nature portfolio

Peer Review File

Quantitative Decoding of Pathway Fluxes that Couple Carbon and Energy Metabolism in Lignin Carbon Utilization

Corresponding Author: Dr Ludmilla Aristilde

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the manuscript “Quantitative decoding of pathway fluxes that couple carbon and energy metabolism in lignin carbon utilization”, Zhou and collaborators conducted a detailed multi-omics study to elucidate how the model soil bacterium Pseudomonas putida KT2440 metabolizes lignin-derived phenolic compounds and balances energy and redox cofactors during the process. By comparing growth on four common aromatics—namely, ferulate, p-coumarate, vanillate, and 4-hydroxybenzoate—to succinate, the authors show a substantial upregulation of transport and catabolic proteins specific to aromatics. Metabolomics revealed bottlenecks in early catabolism that help maintain cellular energy charge, with mutants bypassing these bottlenecks displaying altered energy states. The authors also detected substantial induction of pyruvate carboxylase and glyoxylate shunt enzymes, supported by kinetic 13C-metabolomics and quantified through 13C-fluxomics. This analysis revealed that anaplerotic recycling via pyruvate carboxylase drives tricarboxylic acid cycle fluxes, yielding 50-60% of the NADPH and 60-80% of the NADH required, and achieving ATP surpluses up to 6-fold higher than those observed during succinate metabolism. The glyoxylate shunt was shown to further contribute to NADPH turnover via the malic enzyme. This study is technically robust, scientifically rigorous, and superbly well-written. The quality of the figures is excellent. The authors have covered all angles with a panoply of techniques and approaches in a carefully thought-out experimental design. Furthermore, the results and conclusions of this paper provide a quantitative framework for metabolic engineering towards lignin valorization strategies. The quality of the presentation and the relevance of the findings would warrant an “Accept as is”, and my (very minor) comments below are only meant to serve as suggestions for the authors to improve an otherwise excellent manuscript.

1. Fig. 1: Many dehydrogenase enezymes in central metabolism can accept NADH or NADPH (or their oxidized forms) depending on the conditions (e.g. intracellular concentration of reactants). This is also true for steps where more than dehydrogenase variants are present (e.g. Zwf, see Volke et al., mSystems). Could it be useful to indicate this explicitly, as the authors did for VanAB? This is also true for formaldehyde and formate dehydrogenases (Fig. 3B): some of them are NAD+-dependent and others are NADP+-dependent.

2. I would label the axes in Fig. 1B and C as “Intracellular metabolite concentration (μmol gCDW-1)”. The same applies to other figures where this comment is relevant (e.g. Fig. 2B-F).

3. I would avoid the term “accumulation” (e.g. in Page 4-Line 132) when referring to the intracellular concentration of a metabolite, since the observed concentration is at a dynamic equilibrium.

4. The experimental design underlying Fig. 4 is brilliant. Minor detail: I would use a consistent abbreviation for metabolites across the figures (e.g. acetyl-CoA vs AcCoA).

5. Fig. 6. Yet another example of a self-explanatory way of representing results. Excellent.

6. P17-Line 472: How refreshing it is to see that the authors CORRECTLY call LB lysiogeny broth and not Luria-Bertani!

7. P14-Line 472, P18-L507 and elsewhere: Please specify the type of percentage used for concentrations (w/w, w/v, etc.).

8. P17-Line 474: Is this C-mol or total C atoms? Please clarify.

9. P18-Line 494: Italicize “attB”.

Reviewer #2

(Remarks to the Author)
In this paper, Dr. Aristilde lab presented an outstanding work that use multi-omics investigation of Pseudomonas putida KT2440 grown on four common phenolic substrates. Via 13C labeling, MFA and genome scale modeling, the paper described cellular catabolism of nonmodel substrates. The knowledge has broad impacts and the study is highly innovative. I only have three minor comments.

1. Metabolite extraction for analysis of intracellular metabolite levels and isotope labeling kinetics: The team used cell filtering to quench metabolism. During cell residence on filter paper, cell metabolism and fluxes may be changed which could affect flux outcome. The authors may address this concerns.

2. A similar study has been done in Rhodococcus opacus (Metabolic Engineering, Volume 55, 2019, Pages 120-130). That paper also investigated the hierarchy of carbon source co-utilizations. Since lignocellulosic biomass contains sugars and aromatic substrates, authors may also look at cellular utilization of mixed substrates.

3. Bacteria may use Water-forming NADH oxidase to remove NADH and generate heat. How this reaction impact flux modeling and conclusion?

