nature portfolio

Peer Review File

Biosynthesis of pyomelanin from methanol with engineered Komagataella phaffii and its characterizations

Corresponding Author: Professor Ying Lin

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)
The authors describe a strategy to enhance the biosynthetic yield of pyomelanin—a key raw material used in pharmaceuticals, cosmetics, bioremediation, and energy applications. To improve flux through the pathway, they engineered DAHPS and HPPD, two rate-limiting enzymes, thereby boosting overall production. The final strain achieved the highest pyomelanin titer reported to date. Collectively, the study presents a compelling, stepwise optimization of the production process. The data are robust and the conclusions well supported. Nevertheless, the manuscript still has several shortcomings, detailed below.
Lines 168-171: The manuscript states that the AfHPPD PDB structure from YASARA was used for structural analysis, but this is ambiguous. No experimental structure of AfHPPD has been deposited, so the authors presumably built a homology model. Clarify whether YASARA was used only for visualisation or also for modelling. If the latter, explain why an additional model was generated with MODELLER and AlphaFold2 (lines 417–418). For the AlphaFold2 model, report standard quality metrics (pLDDT, pTM, RMSD to template, etc.) instead of only the Ramachandran plot.
Lines 417-418: Crystal structure of PfHPPD was used as the template for modeling, yet the manuscript offers no justification for this choice. A sequence-alignment comparison of AfHPPD against all HPPD with available crystal structures should be presented to demonstrate that PfHPPD is the most appropriate template (highest identity, best coverage, conserved active-site geometry). Likewise, the final downstream analyses must specify which AfHPPD model (YASARA, MODELLER, or AlphaFold2) was adopted and provide the selection rationale.
Lines 462-464: The authors attribute the improved HGA production to a 0.03-nm decrease in the F338–4-HPPA distance in the variant. This displacement is within the coordinate error of a homology model and is indistinguishable from thermal noise; it is therefore insufficient grounds for claiming enhanced catalysis. In addition, the manuscript states that S224G acts as a “structural bridge,” but never defines what is being bridged or how glycine—lacking a side chain—could fulfill such a role. Provide (i) ensemble docking or MD-derived distance distributions to show the 0.03-nm shift is statistically significant, and (ii) a clear mechanistic explanation of the proposed S224G bridging function, supported by hydrogen-bond maps, etc.
Lines 467-472: The authors assert that a higher RMSD does not imply greater instability for the variant, yet lines 398–401 equate lower RMSD with enhanced structural stability. This is internally inconsistent. Clarify whether RMSD is being used as a proxy for stability in this study; if not, remove the stability claim. If it is, provide evidence—e.g., radius-of-gyration trends, hydrogen-bond counts, or free-energy calculations—to demonstrate that the variant’s increased RMSD does not compromise thermostability.
Figure 2f: Did the authors test the effect of Mn2+? It would be better if the result of Mn2+ was added.
Other minor issues:
1. ‘de novo’ should be italic. Check this thoroughly.
2. Line27, further synthesis of pyomelanin.
3. Line81: constructed
4. The legend of supplementary fig.6, the nine mutated enzymes were not from Komagataella phaffii.

Reviewer #2

(Remarks to the Author)
What are the noteworthy results?
This study engineered Komagataella phaffii for de novo pyomelanin production, achieving a titer of 70.5 g/L. Key advances

include modular pathway optimization, establishment of a color-based screening system, and use of directed evolution and semi-rational design for enzyme improvement.

Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.
This paper reports a titer of 70.5 g/L, representing a significant improvement in pyomelanin production than previously published values. Larroude et al. (doi: 10.3390/microorganisms9040838) reported a pyomelanin titer of 4.5 g/L, Lorquin et al. (https://doi.org/10.1038/s41598-021-87328-2) reported 0.55 g of pyomelanin per liter of medium, and Koch et al. (https://doi.org/10.3389/fmicb.2023.1233740) reported 1.48 g of pyomelanin per liter of medium. The later paper also comparing titer/yield of pyomelanin production in different organisms by different approaches. Thus, this work demonstrates a significant improvement in terms of pyomelanin titers. However, important information on the process are missing to obtain a complete picture of the fermentation process. First of all the description of the fermentation L254 ff. (methods) and L567 ff. (results) is not well described. How was glycerol and methanol fed and which feeding profile was used? How much methanol and glycerol was actually consumed?

