, *Rhodococcus jostii* RHA1 and *Pseudomonas fluorescens* respectively. To further assess the phylogenetic relationship between *vdh*ATCC13032 and *vdh* genes from other bacteria, a multiple-sequence alignment was conducted using ClustalX 1.83 (Fig. 1). The results showed that the *vdh*ATCC13032 from *C. glutamicum* forms an independent cluster on the phylogenetic tree and exhibits clear evolutionary distance with already verified *vdh* genes from other bacteria. These results suggested that *vdh* from *C. glutamicum* may therefore represent a new vanillin dehydrogenase branch and the *vdh*ATCC13032 represents the first vanillin dehydrogenase characterized in detail within this gene family.

Functional characterization of gene *vdh*ATCC13032 in *vivo*. To further characterize the vanillin dehydrogenase activity of VDHATCC13032, a mutant strain (*Avdh*ATCC13032) was obtained by homologous recombination based gene knock-out. Growth analyses of wild type strain and *Avdh*ATCC13032 were conducted in liquid media at 30°C, using different substrates such as vanillin (8 mM), p-hydroxybenzaldehyde (8 mM), 3,4-dihydroxybenzaldehyde (5 mM), 3-hydroxybenzaldehyde (5 mM), ferulic acid (3 mM), caffeic acid (3 mM), p-cresol (5 mM), cinnamyl aldehyde (5 mM) and syringaldehyde (2.5 mM)) as the sole carbon and energy source. Compared to the wild type, the *Avdh*ATCC13032 mutant showed remarkably reduced ability to grow with the above mentioned aromatic compounds (Fig. 2). When complemented with plasmid pXMJ19-vdhATCC13032, the growth ability of the mutant strain could be restored close to that of the wild type (Fig. 2). However, the wild type, the *Avdh*ATCC13032 mutant and the complementary strain showed no difference when grown in p-cresol, cinnamyl aldehyde and syringaldehyde (data not shown).

Heterologous expression and molecular mass (Mr) estimation of VDHATCC13032. To further study the biochemical function of VDHATCC13032, the gene was PCR-amplified and heterologously expressed in *E. coli*. The recombinant strain carrying pET28a-vdhATCC13032 showed vanillin dehydrogenase activity, and the purified recombinant VDH was estimated to have a relative Mr of about 51.1 kDa, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3A), consistent with the predicted Mr value. However, gel filtration chromatography analysis showed four peaks corresponding to molecular mass of 49.5, 92.3, 159.0 and 199.2 kDa, respectively, indicating the native VDHATCC13032 existing as tetramers, trimers and dimers (Fig. 3B). The existence of VDHATCC13032 in tetramer, trimer and dimer was also confirmed by native PAGE analysis (Fig. 3C).

Biochemical properties of VDHATCC13032. Purified enzyme preparations of VDHATCC13032 were found to be stable and could be stored at 4°C for 2 weeks without significant loss of activity. The influence of pH and temperature on the activity of VDHATCC13032 was investigated (Fig. 4). The highest activity was demonstrated to be at 30°C in accordance with the temperature of physiological habitat of *C. glutamicum* whereas the optimum pH observed for reaction was pH 7.0 (in 100 mM potassium phosphate buffer). Several aldehydes were selected as the potential substrates to test the substrate specificity and
Figure 2 | Phenotypic characterization of Avidh and the complemented strain Avidh(pXMJ19-vdh) grown on mineral salts medium containing 8 mM vanillin (A), 5 mM 3-hydroxybenzaldehyde (B), 8 mM p-hydroxy benzaldehyde (C), 5 mM 3,4-dihydroxybenzaldehyde (D), 3 mM ferulic acid (E) and caffeic acid (F), respectively. *WT(pXMJ19), −Avidh(pXMJ19), −Avidh(pXMJ19-vdh).