Reviewer #3

(Remarks to the Author)
This manuscript presents a comprehensive and well-structured study on the aromatic metabolism of Pseudomonas putida KT2440. By combining 13C-metabolic flux analysis, proteomics, intracellular metabolite profiling, and engineered strain comparisons, the authors offer a high-resolution view of how carbon and energy metabolism are coordinated during growth on different lignin-derived phenolic acids. The findings provide quantitative insights into pathway-specific rewiring, energy surplus, and cofactor balancing, which are highly relevant for future metabolic engineering and lignin valorization strategies. The manuscript is of high quality, and the supporting data in the Supplementary Information are thorough and transparent, including detailed proteomic p-values, energy charge data, and flux distributions. Overall, the work is well executed and makes a strong contribution to the field.
Below are some suggestions aimed at further improving clarity, accessibility, and interpretation. These are minor in nature and should be straightforward to address.

Comments
1. Referencing Supplementary Data in Main Text:
Please reference specific supplementary tables (e.g., Table S3 for proteomics statistics) in relevant figure captions or result descriptions for better data traceability.

2. Interpretation of ATP and NAD(P)H Surplus:
The manuscript clearly shows that certain substrates (FER, COU) yield high ATP and NAD(P)H production. A brief comment in the discussion on how this energetic surplus could support synthetic biochemistry (e.g., production of energy-intensive metabolites) would be beneficial.

3. Clarification of TCA remodelling via Pyruvate Carboxylase:
The increased flux through pyruvate carboxylase is a key result. A short explanation linking this to CO_2 assimilation or oxaloacetate demand (e.g., for biosynthesis) would improve biological interpretation.

4. Engineered Strains and Energy Charge:
Reduced energy charge in some overexpression strains is an important observation. A brief note that this might result from metabolic burden, redox imbalance, or high expression cost would strengthen the interpretation. Mentioning future improvements such as RBS tuning or chromosomal integration would add practical value.

5. Flux vs. Protein Abundance Divergence:
Figure 5E shows weak correlation between protein levels and metabolic flux. It would be useful to highlight 1–2 specific cases where flux remains high despite low protein expression, suggesting post-translational regulation or thermodynamic efficiency.

6. Abstract and Introduction Refinement:
The abstract is comprehensive but can be made more accessible by clearly separating the background, objective, methods, key findings, and implications.
Consider briefly reinforcing in the introduction why P. putida KT2440 is particularly well-suited for lignin-derived aromatic metabolism and bow-tie architecture of metabolism in pseudomonas species.

7. Figure Caption Enhancements:

For figures presenting flux maps, energy charge, or kinetic labelling, consider including a brief biological interpretation or highlighting the main trend in the caption.

8. Terminology Consistency:
Ensure consistent use of substrate abbreviations (e.g., FER, VAN), gene/protein names (e.g., vanAB, pobA, pcaHG), and pathway terms (e.g., anaplerosis, cataplerosis).

9. Spelling and Grammar Corrections:
A final proofreading pass to correct minor typographical issues (e.g., “fluzomics” → “fluxomics”, “ene:gy” → “energy”) and ensure grammatical consistency is recommended.

10. Quantitative proteomics sampling procedure:
P18, line 513-518: since the protein changes are happening in the order of 8-10 minutes, one would expect quenching followed by washing steps. Here done in a reverse manner. Please clarify regarding the procedure in the revised MS text and appropriate literature reference.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all my comments.

Reviewer #2

(Remarks to the Author)
My questions have been well-answered.

Reviewer #3

(Remarks to the Author)
The authors have addressed all my concerns in the revised MS. I do not have further comments.

Reviewer #1

In the manuscript “Quantitative decoding of pathway fluxes that couple carbon and energy metabolism in lignin carbon utilization”, Zhou and collaborators conducted a detailed multi-omics study to elucidate how the model soil bacterium Pseudomonas putida KT2440 metabolizes lignin-derived phenolic compounds and balances energy and redox cofactors during the process. By comparing growth on four common aromatics—namely, ferulate, p-coumarate, vanillate, and 4-hydroxybenzoate—to succinate, the authors show a substantial upregulation of transport and catabolic proteins specific to aromatics. Metabolomics revealed bottlenecks in early catabolism that help maintain cellular energy charge, with mutants bypassing these bottlenecks displaying altered energy states. The authors also detected substantial induction of pyruvate carboxylase and glyoxylate shunt enzymes, supported by kinetic 13C-metabolomics and quantified through 13C-fluxomics. This analysis revealed that anaplerotic recycling via pyruvate carboxylase drives tricarboxylic acid cycle fluxes, yielding 50-60% of the NADPH and 60-80% of the NADH required, and achieving ATP surpluses up to 6-fold higher than those observed during succinate metabolism. The glyoxylate shunt was shown to further contribute to NADPH turnover via the malic enzyme. This study is technically robust, scientifically rigorous, and superbly well-written. The quality of the figures is excellent. The authors have covered all angles with a panoply of techniques and approaches in a carefully thought-out experimental design. Furthermore, the results and conclusions of this paper provide a quantitative framework for metabolic engineering towards lignin valorization strategies. The quality of the presentation and the relevance of the findings would warrant an “Accept as is”, and my (very minor) comments below are only meant to serve as suggestions for the authors to improve an otherwise excellent manuscript.

