Microbial Biotechnology

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MICROBIAL BIOTECHNOLOGY Open Access

RESEARCH ARTICLE OPEN ACCESS

Production of Vanillin From Ferulic Acid by *Pseudomonas putida* KT2440 Using Metabolic Engineering and In Situ Product Recovery

Ilona A. Ruhl | Sean P. Woodworth | Stefan J. Haugen | Hannah M. Alt | Gregg T. Beckham | Christopher W. Johnson

Renewable Resources and Enabling Sciences Center, National Renewable Energy Laboratory, Golden, Colorado, USA

Correspondence: Christopher W. Johnson (christopher.johnson@nrel.gov)

Received: 11 November 2024 | **Revised:** 1 April 2025 | **Accepted:** 16 April 2025

Funding: This work was supported by Bioenergy Technologies Office.

Keywords: bioconversion | ferulic acid | in situ product recovery | metabolic engineering | *Pseudomonas putida* KT2440 | vanillin

ABSTRACT

Vanillin is the most in-demand flavouring compound in the world and because vanillin extracted from vanilla pods cannot meet the global demand, most vanillin on the market today is chemically synthesised. Increasing demands by consumers for natural ingredients have inspired efforts to develop vanillin derived from microbial sources. These efforts have been challenged by low titers, likely caused by the toxicity of vanillin to most microbial biocatalysts. In this study, we engineered a *Pseudomonas putida* KT2440-derived strain that accumulated vanillin from ferulic acid to 0.64 g/L. To increase the overall titre, we applied a hydrophobic polystyrene-based resin to vanillin-accumulating cultures, which enabled an increase in total vanillin recovery to an apparent titre of 3.35 g/L. This study demonstrates that *P. putida* can accumulate vanillin from ferulic acid to higher titers when vanillin is removed from the cultivation medium, mitigating its toxicity.

1 | Introduction

As of 2023, the global demand for vanillin was 35,000 tons/year (Persistence Market Research 2023) where it finds use as a flavouring and fragrance (Priefert et al. 2001). Historically, vanillin was derived from the bean pods of vanilla plants, requiring 40,000 vanilla pods to produce 1 kg (reviewed in Ramachandra Rao and Ravishankar 2000). Today, however, almost all vanillin is chemically synthesised (Liu et al. 2023), primarily from guaiacol and eugenol derived from petrochemicals (Martău et al. 2021). The increasing consumer demand for natural products and concerns over the environmental impact of chemical synthesis have motivated the production of vanillin using microbes, as vanillin produced in this way qualifies as natural (Gallage and Møller 2015). Microbially-derived vanillin has been synthesised from a variety of substrates, such as eugenol, isoeugenol, ferulic acid, and glucose (as reviewed in Ramachandra Rao and Ravishankar 2000; Liu et al. 2023).

Ferulic acid is the most commonly used substrate for the production of microbially-derived vanillin (Gallage and Møller 2015), as it has several advantageous characteristics. Firstly, ferulic acid is an abundant aromatic compound found in plants, including both incorporated into and ester-linked to lignin (Escott-Watson and Marais 1992; Priefert et al. 2001; Ralph 2010), so it can be sourced from agricultural byproducts. Secondly, because ferulic acid can be enzymatically extracted from plants with esterase enzymes, it is an attractive substrate for products intended for the food industry, as only extractives obtained from plants by roasting, heating, or enzymolysis can be labelled as a natural flavour according to USDA regulation (21CFR101.22(a)(3) (n.d.); Priefert et al. 2001).

Multiple genera have been used to produce vanillin at titers above 1 g/L, including *Trichosporon* (Ashengroph and Amini 2017), *Lycinibacillus* (Zhao et al. 2005), *Amycolatopsis* (Rabenhorst and Hopp 1996; Muheim and Lerch 1999; Fleige et al. 2013, 2016; Wang et al. 2023), *Streptomyces* (Hua, Ma, Song, et al. 2007), *Pseudomonas* (Overhage, Priefert, Rabenhorst, et al. 1999; Yamada et al. 2007; Kasana et al. 2007; Ashengroph et al. 2010, 2011; Di Gioia et al. 2011; Graf and Altenbuchner 2014; Haridoss et al. 2015; García-Hidalgo et al. 2020), *Serratia* (Rabenhorst and Hopp 1990), *Psychrobacter* (Ashengroph et al. 2012), *Enterobacter* (Saeed et al. 2022), *Escherichia* (Zhao et al. 2018; Han et al. 2019), and *Bacillus* (Zhao et al. 2006; Hua, Ma, Lin, et al. 2007; Haridoss et al. 2015), using isoeugenol, eugenol, and/or ferulic acid as substrates. The highest reported vanillin titre to our knowledge was 32.5 g/L, produced by *Lycinibacillus fusiformis* SW-B9 (formerly classified as *Bacillus*) resting cells in a 60% v/v isoeugenol solvent (Zhao et al. 2005). Additionally, an engineered strain of *Amycolatopsis* sp. ATCC 39116 produced vanillin titers as high as 22.3 g/L from ferulic acid (Fleige et al. 2016); however, the filamentous morphology of *Amycolatopsis* could make its use challenging from a process perspective (Gallage and Møller 2015). *Pseudomonas* has also been extensively pursued for use in vanillin production. The highest titers achieved by *Pseudomonas* were 16.1 g/L vanillin from isoeugenol by resting cells of *P. putida* IE27 (Yamada et al. 2007) and 2.6 g/L vanillin from eugenol by an engineered strain of *Pseudomonas* HR199 (Overhage, Priefert, Rabenhorst, et al. 1999). Lower maximum titers have been reported for *Pseudomonas* producing vanillin from ferulic acid at 1.3 g/L by an engineered *P. putida* KT2440 (Graf and Altenbuchner 2014; García-Hidalgo et al. 2020). The species producing the highest titers tend to be tolerant to aromatic compounds like vanillin and the substrates from which it can be derived, which are toxic to most bacteria (Gallage and Møller 2015). This toxicity has limited vanillin titers in most species (Converti et al. 2010; Gallage and Møller 2015; Li et al. 2020; Xu et al. 2024), but titers have been reported to improve when adsorbent resin is used to remove vanillin from the medium (Hua, Ma, Song, et al. 2007; Yoon et al. 2007; Lee et al. 2009; Ma and Daugulis 2014), a strategy we also apply here.