Figure 3 | Molecular mass determination of the purified VDHATCC13032. (A) SDS-PAGE analysis of VDHATCC13032. M: protein marker. Lane 1: cell extracts of E. coli/pET28a-ngl12578 (not induced); lane 2: cell extracts of E. coli/pET28a-ngl12578 (induced); Lanes 3: purified VDHATCC13032 after Ni-NTA affinity chromatography. (B) Gel filtration analysis of purified VDHATCC13032. Molecular weight standards from large to small weight: Thyroglobulin (bovine)(670 kDa), γ-globulin (bovine)(158 kDa), Ovalbumin (chicken)(44 kDa), Myoglobin (horse)(17 kDa) and Vitamin B12(1.35 kDa). The molecular weight of purified VDHATCC13032 was estimated using the above molecular weight standards. (C) Typical appearance of native-PAGE obtained by loading 40 μg of purified VDHATCC13032. The position of the monomer (V₁), dimer (V₂), trimer (V₃) and tetramer (V₄) of VDH are indicated.
measure the activity of purified VDH_{ATCC13032}. VDH_{ATCC13032} showed catalytic activity toward a broad range of tested substrates (Table S1), including vanillin, 3,4-dihydroxybenzaldehyde, 3-hydroxybenzaldehyde, p-hydroxybenzaldehyde, p-nitrobenzaldehyde, terephthalaldehyde and 2,4-dichlorobenzaldehyde; and VDH_{ATCC13032} showed lower activities (less than 50% of that toward vanillin) toward o-phthalaldehyde, cinnamaldehyde, syringaldehyde, benzaldehyde and benzene propanal. However, phenylacetaldehyde, formaldehyde and aldehyde were not oxidized at detectable rates (rates lower than 5% of the enzyme activity with vanillin).

The catalytic efficiency of VDH_{ATCC13032} toward vanillin, p-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde was further tested by kinetics analysis (Table S2). The experimental results revealed that VDH had a higher affinity toward vanillin than to the other two substrates, and NAD\(^+\) was a required cofactor for this reaction, with the same level of activity as replaced with NADP\(^+\) (Table S3). This suggested that the enzyme could utilize both NAD\(^+\) and NADP\(^+\) as cofactors and show no obvious difference to them.

The catalytic activity of VDH_{ATCC13032} was further confirmed by LC-MS analysis using the selected substrates. Purified VDH_{ATCC13032} enabled the conversion of vanillin to vanillate (as shown in Figure 5). The HPLC results indicated that the production of vanillate in the presence of NAD(P)\(^+\) as a required cofactor for this reaction, with the same level of activity as replaced with NADP\(^+\) (Table S3). This suggested that the enzyme could utilize both NAD\(^+\) and NADP\(^+\) as cofactors and show no obvious difference to them.

In the presence of NAD\(^+\) and NADP\(^+\), the activities of N157A, K180A and C292A decreased to as low as 10% of the wild type enzyme; but for E199A, the catalytic activity still kept at 78% of the wild type enzyme (Table S4). In addition, while other variants showed more than six times higher Km values than the wild type toward NADP\(^+\), the E199A variant showed significantly lower Km (Table S5). Moreover, all the mutations showed decreased affinity to NAD\(^+\) (Table S5). Thus, it could be speculated that E199 have less influence on binding NADP\(^+\). Furthermore, mutation of any of the five residues resulted in increased Km values and decreased k_{cat} values toward vanillin (Table S6). Compared with the wild type enzyme, the deficient mutant of C292A, K180A and E258A had very lower affinities to vanillin (the Km values was 4–9 times higher and the k_{cat}/Km was 10–23 times lower, compared with the wild type enzyme). These results indicate that these residues also played important roles in the VDH_{ATCC13032} to the affinity of substrate (e.g. vanillin).

**Discussion**

The β-keto diapate pathway is the major catabolic route for lignin-derived aromatic compounds in soil bacteria. In the present study, we cloned, expressed and functional characterized a vdh gene from *C. glutamicum*, which channeled a variety of lignin-derived aromatic compounds to the protocatechuate branch of β-keto diapate pathway for further degradation. Based on the genome sequence of *C. glutamicum* ATCC13032, one putative aldehyde dehydrogenase gene, vdh_{ATCC13032}, was identified. But this gene does not show a remarkably high level of homology to those with verified vanillin dehydrogenase activity (Fig. 1). Our study suggests that VDH_{ATCC13032} is probably a unique aldehyde dehydrogenase with special catalytic roles, as discussed in the following.