Response: We greatly appreciated the very positive feedback from the reviewer.

1. Fig. 1: Many dehydrogenase enzymes in central metabolism can accept NADH or NADPH (or their oxidized forms) depending on the conditions (e.g. intracellular concentration of reactants). This is also true for steps where more than dehydrogenase variants are present (e.g. Zwf, see Volke et al., mSystems). Could it be useful to indicate this explicitly, as the authors did for VanAB? This is also true for formaldehyde and formate dehydrogenases (Fig. 3B): some of them are NAD+-dependent and others are NADP+-dependent.

Response: To address this comment regarding the cofactors presented in Fig. 1 and Fig. 3B, we revised the figure captions (see below) and the main text (see **Lines 397-400** and **Lines 452-454**)

In Fig.1, we added: “In A, the white crosses are to represent the reported lack of carbon flux through the oxPPP and Entner–Doudoroff pathway during gluconeogenetic substrate metabolism[48]; any cofactor through these reactions[57] was therefore not included for the metabolism of the phenolic acids. Only NADH is produced during formaldehyde and formate oxidation based on previous studies[58,59].”

Lines 397-400: “Due to reported absence of flux through oxPPP during gluconeogenic growth[48], as illustrated in Fig. 1A and confirmed by our ^{13}C-fluxomics illustrated in Fig. 5A-5D, cofactor production through oxPPP was not a contributing factor in our cofactor balancing for phenolic acid metabolism.”

Lines 452-454: “These findings inform rational design of genetic engineering strategies, particularly when choosing between genes encoding isoenzymes with similar catalytic functions but distinct cofactor preferences.”

48. Wilkes, R. A., Waldbauer, J. & Aristilde, L. Analogous metabolic decoupling in *Pseudomonas putida* and *Comamonas testosteroni* implies energetic bypass to facilitate gluconeogenic growth. *mBio* **12**, e03259-21 (2021).
57. Volke, D. C., Olavarría, K. & Nikel, P. I. Cofactor Specificity of Glucose-6-Phosphate Dehydrogenase Isozymes in Pseudomonas putida Reveals a General Principle Underlying Glycolytic Strategies in Bacteria. *mSystems* **6**, 10.1128/msystems.00014-21 (2021).
58. Tanaka, N., Kusakabe, Y., Ito, K., Yoshimoto, T. & Nakamura, K. T. Crystal Structure of Formaldehyde Dehydrogenase from *Pseudomonas putida*: the Structural Origin of the Tightly Bound Cofactor in Nicotinoprotein Dehydrogenases. *Journal of Molecular Biology* **324**, 519–533 (2002).
59. Zobel, S., Kuepper, J., Ebert, B., Wierckx, N. & Blank, L. M. Metabolic response of Pseudomonas putida to increased NADH regeneration rates. *Engineering in Life Sciences* **17**, 47–57 (2017).

2. I would label the axes in Fig. 1B and C as “Intracellular metabolite concentration (µmol gCDW-1)”. The same applies to other figures where this comment is relevant (e.g. Fig. 2B-F).
Response: The axes in Fig. 1 and Fig.2 were edited accordingly.

3. I would avoid the term “accumulation” (e.g. in Page 4-Line 132) when referring to the intracellular concentration of a metabolite, since the observed concentration is at a dynamic equilibrium.

Response: We revised the text to remove the word “accumulation” in **Line 135**, **Line 142**, and **Line 156**.

Lines 135-138: In the coniferyl branch, bottleneck at the Vdh node was characterized by 20-fold and 4-fold higher intracellular vanillin concentration (4.3 ± 0.5 µmol g_{CDW}^{-1}) relative to its precursor feruloyl-CoA (0.2 ± 0.03 µmol g_{CDW}^{-1}) and its downstream metabolite vanillate (1.1 ± 0.2 µmol g_{CDW}^{-1}), respectively; further downstream, PCA was below the detection limit of 0.1 µM (Fig. 1B).

Lines 142-145: In the *p*-coumaroyl branch, in line with the reported bottleneck node at PobA[21,30,32], there was high intracellular 4HB level (18.1 ± 7.6 µmol g_{CDW}^{-1}) during COU catabolism, while 4-hydroxybenzaldehyde (4HBA) level was 67% lower ($P < 0.05$) and PCA level was below the detection limit (Fig. 1C).

Lines 156-159: Compared to the wild-type strain grown on VAN, the unchanged intracellular PCA level in RW124 and depletion of PCA (by 83%, $P < 0.001$) in RW125 demonstrated efficient PCA cleavage and thus elimination of the bottleneck at PcaHG during VAN catabolism (Fig. 2C).