Pseudomonas putida KT2440 can natively metabolise ferulic acid to protocatechuic acid (Jiménez et al. 2002; Figure 1), which is then further metabolised to β-ketoadipate via the β-ketoadipate pathway (Stanier et al. 1950; Jiménez et al. 2002) and finally to intermediates of the tricarboxylic acid cycle. Vanillin results from ferulic acid via the activity of two enzymes, a feruloyl-CoA synthase, Fcs, and an enoyl-CoA hydratase, Ech (Figure 1; Plaggenborg et al. 2003). A vanillin reductase, AreA (García-Hidalgo et al. 2020), a vanillin dehydrogenase, Vdh (Plaggenborg et al. 2003), and multiple other aldehyde dehydrogenases have activity toward vanillin and together are responsible for its catabolism. To prevent vanillin conversion, the activity of such enzymes must be attenuated or, preferably, eliminated, which is especially challenging for aldehyde dehydrogenases because they are highly promiscuous enzymes (Muñoz-Clares et al. 2023). Multiple previous studies have investigated which genes in *P. putida* encode enzymes that contribute to vanillin oxidation. Notably, in addition to *vdh*, Graf and Altenbuchner (2014) identified *aldb-I*, *aldB-II*, PP_1948, and *modABC* as important to vanillin conversion. Interestingly, the *modABC* operon does not encode an enzyme that acts on vanillin, but rather a molybdate ion transporter. Graf and Altenbuchner (2014) rationalised that many oxidoreductases that could act on vanillin require molybdate ions as cofactors and deletion of this operon likely

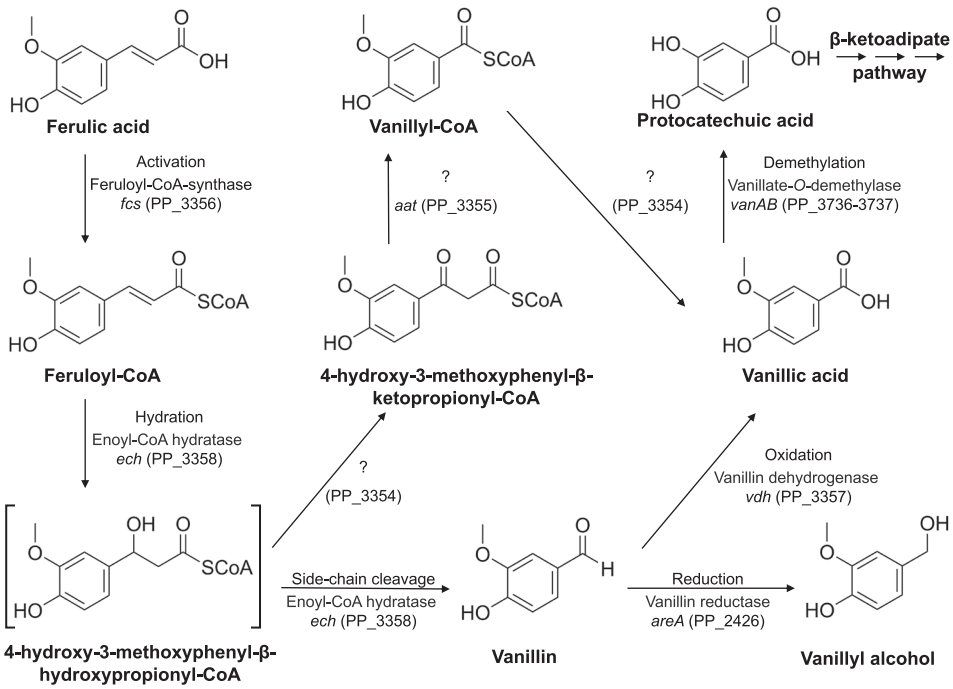

FIGURE 1 | Native ferulic acid metabolism in *P. putida* KT2440. Arrows represent enzymatic reactions and are labelled with the chemical transformation they catalyse, the name of the protein performing the reaction, the name of the gene that encodes the protein, and the genomic location of that gene. Question marks represent an unconfirmed vanillic acid shunt proposed by Overhage, Priefert, Steinbüchel, et al. (1999).

impairs their function. Additionally, in a proteomics study, Simon et al. (2014) reported that the protein abundance of PP_3151, PP_5120, and PP_5258 (encoded by *sad-II*, *calB*, and *amaB*, respectively) increases upon exposure to vanillin, suggesting they might be involved in vanillin catabolism. Finally, the deletion of *areA* (also annotated as *calA*), which encodes a vanillin reductase (Figure 1), has been reported to eliminate all aldehyde reductase activity in *P. putida* (García-Hidalgo et al. 2020).

In this study, we sought to generate a *Pseudomonas putida* strain capable of converting ferulic acid to vanillin. To achieve this, we used a combination of metabolic engineering and in situ product recovery with an adsorbent resin. Ferulic acid catabolism was arrested at vanillin by the deletion of genes that encode enzymes with activity on vanillin, which is toxic to most bacteria. End-product toxicity was overcome by applying an adsorbent resin to vanillin-producing cultures, thus removing vanillin from the medium and permitting continued production of vanillin.

2 | Experimental Procedures

2.1 | Strain Construction

Deletions from the *P. putida* KT2440 (ATCC 47054) genome were performed using the antibiotic/sucrose counter-selection method of gene replacement (Hmelo et al. 2015; Blomfield et al. 1991; Marx 2008; Schäfer et al. 1994), adapted for use in *P. putida* (Johnson and Beckham 2015). Briefly, about 750 bp of DNA upstream and downstream of each targeted gene or gene cluster were PCR-amplified using primers with incorporated EcoRI and HindIII restriction enzyme sites. The upstream and downstream targeting region amplicons were assembled into the suicide vector pK18sB (Jayakody et al. 2018) using the NEBuilder HiFi DNA Assembly kit (New England Biolabs). Assembly was confirmed by restriction digest, and the gene cassette was sequence verified using Genewiz Sanger sequencing (Azenta Life Sciences). Sequence-verified plasmids were transformed into *P. putida* KT2440 via electroporation. The electroporated cells were plated on LB-kanamycin plates containing 50 μg/mL kanamycin for at least two passages, and then sucrose-selection was initiated by plating colonies on 25% w/v sucrose-YPD plates, again, for at least two passages. After sucrose selection, deletion was confirmed via colony PCR. The final strain and all intermediate strains were preserved as glycerol stocks using 1 mL of liquid culture and 500 μL of 60% w/v glycerol. The genotypes of strains utilised in this study are provided in Table 1. Additional details of strain construction, including the plasmids (and Addgene Plasmid#) and PCR primers that were used, are provided in Table S1.