The conclusion that VDH_{ATCC13032} is a vanillin dehydrogenase is based on at least three lines of independent evidence. First, analysis of a vdh deletion mutant revealed a delayed growth when 3, 4-dihydroxy benzaldehyde, 3-hydroxy benzaldehyde, vanillin, or ferulic acid was present as the sole carbon source, suggesting an important role of vdh_{ATCC13032} in assimilation of these compounds. It is known that caffeic acid is not a direct target of VDH. However, when grown in the presence of caffeic acid, the vdh deletion mutant showed a delayed growth as well, supporting the idea that vdh is at the central pathway for catabolism of aromatic compounds. The growth defects of the mutant strain were complemented by expressing wild type vdh (Fig. 2). Thus the resulting phenotype was not due to the polar effects caused by deletion of the vdh gene. The delayed growth observed with the deletion mutant grown on the different substrates may indicate...
minor alternative pathways for catabolism of aromatic compounds. This is consistent with the *Amycolatopsis* sp. strain ATCC 39116, in which VDH also plays a significant role in the course of vanillin degradation, but the biotransformation from vanillin to vanillate were still observed when vdh gene was knocked out.

HPLC-MS was performed to examine the biotransformation products of VDH <sub>ATCC13032</sub> in the presence of vanillin or 3,4-dihydroxybenzaldehyde. As expected, vanillin and 3,4-dihydroxybenzaldehyde were transformed to vanillate and 3,4-dihydroxybenzoic acid, respectively, confirming the activity of VDH <sub>ATCC13032</sub>. Relatively high catalytic activity of purified VDH <sub>ATCC13032</sub> was observed with p-hydroxybenzaldehyde and vanillin, consistent with the fact that vdh gene is at the downstream of ferulate and p-coumarate metabolism pathway. Also, VDH <sub>ATCC13032</sub> was heterologously expressed and its Mr was estimated by SDS-PAGE (Fig. 3A). Interestingly, the native molecular mass determined by gel filtration showed that VDH from *C. glutamicum* existed in the form of tetramers, trimers and dimers (Fig. 3B and C). This is consistent with previous reports that VDHs from *Micrococcus* sp. TA1 and *Burkholderia cepacia* TM1 exist as tetramers and dimers, respectively. The enzyme displayed the highest activity at 30°C, in accordance with the physiological temperature of *C. glutamicum* and the results of in vitro expression assay. VDH from most of the studied species showed specificity against vanillin and benzaldehyde compounds. While purified VDH from *C. glutamicum* ATCC13032 showed catabolic activity toward a relatively broad range of tested substrates (Table S1), oxidation rate for p-hydroxybenzaldehyde, formaldehyde and aldehyde were not detected. The observed substrate specificity is consistent with the substrate specificity observed with vanillin dehydrogenases from *S. paucimobilis* SYK6<sup>4</sup> and *P. fluorescens*. While most VDHs identified so far tend to use NAD<sup>+</sup> as a sole cofactor, VDH <sub>ATCC13032</sub> could utilize both NAD<sup>+</sup> and NADP<sup>+</sup>, which has been observed with the VDH from *P. fluorescens* in a previous study<sup>2</sup>. Taken together, these results have revealed the important roles of VDH in *C. glutamicum* ATCC13032, whereas substrate specificity may vary from one species to another.