21. Salvachúa, D. *et al.* Bioprocess development for muconic acid production from aromatic compounds and lignin. *Green Chem.* **20**, 5007–5019 (2018).
30. Liu, H. *et al.* Engineering *Pseudomonas putida* for lignin bioconversion into *cis-cis* muconic acid. *Chem Eng J* **495**, 153375 (2024).
32. Kuatsjah, E. *et al.* Debottlenecking 4-hydroxybenzoate hydroxylation in *Pseudomonas putida* KT2440 improves muconate productivity from p-coumarate. *Metab Eng* **70**, 31–42 (2022).

4. The experimental design underlying Fig. 4 is brilliant. Minor detail: I would use a consistent abbreviation for metabolites across the figures (e.g. acetyl-CoA vs AcCoA).

Response: Thank you so much for catching this. We edited Fig. 4 to have the same abbreviation for metabolites.

5. Fig. 6. Yet another example of a self-explanatory way of representing results. Excellent.

Response: We greatly appreciated the reviewer's positive feedback on our data presentation.

6. P17-Line 472: How refreshing it is to see that the authors CORRECTLY call LB lysiogeny broth and not Luria-Bertani!

Response: We appreciated the positive feedback from the reviewer.

7. P14-Line 472, P18-L507 and elsewhere: Please specify the type of percentage used for concentrations (w/w, w/v, etc.).

Response: We specified the type of percentage used for concentrations throughout the method section except for Line 472, where we had the concentrations in mM instead of percentage for components of minimal media.

Lines 534-536: "The injection volume was 10 µL and the column was maintained at 25 °C. The mobile phase consisted of 0.1% formic acid (eluent A) and 80:10:10 acetonitrile: methanol: H2O (v/v) (eluent B) with a flow rate of 0.9 mL · min^{-1}."

Lines 559-564: "The peptides were first loaded onto a trap column (PepMap100 C18, 300 µm × 2 mm with 5 µm particle size; Thermo Scientific) then separated through an analytical column (PepMap100 C18, 50 cm × 75 µm with 3 µm particle size; Thermo Scientific) with eluents consisting of 0.1% formic acid in water (v/v) (eluent A) and 0.1% formic acid in acetonitrile (v/v) (eluent B) with a flow rate of 250 nL · min^{-1} using an Ultimate 3000 RSLCnano system with nano electrospray ionization for liquid chromatography mass spectrometry analysis (LC-MS)."

Lines 578-582: "To profile the intracellular metabolites of each of the substrate condition (100 mM C of FER, COU, VAN, 4HB or SUC), cell suspensions (3 mL) of *P. putida* KT2440 during mid-exponential growth phase (OD_{600} = 1.0-1.2) were filtered (0.22 µm nylon membranes; Whatman, 7402-004), then lysed in a cold quenching solution (4 °C) containing methanol, acetonitrile, and water in a 2:2:1 (v/v) ratio."

8. P17-Line 474: Is this C-mol or total C atoms? Please clarify.

Response: We present concentrations of the different substrates as carbon-equivalent concentrations because they have different number of carbons and we wanted to aim to provide the same amount of carbon across the different growth conditions (now Lines **503-505**).

We edited the sentence to make it clear:

"…minimal nutrient medium with the same carbon-equivalent concentration, 100 mM C, of each substrate as a sole source of carbon: ferulate (FER), p-coumarate (COU), vanillate (VAN), 4-hydroxybenzoate (4HB) or succinate (SUC).

9. P18-Line 494: Italicize "attB".

Response: Revised (now **Lines 523**).

Reviewer #2

In this paper, Dr. Aristilde lab presented an outstanding work that use multi-omics investigation of Pseudomonas putida KT2440 grown on four common phenolic substrates. Via 13C labeling, MFA and genome scale modeling, the paper described cellular catabolism of nonmodel substrates. The knowledge has broad impacts and the study is highly innovative. I only have three minor comments.

Response: We greatly appreciated the highly positive feedback from the reviewer.

1. Metabolite extraction for analysis of intracellular metabolite levels and isotope labeling kinetics: The team used cell filtering to quench metabolism. During cell residence on filter paper, cell metabolism and fluxes may be changed which could affect flux outcome. The authors may address this concerns.

Response: We want to point out to that cell metabolism was not quenched by filtration, but by submerging the filter containing the cells immediately in a cold solvent mixture (methanol: acetonitrile:water), which lysed the cells. We revised the text to highlight this in the method (see **Lines 582-586**)

"Metabolism was quenched by submerging the filter containing the cells immediately in the cold solvent mixture, which lysed the cells; there was less than 1 min between the filtering process and the transfer of the filter to the cold solvent. This well-established protocol[83] for metabolite extraction after quenching metabolism was illustrated previously by the senior author[41,48,63,64,84,85] and others[86–88].