Does the work support the conclusions and claims, or is additional evidence needed?
Additional evidence is needed.
The title states “de novo biosynthesis of pyomelanin from methanol,” but the engineered K. phaffii strains actually produce homogentisic acid (HGA) as the final metabolic product. Pyomelanin formation occurs non-enzymatically via oxidation/polymerization of HGA or enzymatically after laccase addition, rather than through an intracellular biosynthetic pathway.
The reported value of 70.5 g/L refers to the titer, not the yield (lines 33 and 95). Titer represents the final concentration of product in the culture medium (often as grams per liter), while yield describes the efficiency of conversion in reference to a given substrate (here: yield on methanol or total carbon source would be appropriate). Additional data on carbon input, substrate consumption, and productivity are needed to support the claims. How reproducible is this fermentation in the 5L setup?
Purity of the reported pyomelanin is also not certain. Should pyomelanin contain N in its structure? Nitrogen detected in the dry weight suggests possible contamination with secreted proteins, meaning not all measured mass is pure pigment.

Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?
Yes, this paper needs revision. It lacks sufficient quantitative and methodological detail to fully support its conclusions.
The authors should clarify how 70 g/L was achieved, meaning what carbon sources were used, feeding rates of methanol and glycerol, and the carbon conversion ratio.
Productivity (g L^{-1} h^{-1}) and specific yield (for example grams of pyomelanin per grams of cell dry weight) are missing and should be compared with literature values.
Some results (for example the reported 6.3 % increase in HGA, lines 548 and 549) don’t have evidence of statistical significance.
OD_{400} readings are presented without justification that the signal detected is only pyomelanin.
Figure 2 and related text imply inconsistent effects of the addition of copper ions, which may both enhance HPPD activity and promote non-enzymatic oxidation of HGA. This needs clarification.
It would be interesting to see the comparison of the native HPPD enzyme from Komagataella phaffii to the engineered one from this paper.
The English in general needs more revision to be clearer and gramatically correct.
Given these issues, the work needs significant revision before publication.

Is the methodology sound? Does the work meet the expected standards in your field?
Not fully. The experimental concept is interesting and the modular metabolic engineering strategy is promising, but methodological details are missing.
There is no clear genetic characterization of strains in this work. Number of integrated copies of some genes is mentioned (lines 338, 499, 501, 547, 700), but there is no information on the way that was achieved.
No plasmid sequences are provided in the supplementary material.
The lack of OD measurements during inoculation (lines 238 and 240), the vague description of adding copper “as needed” (lines 262 and 263) and “pyomelanin solutions of varying concentrations” (lines 291 and 292) make replication and data interpretation difficult.

Is there enough detail provided in the methods for the work to be reproduced?
No. A lot of experimental details are missing or too vague to reproduce, as mentioned above.

Reviewer #4

(Remarks to the Author)
The manuscript “De novo biosynthesis of pyomelanin from methanol with engineered Komagataella phaffii and its characterizations” presents several interesting aspects, such as the production of pyomelanin in a yeast strain, the directed evolution of enzymes to improve biosynthetic processes, the metabolic push-pull strategy and manipulation of K. phaffii for pyomelanin production.
However, we identified several major issues that need to be addressed:
The main observation that I have is that the pigment being produced, pyomelanin, is widely distributed among bacterial species, like those of the genus Pseudomonas, among others. It is not clear what the advantage would be of producing