2.2 | Evaluation of Strains in Shake Flasks

Strains were revived from glycerol stocks in 25 mL of Luria Broth (LB) in 125 mL baffled flasks incubated at 30°C and 250 rpm overnight. Cultures were then diluted to OD_{600} 0.2 in LB and grown to an OD_{600} of 1.5–2.0 at 30°C and 250 rpm. After the second seed culture passage, cultures were spun down and resuspended in M9 minimal medium (6.78 g/L Na_2HPO_4, 3.00 g/L K_2HPO_4, 0.50 g/L NaCl, 1.66 g/L NH_4Cl, 0.24 g/L $MgSO_4$, 0.01 g/L $CaCl_2$, and 0.002 g/L $FeSO_4$). To initiate the growth phase, 1 mL of the resuspended culture was used to inoculate 19 mL of the same medium supplemented with 7.21 g/L (40 mM) glucose (Fisher Scientific) in a 125 mL baffled flask to an OD_{600} of 0.2; this higher starting OD was used for faster initiation of the growth phase. Inoculated flasks were incubated for 17–23 h at 30°C and 250 rpm, at which point glucose was depleted. The conversion phase was initiated by the addition of 1.94 g/L (10 mM) ferulic acid (Sigma Aldrich) and 1.80 g/L (10 mM) glucose in 5 mL of M9 minimal medium, bringing the total volume of the culture to 25 mL. Conversion continued for 24–48 h, varying due to phenotypic differences in vanillin accumulation rate between the strains, with boluses of glucose supplied to a final concentration of 1.80 g/L (10 mM) an additional 1–2 times, depending on the length of the conversion phase. In this study, the term 'bolus' refers to the addition of a single dose of a substrate to the cultivation flask. All conditions were evaluated in at least duplicate. Additional experiments evaluating performance of strains IAR038 and IAR068 are shown in Figure S1.

TABLE 1 | Bacterial strains utilised in this study and their genotypes.

Strain	Genotype
CJ019	*Pseudomonas putida* KT2440
IAR032	*Pseudomonas putida* KT2440 △*vdh* △*aldB-II* △PP_1948 △*aldB-I* △*areA* △*sad-II* △*calB* △*amaB* △*aldA*
IAR038	*Pseudomonas putida* KT2440 △*vdh* △*aldB-II* △PP_1948 △*aldB-I* △*areA* △*sad-II* △*calB* △*amaB* △*aldA* △*modABC*
IAR068	*Pseudomonas putida* KT2440 △*vdh* △*aldB-II* △PP_1948 △*aldB-I* △*areA* △*sad-II* △*calB* △*amaB* △*aldA* △*modABC* △*crc* △*vanAB* △*iorAB* △PP_3354–3355

2.3 | Removal of Vanillin With Adsorbent Resins

Vanillin was removed from culture broth via the application of Amberlite resin XAD-2, a polystyrene-divinylbenzene copolymer, with the divinylbenzene acting as a crosslinking agent between polystyrene chains (product number 10357, Sigma Aldrich). The affinity of XAD-2 toward both vanillin and ferulic acid was tested as follows: Solutions of known concentrations (verified by High Performance Liquid Chromatography (HPLC)) of each substrate were prepared in M9 minimal medium to a final volume of 6 mL. XAD-2 was added to these solutions at a concentration of 0.5 g/mL and adsorption was allowed to proceed overnight at 30°C and 250 rpm. Solutions were then sampled for HPLC to determine the change in vanillin or ferulic acid concentration, which was assumed to indicate adsorption to the resin.

IAR068 was revived from a glycerol stock as described above. Overnight cultures were centrifuged and the cell pellets were resuspended in M9 minimal medium. To initiate the growth

phase, 1 mL of the resuspended culture was used to inoculate 24 mL of M9 minimal medium supplemented with 7.21 g/L (40 mM) glucose to an OD_{600} of 0.1 in a 125 mL baffled flask; inoculated flasks were incubated for 22 h at 30°C and 250 rpm. The conversion phase was initiated by the addition of 1.7 mL of a ferulic acid and glucose solution to a final concentration of 3.88 g/L (20 mM) ferulic acid and 1.80 g/L (10 mM) glucose. Conversion continued for a total of 59.5 h with additional boluses of ferulic acid and glucose supplied twice per day, as follows: after around 12 h of conversion, cultures were sampled for HPLC, decanted into a 50 mL centrifuge tube, and centrifuged to separate cells from the medium. The supernatant was decanted into another 50 mL centrifuge tube containing 12.5 g (0.5 g/mL culture) of prepped Amberlite XAD-2 resin for adsorption.

Resins had been prepared for use before the start of the experiment as follows: resins were autoclaved using a dry cycle, dried in a 105°C oven overnight, placed in a desiccator for 2 h, soaked in 90% v/v ethanol for 1 day, and decanted and rinsed with MilliQ water twice. To remove any residual ethanol, resins were washed by passing about 3 L of MilliQ water over the resins through a filter with frequent stirring of the resin. Next, the same method was used to rinse the resins with M9 minimal medium; finally, resins were scraped off the filter into a sterile bottle. Prepped resins were used for a maximum of two extraction cycles before being discarded. Cell pellets were set aside at room temperature for the duration of resin application. Adsorption was carried out on a Labnet Orbit LS rotator (Labnet International Inc) at maximum rpm (60) for 1 h and the supernatant was sampled for HPLC post adsorption. Resins were separated from the treated supernatant by filtration, and the used resins were either returned to a 50 mL centrifuge tube or discarded. Filter-sterilised supernatant was returned to the falcon tube containing the cell pellet and the pellet was resuspended by vortexing. Ferulic acid was resupplied to the cell culture to a final concentration of ~3.88 g/L (20 mM), using estimates of conversion and adsorption efficiencies for ferulic acid obtained from prior experiments (which showed that around 15% of ferulic acid was utilised at each timepoint and around 73% of the unutilized ferulic acid was adsorbed by the resin). Glucose was also supplied with each bolus of ferulic acid to a final concentration of 1.80 g/L (10 mM). The supernatant was sampled for HPLC following the addition of ferulic acid and glucose. Conversion was allowed to proceed for another 12 h, approximately, when the adsorption procedure described above was repeated. A total of four rounds of adsorption were carried out over the 59.5 h of conversion. All conditions were evaluated in duplicate.