83. Bennett, B. D., Yuan, J., Kimball, E. H. & Rabinowitz, J. D. Absolute quantitation of intracellular metabolite concentrations by an isotope ratio-based approach. *Nat Protoc* **3**, 1299–1311 (2008).

41. Kukurugya, M. A. *et al.* Multi-omics analysis unravels a segregated metabolic flux network that tunes co-utilization of sugar and aromatic carbons in *Pseudomonas putida*. *J Biol Chem* **294**, 8464–8479 (2019).
48. Wilkes, R. A., Waldbauer, J. & Aristilde, L. Analogous metabolic decoupling in *Pseudomonas putida* and *Comamonas testosteroni* implies energetic bypass to facilitate gluconeogenic growth. *mBio* **12**, e03259-21 (2021).
63. Mendonca, C. M. *et al.* Hierarchical routing in carbon metabolism favors iron-scavenging strategy in iron-deficient soil *Pseudomonas* species. *Proc Natl Acad Sci USA* **117**, 32358–32369 (2020).
64. Wilkes, R. A. *et al.* Complex regulation in a *Comamonas* platform for diverse aromatic carbon metabolism. *Nat Chem Biol* 1–12 (2023) doi:10.1038/s41589-022-01237-7.
84. Aristilde, L. *et al.* Glyphosate-induced specific and widespread perturbations in the metabolome of soil *Pseudomonas* species. *Front Environ Sci* **5**, (2017).
85. Mendonca, C. M., Zhang, L., Waldbauer, J. R. & Aristilde, L. Disproportionate Carbon Dioxide Efflux in Bacterial Metabolic Pathways for Different Organic Substrates Leads to Variable Contribution to Carbon-Use Efficiency. *Environ. Sci. Technol.* **58**, 11041–11052 (2024).

86. Munger, J. *et al.* Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. *Nat Biotechnol* **26**, 1179–1186 (2008).
87. Park, J. O. *et al.* Metabolite concentrations, fluxes and free energies imply efficient enzyme usage. *Nat Chem Biol* **12**, 482–489 (2016).
88. Pisithkul, T. *et al.* Metabolic Remodeling during Biofilm Development of Bacillus subtilis. *mBio* **10**, 10.1128/mbio.00623-19 (2019).

2. A similar study has been done in Rhodococcus opacus (Metabolic Engineering, Volume 55, 2019, Pages 120-130). That paper also investigated the hierarchy of carbon source co-utilizations. Since lignocellulosic biomass contains sugars and aromatic substrates, authors may also look at cellular utilization of mixed substrates.

Response: As pointed out in **Lines 94-97** in the Introduction, the focus of the current study is on aromatic substrate catabolism, by investigating four different types of phenolic acids known to be derived from the lignin component of lignocellulose hydrolysates (see). The co-utilization of sugars and aromatic substrates was not part of the intended scope of the current study. However, cellular utilization of mixed substrates has been investigated by the research group of the senior author (Aristilde) in previous studies, which addressed the co-utilization of benzoate and glucose[3], ferulate and glucose[7], or *p*-coumarate and glucose[7].n *P. putida*.

3. Bacteria may use Water-forming NADH oxidase to remove NADH and generate heat. How this reaction impact flux modeling and conclusion?

Response: Indeed, water-forming NADH oxidase has been shown to alter bacterial metabolism in certain species, such as *Escherichia coli* and *Clostridium acetobutylicum*. For instance, overexpression of water-forming NADH oxidase increased the glucose consumption rate by 50% in aerobically grown *E. coli* [11] and decreased anaerobic fermentation product yields from glucose by up to 79% in *C. acetobutylicum* [12]. However, a previous study demonstrated that overproduction of a water-forming NADH oxidase had **minimal to no effect** on either glucose consumption rate or metabolic flux distribution in *P. putida* KT2440[13]. Therefore, we conclude that water-forming NADH oxidase activity was not likely to impact our flux modeling or conclusions, as we now stated in **Line 401-403**).
“Furthermore, water-forming NADH oxidase was not included in the cofactor balancing, based on a previous report of minimal to no effect on metabolic flux distribution in *P. putida* KT2440[42].”

11. Vemuri, G. N., Altman, E., Sangurdekar, D. P., Khodursky, A. B. & Eiteman, M. A. Overflow metabolism in Escherichia coli during steady-state growth: transcriptional regulation and effect of the redox ratio. *Appl Environ Microbiol* **72**, 3653–3661 (2006).
12. Shi, X.-C. *et al.* A water-forming NADH oxidase regulates metabolism in anaerobic fermentation. *Biotechnol Biofuels* **9**, 103 (2016).
13. Ebert, B. E., Kurth, F., Grund, M., Blank, L. M. & Schmid, A. Response of *Pseudomonas putida* KT2440 to Increased NADH and ATP Demand. *Appl Environ Microbiol* **77**, 6597–6605 (2011).
42. Ebert, B. E., Kurth, F., Grund, M., Blank, L. M. & Schmid, A. Response of *Pseudomonas putida* KT2440 to Increased NADH and ATP Demand. *Appl Environ Microbiol* **77**, 6597–6605 (2011).