pyomelanin in a eukaryotic organism. On one hand, achieving high pigment production requires a rather complex fed-batch fermentation scheme, which is not inexpensive. Although methanol is a low-cost substrate, bacteria can utilize waste compounds from agro-industrial or fuel industries, for example. If the advantage lies in cost, this point has not been thoroughly discussed. Moreover, the fermentation lasts five days (example in line 205), which is quite long, and no comparison is made to yields obtained in other studies using fast-growing bacterial producers. In the Results section (line 320), no measurement uncertainty or deviation is indicated, which prevents assessing reproducibility. Considering the importance of genetic stability in organisms intended for biotechnological use, it would be highly relevant to discuss the stability of this yeast strain, which carries multiple genetic modifications, even the use of Crispr-Cas9 can lead to off-targets editions that should be analyzed. More importantly, while the reported yield of 1.65 g/L is high, productivity (yield per unit time) is not expressed, so it is unclear whether this microorganism is actually a better producer than bacterial systems, either natural producers or recombinant, fast growing strains. For example, line 573 states that fermentation was completed after 168 hours (seven days), yet this aspect is not discussed at all.
Regarding the chemical characterization, we found significant issues:
First, in the M-M section (line 210), the purification method described appears to yield poorly purified material, potentially containing impurities such as proteins and polysaccharides. These contaminants could have influenced some of the reported scavenging results. Moreover, the reported production values might be highly overestimated due to this purification protocol.
On the other hand, all experiments should have included, at least as a control, the best-characterized pyomelanin from Pseudomonas aeruginosa ΔhmgA. While it is true that commercial melanin from Sigma (derived from alkaline treatment of tyrosine) is available, the authors could have used P. aeruginosa ΔhmgA pyomelanin as reference, which has been well characterized by several research groups over time.
Furthermore, in the M-M (lines 269 and 290), in the antioxidant and cell assays, it is not stated how the pyomelanin solutions were prepared, given that the natural pyomelanin is insoluble in water, buffer, or ethanol therefore is crucial to understand how the experiments were made. Additionally, there is a lack of controls with pyomelanins from bacterial. Antioxidant, cosmetic, and UV-protective properties of pyomelanin have already been extensively described in the literature, so many of the authors' statements overlook the substantial existing body of work (lines 623–627). The characterization relies mainly on IR spectroscopy, which provides limited structural information for polymers as complex and heterogeneous as melanins. Even for pyomelanin, key structural differences have been revealed primarily by NMR analysis. Therefore, the claims made in lines 607–609 cannot supported by IR data alone.