2.4 | Quantification of Metabolites in Fermentation Broth by HPLC

Aromatic compounds (vanillin, vanillic acid, vanillyl alcohol, ferulic acid) were quantified as described in the protocol "Muconic acid isomers and aromatic compounds analyzed by UHPLC-DAD V.2" (Woodworth et al. 2024). Glucose was quantified as described in the protocol "Analysis of sugars, small organic acids, and alcohols by HPLC-RID V.2" (Alt et al. 2024).

3 | Results

3.1 | Engineered *P. putida* Can Accumulate Vanillin

Genetic targets for deletion from the *P. putida* KT2440 genome were identified using insights from previously published work, such as engineering and conversion (Graf and Altenbuchner 2014; García-Hidalgo et al. 2020), proteomic (Simon et al. 2014), and transcriptomic (Price et al. 2018; Incha et al. 2020) studies. Additionally, some targets were identified based on genome annotation in BioCyc, such as *aldA* (Karp et al. 2019), or by rational choice, such as *vanAB* (Brunel and Davison 1988) and *crc* (Magasanik 1970; Morales et al. 2004; Johnson et al. 2017). A list of each genetic target identified and the rationale for its deletion is provided in Table 2.

As described above, vanillin is an intermediate in ferulic acid catabolism in wild-type *P. putida* KT2440, and as expected, vanillin does not accumulate during the metabolism of ferulic acid under the conditions tested here (Figure S2). Deletion of the vanillin reductase gene *areA* and the genes encoding the eight aldehyde dehydrogenases mentioned above, including *vdh* (Table 2), also did not lead to the accumulation of vanillin (strain *P. putida* IAR032, Table 1, Figure 2A, Table S2), suggesting that additional genes encoding enzymes with affinity toward vanillin were still present in this strain.

Vanillin accumulation was observed, however, with an additional deletion of the *modABC* operon (*vide infra*), encoding a molybdate ion transporter, from strain IAR032 (resulting in strain *P. putida* IAR038 (Table 1)), an observation also reported by Graf and Altenbuchner (2014). In fact, deletion of the *modABC* operon also enabled vanillin to accumulate to 0.52 g/L in IAR035, a strain with only *vdh*, *aldB-II*, PP_1948, and *aldB-I* deleted (Figure S3B). To further explore the role of the *modABC* operon in vanillin metabolism, we evaluated strains IAR032 and IAR038, which only differed in the deletion of the *modABC* operon in strain IAR038 (Table 1), for their ability to grow on either ferulic acid or vanillin as a sole carbon source. IAR032 was able to grow on both substrates as a sole carbon source, but IAR038 was not (Table S4), further highlighting the critical role *modABC* plays in vanillin metabolism in *P. putida* KT2440, at least under these cultivation conditions.

The ability of strain IAR032 (Table 1) to grow on vanillin (Table S4) implies that this strain still possesses enzymes with affinity toward vanillin, the activity of which the deletion of *modABC* interrupts by preventing the import of molybdate ions that these enzymes presumably require (Graf and Altenbuchner 2014). One such enzyme may be an alternate vanillin dehydrogenase in *P. putida*, encoded by the *iorAB* operon (PP3621-3623), which has been reported to use molybdenum as a cofactor (Price et al. 2019). This operon was identified via Tn-Seq studies that showed reduced fitness in cells with deletions in these genes when growing on vanillin (Price et al. 2018; Incha et al. 2020). Though the deletion of the *modABC* operon to generate strain IAR038 was sufficient to allow vanillin to accumulate (Figure 3A, Table 1), we nevertheless deleted the *iorAB* operon (Figure 2D, Table S2) in case the vanillin dehydrogenase

TABLE 2 | Genes targeted for deletion. Note, the gene *areA* is alternately annotated as "*calA*".

Locus	Name	Annotation	Rationale	Publication suggesting deletion
PP_3357	*vdh*	Vanillin dehydrogenase	Deletion has been shown to contribute to the accumulation of vanillin	Graf and Altenbuchner (2014)
PP_0545	*aldB-I*	Aldehyde dehydrogenase		
PP_1948	*bdh*	Benzaldehyde dehydrogenase		
PP_2680	*aldB-II*	Aldehyde dehydrogenase		
PP_3151	*sad-II*	NAD+-dependent succinate semialdehyde dehydrogenase	Found to be upregulated on exposure to vanillin	Simon et al. (2014)
PP_5120	*calB*	Coniferyl aldehyde dehydrogenase		
PP_5258	*amaB*	L-aminoadipate-semialdehyde dehydrogenase		
PP_2487	*aldA*	Aldehyde dehydrogenase	Predicted aldehyde dehydrogenase	BioCyc annotation (Karp et al. (2019)
PP_2426	*areA*	Vanillin reductase	Accepts vanillin as a substrate	García-Hidalgo et al. (2020)
PP_3827–3832	*modABC*	Molybdate ABC transporter	Molybdate ions are cofactors for oxidoreductases	Graf and Altenbuchner (2014)
PP_3736–3737	*vanAB*	Vanillate monooxygenase	Prevents further breakdown of vanillic acid	Brunel and Davison (1988)
PP_5292	*crc*	Catabolite repression control protein	To enable simultaneous FA and glucose uptake	Johnson et al. (2017)
PP_3621–3623	*iorAB*	Vanillin dehydrogenase	Mutants have reduced fitness on vanillin	Price et al. (2018); Incha et al. (2020)
PP_3354 PP_3355	*aat*	β-ketothiolase acetyl-CoA dehydrogenase	Could act on an intermediate in the vanillin production pathway	Graf and Altenbuchner (2014)

it encodes retains some activity, despite deletion of *modABC*, *vide infra*.