Reviewer #3

This manuscript presents a comprehensive and well-structured study on the aromatic metabolism of Pseudomonas putida KT2440. By combining 13C-metabolic flux analysis, proteomics, intracellular metabolite profiling, and engineered strain comparisons, the authors offer a high-resolution view of how carbon and energy metabolism are coordinated during growth on different lignin-derived phenolic acids. The findings provide quantitative insights into pathway-specific rewiring, energy surplus, and cofactor balancing, which are highly relevant for future metabolic engineering and lignin valorization strategies.
The manuscript is of high quality, and the supporting data in the Supplementary Information are thorough and transparent, including detailed proteomic p-values, energy charge data, and flux distributions. Overall, the work is well executed and makes a strong contribution to the field.
Below are some suggestions aimed at further improving clarity, accessibility, and interpretation. These are minor in nature and should be straightforward to address.
Response: We greatly appreciated the highly positive feedback from the reviewer.

Comments
1. Referencing Supplementary Data in Main Text:
Please reference specific supplementary tables (e.g., Table S3 for proteomics statistics) in relevant figure captions or result descriptions for better data traceability.
Response: We went through the manuscript text and figure captions to ensure proper reference to specific supplementary tables.

2. Interpretation of ATP and NAD(P)H Surplus:
The manuscript clearly shows that certain substrates (FER, COU) yield high ATP and NAD(P)H production. A brief comment in the discussion on how this energetic surplus could support synthetic biochemistry (e.g., production of energy-intensive metabolites) would be beneficial.

Response: We added one sentence in the discussion to address how the benefits of cofactor surplus can be beneficial to overproduction in synthetic biology (see **Lines 452-456**).

Lines 452-456: “These findings inform rational design of metabolic engineering strategies, particularly when targeting fluxes through isoenzymes with similar catalytic functions but with distinct cofactor preferences. The surplus of NAD(P)H can benefit bioproduction of free fatty acids[66] and polyhydroxyalkanoates[67], while ATP overproduction can be employed in improving yield of biopolymers that require energy input, such as ε-Poly-L-lysine[68].”

66. Javidpour, P. *et al.* Biochemical and Structural Studies of NADH-Dependent FabG Used To Increase the Bacterial Production of Fatty Acids under Anaerobic Conditions. *Appl Environ Microbiol* **80**, 497–505 (2014).
67. Xu, Z. *et al.* Enhancement of polyhydroxyalkanoate production by co-feeding lignin derivatives with glycerol in Pseudomonas putida KT2440. *Biotechnol Biofuels* **14**, 11 (2021).
68. Yang, H. *et al.* Engineering Streptomyces albulus to enhance ε-poly-L-lysine production by introducing a polyphosphate kinase-mediated ATP regeneration system. *Microb Cell Fact* **22**, 51 (2023).

3. Clarification of TCA remodelling via Pyruvate Carboxylase:
The increased flux through pyruvate carboxylase is a key result. A short explanation linking this to CO_2 assimilation or oxaloacetate demand (e.g., for biosynthesis) would improve biological interpretation.

Response: Addressing the reviewer's suggestion, we added a short biological interpretation in the Discussion to link pyruvate carboxylase with oxaloacetate demand (see **Lines 461-465**).

"Notably, the enhanced carbon flux through malic enzyme was critical in sustaining the necessary NADPH yield, but resulted in decreasing flux to OAA through malate dehydrogenase. As a consequence, one important role of the anaplerotic carbon recycling to the TCA cycle via pyruvate carboxylase was to satisfy OAA demand for biosynthesis."

4. Engineered Strains and Energy Charge:
Reduced energy charge in some overexpression strains is an important observation. A brief note that this might result from metabolic burden, redox imbalance, or high expression cost would strengthen the interpretation. Mentioning future improvements such as RBS tuning or chromosomal integration would add practical value.
Response: We had a edited the interpretation of the reduced energy charge and added the suggestions from the reviewer (see **Lines 168-172**).

Lines 168-172: "In sum, alleviation of native metabolite buildups in initial substrate catabolism led to adverse change in the cellular energy charge, thereby implying an interplay between the regulation of phenolic carbon influx and cofactor balance and a potential energy burden caused by gene overexpression[50] "It remains to be investigated whether this metabolic burden could be overcome through alternative genetic engineering approaches such as tuning of ribosomal binding site or chromosomal integration."