Extensive works have been done to examine the active amino acid residues in the ALDH enzymes, and the examined residues have been well documented<sup>15,16,23</sup>. Sequence alignment revealed conserved sequences in VDH <sub>ATCC13032</sub> with relatively high amino acid similarity with the active residues identified in ALDH and PaBADH. One strictly conserved residue is Cys-292 in VDH <sub>ATCC13032</sub>, which has been demonstrated to be responsible for the dehydrogenase as well as esterase activities of aldehyde dehydrogenase<sup>16,24</sup>. In *P. aeruginosa*, the PaBADH catalytic cysteine (C286, corresponding to C292 in VDH <sub>ATCC13032</sub>) is oxidized to sulfenic acid or forms a mixed disulfide with 2-mercaptoethanol<sup>16</sup>. The second active residue identified in VDH <sub>ATCC13032</sub> was Glu-258, which is in the vicinity of the catalytic cysteine, corresponding to E268 in ALDH2, E252 in PaBADH and E258 in VDH. This conserved residue probably functions as a general base in ALDH catalysis<sup>25</sup>. Lys-180 is another conserved residue in VDH <sub>ATCC13032</sub> and may have functions similar to that of ALDH (K78) and PaBADH (K162)<sup>16,23</sup>. Glu-199 in the VDH <sub>ATCC13032</sub> may also be an conserved residue that would produce a steric clash with the 2'-phosphate of NAD(P)<sup>+</sup>, resulting in a low affinity of ALDH2 for NAD(P)<sup>+</sup>. Therefore we speculated that these five conserved sites may be important for the catalytic activity of VDH <sub>ATCC13032</sub>, and they were subjected to further characterization. Mutations of these residues decreased catalytic activities by more than 50% compared with wild type when NAD<sup>+</sup> was used as a cofactor. However, when NADP<sup>+</sup> was used as a cofactor, mutations of N157A, E258A and C292A caused 90% reduction of catalytic activities compared with wild type, while E199A mutation only reduced the activity by 12%. This indicates that Glu-199 may have less influence on NADP<sup>+</sup> binding compared with the other tested residues, whereas all these residues play roles in NAD<sup>+</sup> binding. Consistently, Glu-199 showed no effect on the affinity of VDH <sub>ATCC13032</sub> to NADP<sup>+</sup>, but affected the affinity of VDH <sub>ATCC13032</sub> for the substrates such as vanillin. Taken together, the examined residues play critical roles in VDH catalysis, but their mechanisms may be different.

Lignocellulosic hydrolysates for biofuel production usually contain not only fermentable sugars but also non-fermentable growth inhibitors, including furan, weak acids, and various lignin-derived aromatic compounds (such as vanillin, ferulic acid, p-coumaric acid, 4-hydroxybenzoic acid, vanillic acid, etc.), which inhibit microbial
fermentation to the desired products.\textsuperscript{26,27} \textit{C. glutamicum} could be applied to both detoxify and assimilate lignin-derived aromatic inhibitors as an alternative source to sugars for carbon and energy. The functional characterization of VDH\textsubscript{ATCC13032} contributes not only to a systematical understanding of aromatic compound assimilation, but also to develop \textit{C. glutamicum} as an efficient strain to convert lignocellulose to bioproducts, such as biofuels.

**Methods**

**Bacterial strains, plasmids, and culture conditions.** All bacterial strains and plasmids used in this study are listed in Table S7. Escherichia coli was cultivated at 37°C in Luria–Bertani (LB) medium.\textsuperscript{28} \textit{C. glutamicum} strains were routinely grown at 30°C in LB or in mineral salts medium (MM), which was adjusted to pH 8.4 and supplemented with yeast extract (0.05 g L\textsuperscript{-1}).\textsuperscript{29} For generation of mutants and maintenance of \textit{C. glutamicum}, BHIS (brain heart broth with 0.5 M sorbitol) medium was used.\textsuperscript{30} \textit{C. glutamicum} RES167, a restriction-deficient strain derived from \textit{C. glutamicum} ATCC 13032 was the parent of all derivatives used in this study. Aromatic compounds were added to the MM medium at suitable final concentrations. Cell growth was monitored photometrically at 600 nm.\textsuperscript{31,32} Kanamycin, chloramphenicol were added at final concentrations of 20 and 10 µg ml\textsuperscript{-1}, respectively for both \textit{E. coli} and \textit{C. glutamicum}, and ampicillin of 100 µg ml\textsuperscript{-1} for \textit{E. coli}, whereas nalidixic acid of 40 µg ml\textsuperscript{-1} for \textit{C. glutamicum}.