We also deleted two genes proposed to encode enzymes participating in a vanillic acid shunt (PP 3354_3355), in which 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA, a transient intermediate in the conversion of ferulic acid to vanillin, is converted directly to vanillic acid, bypassing vanillin entirely (Overhage, Priefert, Steinbüchel, et al. 1999; Table 2, Figure 1). Additionally, we deleted *vanAB*, an operon encoding a vanillate-*O*-demethylase which converts vanillic acid to protocatechuate (Figure 2C, Table S2); with this deletion, any vanillic acid that may be generated by promiscuous aldehyde dehydrogenase activity would not be able to be further utilised for growth. The accumulation of vanillic acid in this strain would also serve to reveal the presence of not-yet-characterised aldehyde dehydrogenases with activity on vanillin.

In *Pseudomonas*, catabolic preferences for organic acids and sugars over aromatic substrates are imparted by the Catabolite Repression Control (Crc) protein, a global regulator (Rojo 2010). Expression of Ech, an enoyl-CoA hydratase involved in the conversion of ferulic acid to vanillin, has been predicted to be regulated by Crc (Browne et al. 2010) and deletion of *crc* improves catabolism of ferulic acid in the presence of glucose (Johnson et al. 2017). Thus, to promote the conversion of ferulic acid to vanillin in the presence of glucose, which was provided as a source of carbon and energy during conversion of ferulic acid to vanillin in our study, we deleted *crc* from our strain (Figure 2B Table S2). The performance of these intermediate strains converting ferulic acid to vanillin is shown in Figure 2, their genotypes are shown in Table S2, and the rationale for various experimental conditions employed is explained in Table S3. The genotype of the final strain, *P. putida* KT2440 IAR068, is shown in Table 1.

Conversion of ferulic acid to vanillin by *P. putida* KT2440 IAR038 and IAR068 was compared in shake flasks to determine if the additional deletions in IAR068 improved vanillin production (Figure 3). In prior experiments, we observed that vanillin titers are higher when the bioprocess consists of separate growth and conversion phases (Figure S4), as has also been noted by others (Graf and Altenbuchner 2014); in fact, vanillin accumulates

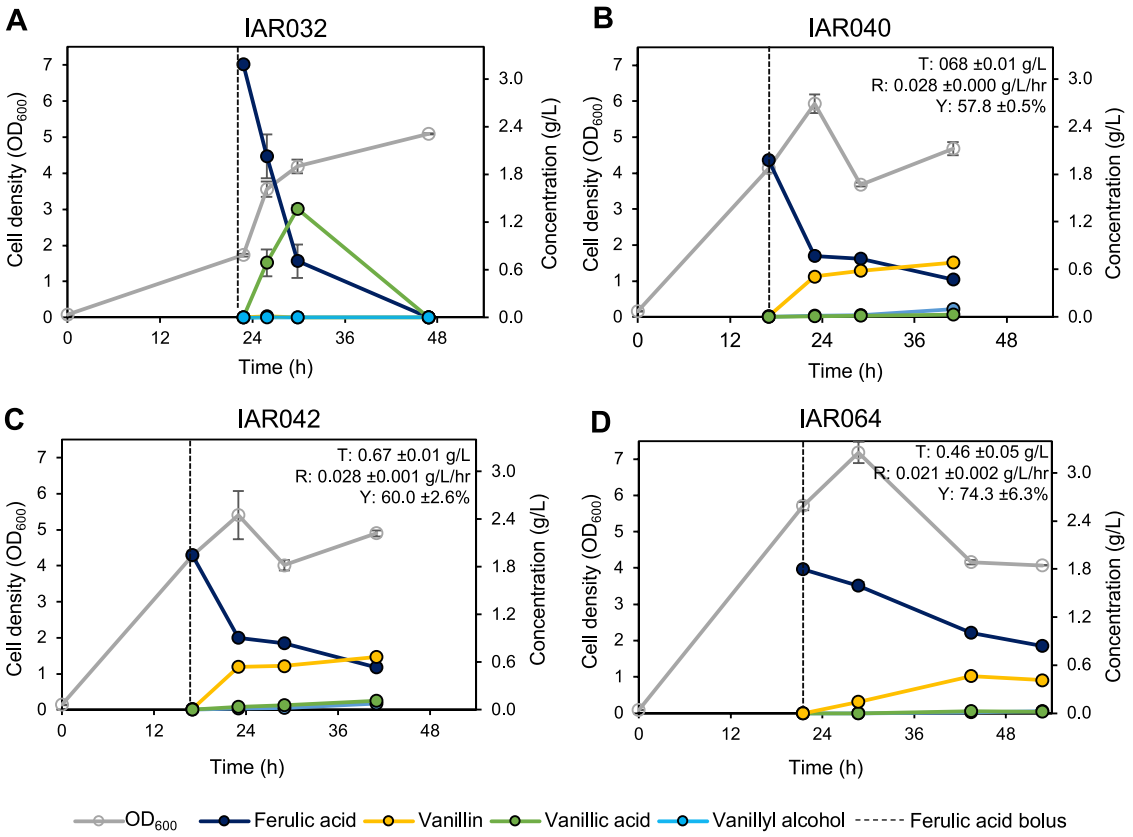

FIGURE 2 | Growth and ferulic acid conversion by *P. putida* IAR032 (A), IAR040 (B), IAR042 (C), and IAR064 (D). Before the dashed lines, cultures were grown on 3.60 g/L (20 mM) glucose (A) or 7.21 g/L (40 mM) glucose (B-D). At the dashed lines, ferulic acid was added to the medium to a final concentration of 1.94 g/L (10 mM) (B–D) or 3.88 g/L (20 mM) (A). Either 1.8 g/L (10 mM) of glucose (A–C) or 3.60 (20 mM) of glucose (D) was provided to cultures at the time of ferulic acid addition; additional glucose was provided to cultures as follows: 1.80 g/L (10 mM) 12 h after initiation of the conversion phase (B, C), or 3.60 g/L (20 mM) 22 h after initiation of the conversion phase (D). Error bars show absolute difference between two biological replicates (B, C) or ± SEM of three biological replicates (A, D). For those strains that accumulated vanillin (B–D), "T" refers to maximum titre; "R" refers to apparent production rate at the time of maximum titre, and "Y" refers to percent molar yield at the time of maximum titre. Genotypes of these strains are provided in Table S2. Rationale for varying experimental conditions is summarised in Table S3 Methods.