5. Flux vs. Protein Abundance Divergence:
Figure 5E shows weak correlation between protein levels and metabolic flux. It would be useful to highlight 1–2 specific cases where flux remains high despite low protein expression, suggesting post-translational regulation or thermodynamic efficiency.

Response: To address the reviewer's comment, we specifically described the change of protein level and metabolic flux for the isocitrate dehydrogenase node (see **Lines 389-393**):

"For instance, although carbon flux through isocitrate dehydrogenase increased less than 5-fold to 8-fold during growth on the phenolic acids compared to SUC, there was less than 2-fold change in the corresponding enzyme abundance. Serine phosphorylation was previously implicated in modulating flux through isocitrate derhydrogenase in *P. putida* KT2440 grown on a glucose:benzoate mixture versus glucose alone[41]."

41. Kukurugya, M. A. *et al.* Multi-omics analysis unravels a segregated metabolic flux network that tunes co-utilization of sugar and aromatic carbons in *Pseudomonas putida*. *J Biol Chem* **294**, 8464–8479 (2019).

6. Abstract and Introduction Refinement:
The abstract is comprehensive but can be made more accessible by clearly separating the background, objective, methods, key findings, and implications.
Consider briefly reinforcing in the introduction why P. putida KT2440 is particularly well-suited for lignin-derived aromatic metabolism and the bow-tie architecture of metabolism in Pseudomonas species.

Response: Addressing the reviewer's suggestion, we revised the introduction to connect explicitly the suitability of *P. putida* KT2440 as a host for bioprocessing lignin-derived aromatics with its relevant metabolic pathways. (see **Lines 41-43**).

"Of particular interest is *Pseudomonas putida* KT2440, which is extensively explored as a chassis for biotechnology[7–11], including notably the bioconversion of lignin-derived aromatics[12,13]."

Regarding the abstract, we decided our original abstract, which was written in a manner to keep each method used to specific findings, rather than separating them, as we believe this enhances readers to understand how results were obtained. To adhere to the abstract word limit of Communication Biology, we kept the generalized implication sentence in our original abstract, but we have made effort as indicated above to expand on the implication of our study in the Discussion section.

7. Nikel, P. I. & de Lorenzo, V. *Pseudomonas putida* as a functional *chassis* for industrial biocatalysis: From native biochemistry to *trans*-metabolism. *Metab Eng* **50**, 142–155 (2018).
8. Schwanemann, T., Otto, M., Wierckx, N. & Wynands, B. *Pseudomonas* as Versatile Aromatics Cell Factory. *Biotechnol J* **15**, 1900569 (2020).
9. de Lorenzo, V., Pérez-Pantoja, D. & Nikel, P. I. *Pseudomonas putida* KT2440: the long journey of a soil-dweller to become a synthetic biology chassis. *J Bacteriol* **0**, e00136-24 (2024).
10. Silva, M., Donati, S. & Dvořák, P. Advances in engineering substrate scope of *Pseudomonas* cell factories. *Current Opinion in Biotechnology* **92**, 103270 (2025).
11. Kim, G. B., Kim, H. R. & Lee, S. Y. Comprehensive evaluation of the capacities of microbial cell factories. *Nat Commun* **16**, 2869 (2025).
12. Ravi, K., García-Hidalgo, J., Gorwa-Grauslund, M. F. & Lidén, G. Conversion of lignin model compounds by *Pseudomonas putida* KT2440 and isolates from compost. *Appl Microbiol Biotechnol* **101**, 5059–5070 (2017).
13. Wada, A. *et al.* Characterization of aromatic acid/proton symporters in *Pseudomonas putida* KT2440 toward efficient microbial conversion of lignin-related aromatics. *Metab Eng* **64**, 167–179 (2021).

7. Figure Caption Enhancements:
For figures presenting flux maps, energy charge, or kinetic labelling, consider including a brief biological interpretation or highlighting the main trend in the caption.
Response: We edited the figure captions of Fig. 2, Fig. 4 and Fig. 5 to include brief interpretations as the reviewer suggested (see below).

"Fig.2. Resolved bottleneck nodes in initial catabolism and unfavorable cellular energy state indicated by decreased energy charge values.

Fig.4. Kinetic ^{13}C-profiling and carbon mapping of metabolic flux partitioning revealed metabolic remodeling nodes in the central carbon metabolism.

Fig. 5. Quantitative flux analysis of the reaction network in phenolic acid metabolism revealed structure of the cataplerosis-anaplerosis nodes to sustain high flux in the TCA cycle."

8. Terminology Consistency:
Ensure consistent use of substrate abbreviations (e.g., FER, VAN), gene/protein names (e.g., vanAB, pobA, pcaHG), and pathway terms (e.g., anaplerosis, cataplerosis).
Response: We went through the whole text to ensure the terminology was consistent.