In line 629, the authors discuss the use of pyomelanin for electrode fabrication in sodium-ion batteries. However, it is unclear what the advantage would be of using such an expensive compound for this purpose. Without a clear rationale, this part of the study lacks relevance. There are numerous inexpensive precursors for synthesizing hard carbon anodes for SIBs, including agricultural biomass/waste (lignin included), industrial pitch, and waste plastics (e.g., PET), all of which have demonstrated competitive performance (DOI: 10.1016/j.jechem.2018.01.025; DOI: 10.1038/s41467-023-39637-5; DOI: 10.1039/C5TA08601A).
Finally, the Discussion contains numerous claims about the advantages and novelty of the study while overlooking prior research that had already addressed many of these points. For example, the comparison between laccase-synthesized and chemically synthesized pyomelanins (under alkaline conditions) has already been thoroughly studied, including NMR analysis, as reported in Lorquin et al., Scientific Reports (2021): 10.1038/s41598-021-87328-2. Given that this work is even cited by the authors themselves in the present manuscript, the claimed novelty is practically null and contradictory in this regard. Lorquin's study provides a complete structural analysis with NMR data.
Lastly, the statement that this melanin could serve as a reference pyomelanin standard is not well justified, since naturally producing bacterial strains already exist and are routinely used as reference sources in various studies.
Some important publications:
Rodríguez-Rojas A, Mena A, Martín S, Borrell N, Oliver A, Blázquez J. Inactivation of the hmgA gene of Pseudomonas aeruginosa leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. Microbiology (Reading). 2009 Apr;155(Pt 4):1050-1057. doi: 10.1099/mic.0.024745-0. PMID: 19332807.
Lorquin, F., Ziarelli, F., Amouric, A. et al. Production and properties of non-cytotoxic pyomelanin by laccase and comparison to bacterial and synthetic pigments. Sci Rep 11, 8538 (2021). https://doi.org/10.1038/s41598-021-87328-2
Lorquin F, Piccerelle P, Orneto C, Robin M, Lorquin J. New insights and advances on pyomelanin production: from microbial synthesis to applications. J Ind Microbiol Biotechnol. 2022 Jul 30;49(4):kuac013. doi: 10.1093/jimb/kuac013. PMID: 35482661; PMCID: PMC9338888.
Diaz Appella MN, Kolender A, Oppezzo OJ, López NI, Tribelli PM. The structural complexity of pyomelanin impacts UV shielding in Pseudomonas species with different lifestyles. FEBS Lett. 2024 Nov;598(21):2702-2716. doi: 10.1002/1873-3468.15000. Epub 2024 Aug 16. PMID: 39152523.
Urbaniak MM, Gazińska M, Rudnicka K, Płociński P, Nowak M, Chmiela M. In Vitro and In Vivo Biocompatibility of Natural and Synthetic Pseudomonas aeruginosa Pyomelanin for Potential Biomedical Applications. Int J Mol Sci. 2023 Apr 25;24(9):7846. doi: 10.3390/ijms24097846. PMID: 37175552; PMCID: PMC10178424.
Ben Tahar I, Kus-Liśkiewicz M, Lara Y, Javaux E, Fickers P. Characterization of a nontoxic pyomelanin pigment produced by the yeast Yarrowia lipolytica. Biotechnol Prog. 2020 Mar;36(2):e2912. doi: 10.1002/btpr.2912. Epub 2019 Nov 4. PMID: 31525285.
Al Khatib M, Costa J, Spinelli D, Capecchi E, Saladino R, Baratto MC, Pogni R. Homogentisic Acid and Gentisic Acid Biosynthesized Pyomelanin Mimics: Structural Characterization and Antioxidant Activity. Int J Mol Sci. 2021 Feb 9;22(4):1739. doi: 10.3390/ijms22041739. PMID: 33572316; PMCID: PMC7916096.
Bayram S. Production, purification, and characterization of Streptomyces sp. strain MPPS2 extracellular pyomelanin pigment. Arch Microbiol. 2021 Sep;203(7):4419-4426. doi: 10.1007/s00203-021-02437-w. Epub 2021 Jun 14. PMID: 34128104.
Urbaniak MM, Gazińska M, Rudnicka K, Płociński P, Nowak M, Chmiela M. In Vitro and In Vivo Biocompatibility of Natural

and Synthetic Pseudomonas aeruginosa Pyomelanin for Potential Biomedical Applications. Int J Mol Sci. 2023 Apr 25;24(9):7846. doi: 10.3390/ijms24097846. PMID: 37175552; PMCID: PMC10178424.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have almost addressed all the issues. One remaining major issue: In response figure 2d, Mn2+ promoted nonenzymatic oxidation of HGA. Why in response figure 2b, no nonenzymatic oxidation phenonmenon was observed in the presence of Mn2+.

Another minor issue in supplementary figure 6: for the sequence alignment, the name for each sequence should be clear. In the present version, I don't know which sequence they used.

Reviewer #2

(Remarks to the Author)
The authors carefully revised the manuscript, clarified the methodology, improved the representation of their data and corrected some inconsistencies. Thus, the manuscript appears ready for publication.

Reviewer #4

(Remarks to the Author)
The manuscript has been substantially improved and is now supported by a much stronger scientific framework. The addition of key structural experiments significantly strengthens the main conclusions, and the discussion is more rigorous and better integrated with previously published data from multiple independent groups.
The purification of the material, however, remains inherently complex: although the nitrogen content is reported to be approximately 5%, the nitrogen-containing components may differ in molecular mass, and their contribution to the final purified melanin preparation is therefore not necessarily linear. However, this limitation reflects a broader methodological challenge in the field rather than a specific weakness of this study. Overall, and within my area of expertise all our questions were addressed.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

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

Responses to four reviewers' comments (NCOMMS-25-76159A)

Dear Reviewers

We appreciate very much all your constructive comments and suggestions, which definitely gave us helpful guidance to improve our manuscript. We have now revised our manuscript accordingly, and for your reference, the changes in the revised manuscript are highlighted in red. We have also provided a point-by-point response (below) with detailed explanations. We are willing to make further changes upon your request.