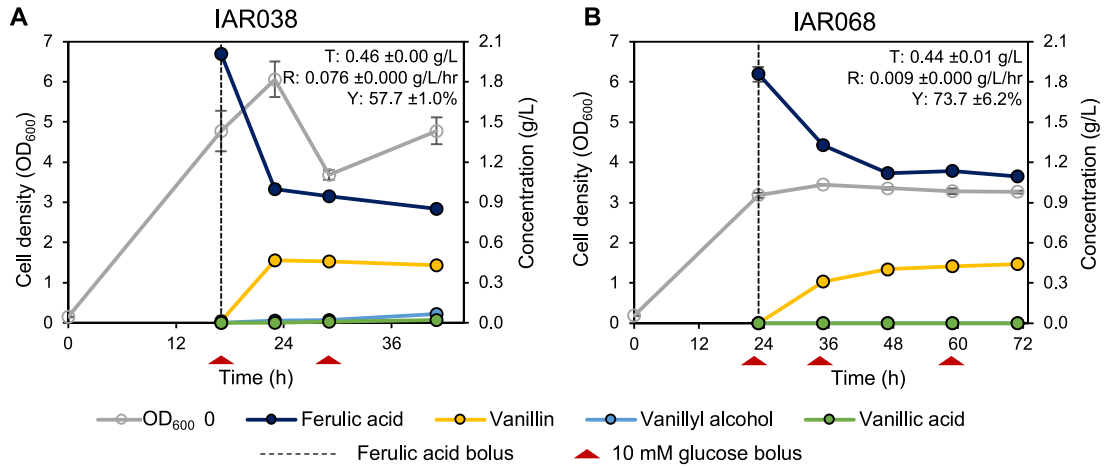

FIGURE 3 | Conversion of 1.94 g/L (10 mM) ferulic acid to vanillin by (A) *P. putida* KT2440 IAR038 and (B) *P. putida* KT2440 IAR068. Dashed lines indicate the addition of ferulic acid to the culture medium and represent the transition of the cell culture from growth on 7.21 g/L (40 mM) glucose to vanillin production from ferulic acid. Red triangles represent the addition of 1.80 g/L (10 mM) glucose as an energy source, with a total of 3.60 (for IAR038) or 5.40 (for IAR068) g/L glucose (20–30 mM) added per cultivation. Circles represent the mean of two biological replicates; error bars show absolute difference between duplicates but are not always visible due to small differences between replicate datapoints. "T" refers to maximum titre; "R" refers to apparent maximum production rate at the time of maximum titre, and "Y" refers to percent molar yield at the time of maximum titre. Experimental data for these experiments are shown in the supplementary data file.

only transiently in the simultaneous growth and conversion regime before conversion to vanillic acid (Figure S4A), suggesting potential differences in the regulation of *vanAB* during growth and stationary phases. Given these data, both strains were grown on glucose for 18–24 h to accumulate biomass; at stationary phase, conversion was initiated by the addition of 1.94 g/L (10 mM) of ferulic acid (note that the optimal duration between reaching stationary phase and the addition of ferulic acid was not investigated). At the same time, and periodically throughout the conversion phase, 1.80 g/L (10 mM) of glucose was added for the purpose of providing cells with additional energy, as conversion of ferulic acid to feruloyl-CoA imposes an energetic burden on the cell. Conversion continued for 1–2 days, though most of the vanillin (91% of final titre for strain IAR068) was generated within the first 24 h of conversion (Figure 3). The final titers of the strains were not statistically different, which we suspected might be related to the toxicity associated with vanillin accumulation, since the ferulic acid provided was not completely converted by either strain. Substantial accumulation of vanillic acid and vanillyl alcohol was not observed in either strain, even IAR068, in which vanillic acid, if produced, would have accumulated due to the additional deletion of *vanAB*. Interestingly, IAR038 accumulated vanillin faster than IAR068 (Figure 3): the maximum productivity of IAR038 was 0.078 g/L/h, observed within the first 6 h, while that of IAR068 was 0.037 g/L/h, within the first 12 h of conversion (note, productivity at the time of maximum titre for strain IAR068 was 0.009 g/L [Figure 3B]). Reduced productivity was also observed by Graf and Altenbuchner (2014) in strains in which PP_3354–3355 had been deleted; they proposed that the deletion of these two genes, which are immediately downstream of *fcs* (PP_3356) and *ech* (PP_3358), may result in a less-stable mRNA of the remaining *fcs-ech* genes, possibly reducing their activity. However, it is worth noting that a slower conversion rate was also observed in the parent of IAR068 (IAR064, Figure 2D), which had *iorAB* deleted. While vanillin titers and rates were not improved, the molar yield (calculated as moles vanillin produced from moles ferulic acid consumed) significantly increased from 59.2% in IAR038 to 73.7% in IAR068 ($p < 0.05$), so we proceeded with IAR068. Glucose consumption was not considered when calculating yields as glucose is used for cell growth and does not contribute to vanillin synthesis.