9. Spelling and Grammar Corrections:
A final proofreading pass to correct minor typographical issues (e.g., “fluzomics” → “fluxomics”, “ene:gy” → “energy”) and ensure grammatical consistency is recommended.

Response: We did careful proofreading to correct the typographical issues.

10. Quantitative proteomics sampling procedure:
P18, line 513-518: since the protein changes are happening in the order of 8-10 minutes, one would expect quenching followed by washing steps. Here done in a reverse manner. Please clarify regarding the procedure in the revised MS text and appropriate literature reference.

Response: For the proteomics sampling procedure, we followed an established protocol shared by the core facility that performed the proteomics [14]. To minimize the protein changes happening during the sample processing, we minimized the time in cell washing to be within 10 min, and kept samples on ice all the time. We have now added the details of this procedure and reference into the revised MS text (see **Lines 544-547**).

“After centrifugation of 2-mL cultures (10000 *g* for 5 min at 4 °C), the cell pellets were washed twice with phosphate-buffered solution (pH = 7.4) within 10 min to remove extracellular components followed by quenching with cold methanol (4 °C) and incubated on ice for 30 min following a previously established protocol[78]. Samples were kept on ice to minimize protein change during the processing.”

78. Elsayyid, M., Tanis, J. E. & Yu, Y. Simple In-Cell Processing Enables Deep Proteome Analysis of Low-Input Caenorhabditis elegans. *Anal. Chem.* **97**, 9159–9167 (2025).

References

1. Bennett, B. D., Yuan, J., Kimball, E. H. & Rabinowitz, J. D. Absolute quantitation of intracellular metabolite concentrations by an isotope ratio-based approach. *Nat Protoc* **3**, 1299–1311 (2008).
2. Aristilde, L. *et al.* Glyphosate-induced specific and widespread perturbations in the metabolome of soil *Pseudomonas* species. *Front Environ Sci* **5**, (2017).
3. Kukurugya, M. A. *et al.* Multi-omics analysis unravels a segregated metabolic flux network that tunes co-utilization of sugar and aromatic carbons in *Pseudomonas putida*. *J Biol Chem* **294**, 8464–8479 (2019).
4. Mendonca, C. M. *et al.* Hierarchical routing in carbon metabolism favors iron-scavenging strategy in iron-deficient soil *Pseudomonas* species. *Proc Natl Acad Sci USA* **117**, 32358–32369 (2020).
5. Wilkes, R. A., Waldbauer, J. & Aristilde, L. Analogous metabolic decoupling in *Pseudomonas putida* and *Comamonas testosteroni* implies energetic bypass to facilitate gluconeogenic growth. *mBio* **12**, e03259-21 (2021).
6. Wilkes, R. A. *et al.* Complex regulation in a *Comamonas* platform for diverse aromatic carbon metabolism. *Nat Chem Biol* 1–12 (2023) doi:10.1038/s41589-022-01237-7.
7. Mendonca, C. M., Zhang, L., Waldbauer, J. R. & Aristilde, L. Disproportionate Carbon Dioxide Efflux in Bacterial Metabolic Pathways for Different Organic Substrates Leads to Variable Contribution to Carbon-Use Efficiency. *Environ. Sci. Technol.* **58**, 11041–11052 (2024).
8. Munger, J. *et al.* Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. *Nat Biotechnol* **26**, 1179–1186 (2008).
9. Park, J. O. *et al.* Metabolite concentrations, fluxes and free energies imply efficient enzyme usage. *Nat Chem Biol* **12**, 482–489 (2016).
10. Pisithkul, T. *et al.* Metabolic Remodeling during Biofilm Development of Bacillus subtilis. *mBio* **10**, 10.1128/mbio.00623-19 (2019).
11. Vemuri, G. N., Altman, E., Sangurdekar, D. P., Khodursky, A. B. & Eiteman, M. A. Overflow metabolism in Escherichia coli during steady-state growth: transcriptional regulation and effect of the redox ratio. *Appl Environ Microbiol* **72**, 3653–3661 (2006).
12. Shi, X.-C. *et al.* A water-forming NADH oxidase regulates metabolism in anaerobic fermentation. *Biotechnol Biofuels* **9**, 103 (2016).
13. Ebert, B. E., Kurth, F., Grund, M., Blank, L. M. & Schmid, A. Response of *Pseudomonas putida* KT2440 to Increased NADH and ATP Demand. *Appl Environ Microbiol* **77**, 6597–6605 (2011).
14. Elsayyid, M., Tanis, J. E. & Yu, Y. Simple In-Cell Processing Enables Deep Proteome Analysis of Low-Input Caenorhabditis elegans. *Anal. Chem.* **97**, 9159–9167 (2025).