**Gene prediction in the \textit{C. glutamicum} genome.** Translated BLAST search (blasts) from the National Center for Biotechnology Information (NCBI) was used to identify genes involved in aromatic metabolism in \textit{C. glutamicum} ATCC 13032 (Accession no. NC 003450). Through the BLAST analyses, homologues of the putative genes \textit{C. glutamicum} genes from other bacteria was constructed by ClustalX 1.83 and Molecular Evolutionary Genetics Analysis (MEGA), and the calculation were determined by molecular mass calculations with an extinction coefficient of 6,220 M\textsuperscript{-1} cm\textsuperscript{-1}. The reaction mixture (1 ml) contained substrate (1 mM), NAD(P)\textsuperscript{+} (0.5 mM) and an appropriate amount of enzyme in potassium phosphate buffer (0.5 mM, pH 7.0). The reaction was performed in various pHs of 100 mM and an appropriate amount of substrate in potassium phosphate buffer (pH 7–4). Protein concentrations were determined using the Bradford assay with bovine serum albumin as standard.

**Site-directed mutagenesis.** To identify the potential active residues in the VDH from \textit{C. glutamicum} ATCC13032, genes from ALDH (aldehyde dehydrogenase) family with known active sites, such as ALDH2 and P450DDH (\textit{Pseudomonas aeruginosa} bacterial aldehyde) were used as references for alignment analysis. The alignment suggested that the five known residues were also present in the VDH \textit{C. glutamicum}.

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**DNA manipulations and plasmid construction.** Genomic DNA of \textit{C. glutamicum} was isolated according to the method described by Tauch \textit{et al.} Plasmids were isolated with plasmid DNA mini prep spin columns (TIANGEN, Beijing, China), and DNA fragments were amplified from agarose gels by EasyPure Quick Gel Extraction Kit (TransGen Biotech, Beijing, China). \textit{C. glutamicum} DNA was transformed by electroporation according to the method of Tauch \textit{et al.} Competent cells of \textit{E. coli} were prepared and transformed using the CaCl\textsubscript{2} procedure.\textsuperscript{29} Target DNA fragments were amplified and digested by standard methods. All plasmids were constructed based on pK18mobac8c, pXM19, or pET28a. Plasmids used are listed in Table S8. To construct the plasmid for ncgl2578 knockout, the upstream and downstream DNA fragments were amplified using primer pairs ncgl2578upFBamHI/ncgl2578downFBamHI (Table S8). The downstream PCR fragments were linked together by overlap PCR and then inserted into pK18mobac8c to generate pK18mobac8c-ncgl2578 through restriction enzyme digestion. For complementation, plasmid pXM19-ncgl2578 was created by insertion of the PCR-amplified ORF into pXM19. Plasmid for expression of the target gene in \textit{E. coli} was constructed from PCR-amplified gene and pET28a. All the constructed plasmids were confirmed by DNA sequencing.

**Constitution of the vdh mutant and complementary strain in \textit{C. glutamicum}.** To construct the vdh mutant, the plasmid pK18mobac8c-BadH\textsubscript{ATCC13032} was transformed into \textit{C. glutamicum} RES167 by electroporation, and chromosomal integration was selected by plating on LB agar plates supplemented with kanamycin. The BadH\textsubscript{ATCC13032} deletion mutant was subsequently screened on LB agar plates containing 10% sucrose and confirmed by PCR and sequencing as previously described.\textsuperscript{30} For complementation, pXM19-ncgl2578 was transformed into the mutant strain and vdh gene was amplified in \textit{C. glutamicum} was induced by addition of 0.5 mM isopropyl-D-thiogalactopyranoside ( IPTG) to the culture broth. Expression and purification of recombinant VDH in \textit{E. coli}. To express the His\textsubscript{6}-VDH protein, recombinant plasmid pET28a-ncgl2578 was electroplated into \textit{E. coli} BL21 (DE3). When the growth of recombinant \textit{E. coli} reached OD\textsubscript{600} = 0.4, the expression of recombinant protein was initiated by addition of 0.5 mM IPTG, and the culture was then shaken overnight at 22°C. Cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C, washed twice with ice-cold phosphate-buffered saline (PBS). Harvested cells were disrupted by sonication and purified with the His-Bind Ni-NTA resin (Novagen, WI, USA) following the manufacturer's instructions. Purified recombinant protein was dialyzed against PBS overnight at 4°C and stored at –80°C until use. Protein concentrations were determined using the Bradford assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