3.2 | Removal of Vanillin From Culture Broth Increases Vanillin Titers

Vanillin titers were ~0.5 g/L and unconverted ferulic acid remained when *P. putida* IAR038 or IAR068 converted ferulic acid to vanillin (Figure 3B), suggesting that this concentration of vanillin represented the maximum tolerable concentration for these strains. While we have not investigated what proportion of total vanillin is represented by extracellular vanillin, relatively small, nonpolar, and hydrophobic molecules like vanillin are expected to be able to diffuse across cell membranes (Vermaas et al. 2022) and thus are susceptible to adsorption to the resin even in the absence of active excretion. Thus, to alleviate vanillin toxicity, we explored periodically removing vanillin from the supernatants of cultures with a hydrophobic adsorbent resin, XAD-2. While the XAD-2 resin exhibits stronger affinity to vanillin than to ferulic acid, ferulic acid is nevertheless adsorbed by the resin; for example, when either 20 mM of ferulic acid (3.88 g/L) or vanillin (3.04 g/L) is loaded with XAD-2, 70.0% and 100.0% of the compound are adsorbed, respectively (Figure S5). While resin choice would require optimisation for an industrial process, we reasoned that the application of XAD-2 would still be useful towards understanding if removal of vanillin from cultures would promote an improved "apparent titre" of vanillin, defined as the cumulative amount of vanillin produced by a single bacterial culture throughout multiple rounds of product extraction. The affinity of XAD-2 to ferulic acid required that it be resupplied to the supernatant after each round of adsorption to maintain ~3.88 g/L (20 mM) of substrate; subsequent HPLC analyses revealed that actual concentrations of ferulic acid ranged from 3.42 to 4.54 g/L (17.6–23.4 mM; Figure 4C). The application of resin XAD-2 (Figure 4A, Figure S6) allowed the cells to produce a cumulative 3.35 g/L vanillin from a total of 5.53 g/L ferulic acid consumed (Figure 4D) in 59.5 h of conversion (77.5% ± 2.6% molar yield; 0.056 ± 0.001 g/L/h productivity), while the control, in which vanillin was not periodically removed, reached a maximum titre of 0.64 g/L in 46.7 h of conversion (69.1% ± 2.2% molar yield; 0.014 g/L/h ± 0.000 productivity; Figure 4B), supporting the hypothesis that titers had been previously limited by vanillin toxicity and demonstrating the utility of its removal as a means of improving its production. An additional experiment evaluating the effect of resin addition on conversion is shown in Figure S6. In addition to improved apparent titers, IAR068 was able to produce higher titers of vanillin during each cycle of conversion and resin application (Figure 4A, dashed purple lines). It is possible that the lower affinity of the resin to ferulic acid than vanillin contributed to this effect. When ferulic acid is first applied to the cultures post-extraction, some of it is adsorbed to the resin; as conversion begins, vanillin outcompetes ferulic acid in binding to the resin, causing the release of previously bound ferulic acid from the resin. It is possible that this gradual release of ferulic acid in response to vanillin production contributed to the higher titers observed during each conversion cycle.

4 | Discussion

4.1 | *P. putida* Is a Suitable Biocatalyst for the Production of Vanillin From Ferulic Acid

From the genus *Pseudomonas*, both *P. putida* KT2440 and *P. fluorescens* BF13 have previously been engineered for the production of vanillin from ferulic acid, with both strains achieving titers of around 1.3 g/L (Graf and Altenbuchner 2014; García-Hidalgo et al. 2020; Di Gioia et al. 2011). In this study, *P. putida* IAR068 was able to produce up to 0.64 g/L vanillin in shake flasks and up to 3.35 g/L vanillin when vanillin was periodically removed from the cultivation medium by adsorbent hydrophobic resin XAD-2. High vanillin titers have been achieved in *Pseudomonas* using other substrates like eugenol (Overhage, Priefert, Rabenhorst, et al. 1999) and isoeugenol (Yamada et al. 2007). We have not tested the ability of IAR068 to convert eugenol or isoeugenol to vanillin; however, we anticipate that the deletion of *calB* (PP_5120) would prevent this strain from metabolising eugenol (Overhage et al. 2002), while *iem*, encoding an isoeugenol monooxygenase responsible for the conversion of isoeugenol to vanillin, is present in this strain and should enable the conversion of isoeugenol to vanillin.

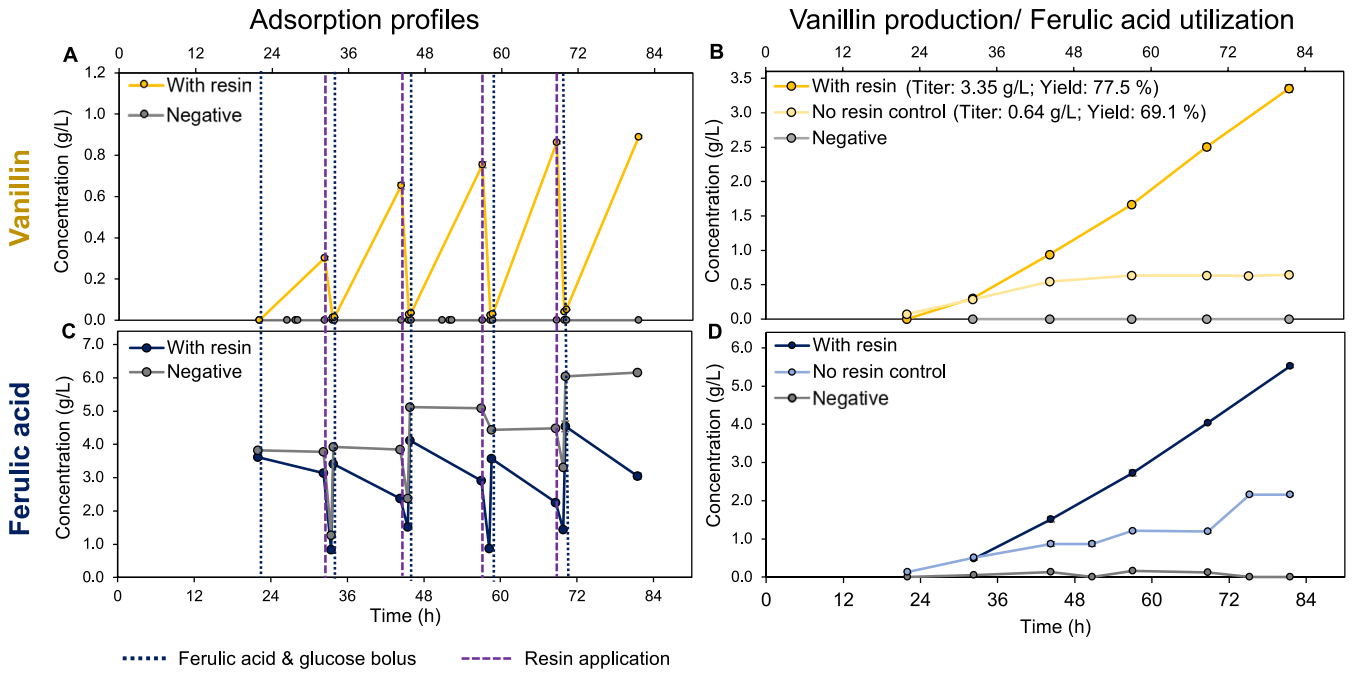

FIGURE 4 | Adsorption profiles (A, C) and cumulative vanillin production (B) and ferulic acid utilisation (D) curves of *P. putida* KT2440 IAR068 with and without the application of resin XAD-2 to culture supernatants. Addition of ferulic acid and XAD-2 is indicated by blue and purple dashed lines, respectively. Circles represent the mean of two biological replicates; error bars show absolute differences between duplicates but are not always visible due to small differences between replicate datapoints. Experimental data for these experiments are shown in the supplementary data file.

4.2 | Yields Lower Than 100% Indicate Some Carbon Is Unaccounted for

Maximum vanillin yields in *P. putida* IAR068 were ~74% (Figure 3), suggesting that some ferulic acid is being converted to untargeted products or metabolised. The two metabolites known to be immediately downstream of vanillin, vanillic acid and vanillyl alcohol, were below the threshold of detection in cultivation broth; thus, neither significantly contributed to the product pool, but their accumulation within cells cannot be ruled out. Ferulic acid could have been metabolised via non-specific enzymatic activity of aldehyde dehydrogenases still present in the genome of strain IAR068, though the product of such reactions would be vanillic acid, and the deletion of *vanAB* should prevent further metabolism of vanillic acid unless by unidentified enzymes. Additionally, the metabolism of over 25% of the provided substrate should result in the growth of this engineered strain on ferulic acid as a sole carbon source—a phenotype that we did not observe; this suggests that the unaccounted-for carbon was accumulating in the form of an intermediate that is not contributing to growth. One possibility is feruloyl-CoA (Figure 1), an intermediate upstream of vanillin. While we were unable to quantify intracellular feruloyl-CoA, this does not rule out feruloyl-CoA accumulation since CoA-conjugated intermediates tend to be labile and could have broken down during extraction. Careful protocol development to confidently extract this intermediate would be useful toward understanding if it is accumulating in these strains.

4.3 | Vanillin Toxicity Likely Limits Vanillin Titers

Wild-type *P. putida* KT2440 is able to grow in up to 3.80 g/L (25 mM) of exogenous vanillin (Figure S7), though this tolerance is in large part enabled by the catabolism of vanillin, a tolerance mechanism not available to strains engineered to accumulate it. As discussed above, the ~0.5 g/L limit of vanillin titers observed in our shake flask experiments is likely due to product toxicity. Graf and Altenbuchner (2014) have observed that supplying additional vanillin to cultures of vanillin-accumulating engineered strains of *P. putida* KT2440 resulted in a decrease in vanillin titers, further suggesting that there is a physiological limit to vanillin production that could be alleviated by reducing the concentration of vanillin in culture broth. This hypothesis is supported by the ability of *P. putida* IAR068 to produce up to 3.35 g/L of vanillin over the course of a 59.5-h cultivation when vanillin was periodically removed from the culture broth by an adsorbent resin. In this way, the use of adsorbent resin serves as a proof-of-concept that higher titers are possible with the removal of vanillin. Further performance optimisation could be achieved with other product-removal strategies like a solid–liquid two-phase partitioning bioreactor, as has been used to enhance vanillin production in *Amycolatopsis* (Ma and Daugulis 2014). Further, if the use of product-removal strategies alleviates product or intermediate build-up, additional strain engineering to overexpress *fcs-ech* may be beneficial. It is also possible that the accumulation of feruloyl-CoA could contribute to the toxic effects of converting ferulic acid to vanillin, as the accumulation of coumaroyl-CoA, the product of Fcs when *p*-coumarate rather than ferulate is metabolised by *P. putida*, was found to be toxic, perhaps due to the sequestration of CoA required for other metabolic processes (Incha et al. 2020).

5 | Conclusion

In this study, *P. putida* KT2440 IAR068 produced a total of 3.35 g/L vanillin when vanillin was removed from the medium

with a hydrophobic adsorbent resin, XAD-2; to our knowledge, this is the highest effective titre reported to date using *Pseudomonas* to convert ferulic acid to vanillin. The increase in vanillin titre observed from the application of adsorbent resin to vanillin-accumulating cultures serves as a proof-of-concept that removal of vanillin from the cultivation medium facilitates increased production of vanillin. Advances in in situ product recovery and/or additional strain development would help to enable the development of an industrial-scale process for production of microbially-derived vanillin to meet consumer demand for natural vanillin and reduce reliance on chemical synthesis strategies.

Author Contributions

Ilona A. Ruhl: methodology, investigation, formal analysis, validation, writing – original draft, writing – review and editing. **Sean P. Woodworth:** methodology, investigation, writing – review and editing. **Stefan J. Haugen:** methodology, investigation. **Hannah M. Alt:** methodology, investigation. **Gregg T. Beckham:** conceptualization, funding acquisition, supervision, writing – review and editing. **Christopher W. Johnson:** conceptualization, funding acquisition, supervision, methodology, writing – review and editing, writing – original draft.

Acknowledgements

Strain GN442 was provided as a kind gift from the Altenbuchner group. We also thank William Michener for the development of the feruloyl-CoA HPLC method and Lucas Friedberg for preparing and submitting plasmids to Addgene. This work was authored by the National Renewable Energy Laboratory for the U.S. Department of Energy (DOE) under Contract No. DE-AC36-08GO28308. Funding was provided by the U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, Bioenergy Technologies Office. The views expressed in the article do not necessarily represent the views of the DOE or the U.S. Government. The U.S. Government retains and the publisher, by accepting the article for publication, acknowledges that the U.S. Government retains a nonexclusive, paid-up, irrevocable, worldwide licence to publish or reproduce the published form of this work, or allow others to do so, for U.S. Government purposes.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Experimental data is either presented in the manuscript or provided in the Supporting Information document. The plasmids generated for this study have been deposited with Addgene under the Catalogue numbers listed in the Supporting Information document.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.