**SDS-PAGE and determination of molecular mass of the purified enzyme.** SDS-PAGE was conducted with 3% stacking gels and 10% resolving gel and run in a Mini-PROTEAN III Electrophoresis Cell (Bio-Rad) according to the manufacturer’s instructions. After electrophoresis, the protein bands were visualized by Coomassie brilliant blue staining. Apparent molecular mass was estimated according to the relative mobility of Blue Plus II protein markers, with molecular masses ranging from 14 to 120 kDa. The native molecular mass of the VDH was estimated by gel filtration chromatography on a Superdex 200 10/300 GL column (1.0 cm × 30 cm) (Amersham Biosciences) eluted at a flow rate of 0.25 ml min\textsuperscript{-1} with 100 mM potassium phosphate buffer containing 150 mM NaCl (pH 7.2). Molecular weight standards used were: hyroglobulin (bovine) (670 kDa), γ-globulin (bovine) (158 kDa), Ovalbumin (chicken) (44 kDa), Myoglobin (horse) (17 kDa) and Vitamin B12 (1350 kDa). To characterize the molecular configuration of the purified VDH, we employed PAGE under non-denaturing conditions. Gels consisted of a separating gel (15% acrylic) and a stacking gel (5% acrylic) and the loading buffer was also under non-denaturing condition, which contained 0.5 M Tris-HCl buffer (pH 6.8), 0.1% bromophenol blue and 10% glycerol. The Sample containing 40 µg protein was loaded, and carried out at 4°C (125 V, 250 mA Tris-base, 250 mM Glycine). Apparent molecular mass was estimated according to the mobility protein markers Protein Rule IV (TransGen Biotech, Beijing, China), with molecular masses ranging from 30 to 200 kDa.

**Enzyme assays.** The VDH enzyme assay was performed based on described methods.\textsuperscript{33} To 100 mM potassium phosphate buffer (pH 7.0). The activity of vanillin dehydrogenase was monitored spectrophotometrically by measuring the rate of decrease in absorption at 340 nm due to oxidation of NAD(P)H and was calculated with an extinction coefficient of 6,220 M\textsuperscript{-1} cm\textsuperscript{-1}. The reaction mixture (1 ml) contained substrate (1 mM), NAD(P)\textsuperscript{+} (0.5 mM) and an appropriate amount of enzyme in potassium phosphate buffer (0.5 mM, pH 7.0). The reaction was performed in various pHs of 100 mM and an appropriate amount of substrate in potassium phosphate buffer (pH 3–4). Protein concentrations were determined using the Bradford assay with bovine serum albumin as standard.

**Biotransformation of aromatic compounds with recombinant VDH.** To further analyze the biotransformation of aromatic compounds, the purified VDH from \textit{C. glutamicum} strains was added to the reaction mixture (1 ml) containing substrate (1 mM) and NAD\textsuperscript{+} (0.5 mM) as a cofactor for reaction analysis. The alignment suggested that the five known residues were also present in the VDH \textit{C. glutamicum}.

**Enzyme assays.** The VDH enzyme assay was performed based on described methods.\textsuperscript{33} To 100 mM potassium phosphate buffer (pH 7.0). The activity of vanillin dehydrogenase was monitored spectrophotometrically by measuring the rate of decrease in absorption at 340 nm due to oxidation of NAD(P)H and was calculated with an extinction coefficient of 6,220 M\textsuperscript{-1} cm\textsuperscript{-1}. The reaction mixture (1 ml) contained substrate (1 mM), NAD(P)\textsuperscript{+} (0.5 mM) and an appropriate amount of enzyme in potassium phosphate buffer (0.5 mM, pH 7.0). The reaction was performed in various pHs of 100 mM and an appropriate amount of substrate in potassium phosphate buffer (pH 3–4). Protein concentrations were determined using the Bradford assay with bovine serum albumin as standard.
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22. W.D., W.Z., S.C. and X.S. wrote the main manuscript. W.D., M.S., W.Z., Y.Z., C.C., L.Z., Z.L. and X.S. designed the experiments and performed the experiments. W.D., M.S., W.Z., S.C. and X.S. analyzed the data. W.D. and C.C. prepared samples. All authors discussed and reviewed the manuscript.

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