Responses to Reviewer 1

Reviewer #1 (Remarks to the Author):

The authors describe a strategy to enhance the biosynthetic yield of pyomelanin—a key raw material used in pharmaceuticals, cosmetics, bioremediation, and energy applications. To improve flux through the pathway, they engineered DAHPS and HPPD, two rate-limiting enzymes, thereby boosting overall production. The final strain achieved the highest pyomelanin titer reported to date. Collectively, the study presents a compelling, stepwise optimization of the production process. The data are robust and the conclusions well supported. Nevertheless, the manuscript still has several shortcomings, detailed below.

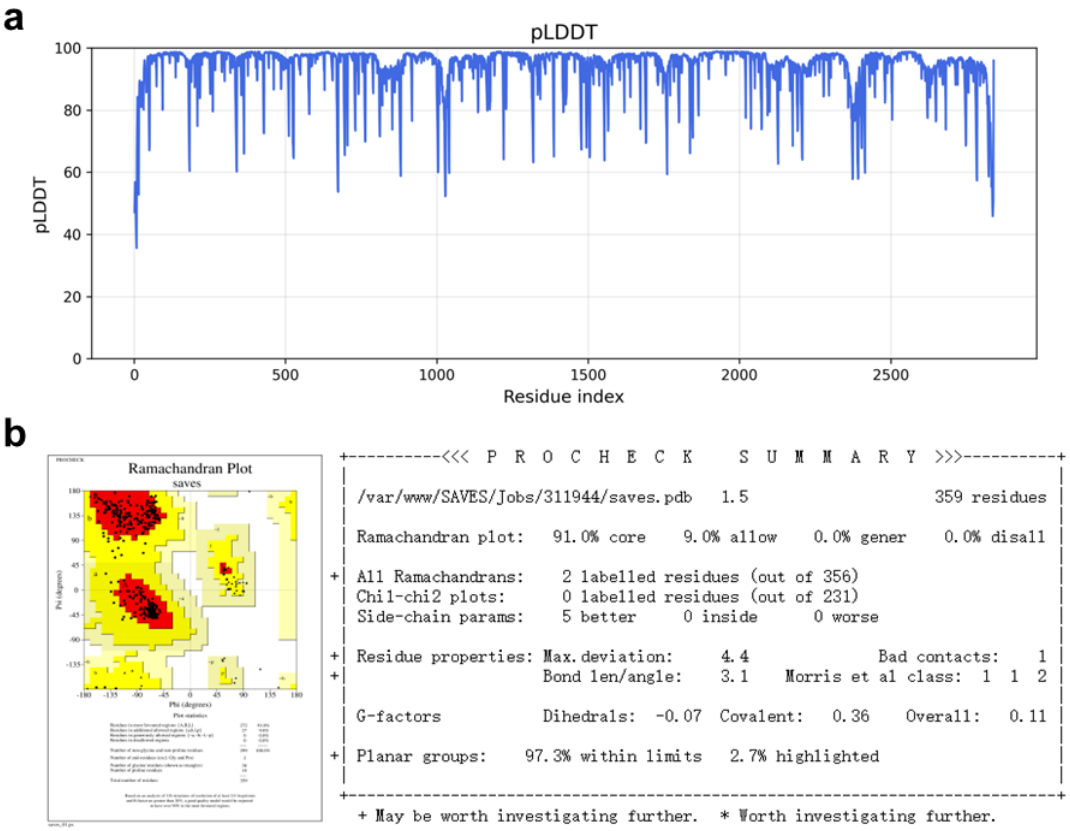
Response: Thank you for the positive comments. We have now revised the manuscript according to your suggestions.

Lines 168-171: The manuscript states that the *Af*HPPD PDB structure from YASARA was used for structural analysis, but this is ambiguous. No experimental structure of *Af*HPPD has been deposited, so the authors presumably built a homology model. Clarify whether YASARA was used only for visualization or also for modelling. If the latter, explain why an additional model was generated with MODELLER and AlphaFold2 (lines 417–418). For the AlphaFold2 model, report standard quality metrics

(pLDDT, pTM, RMSD to template, etc.) instead of only the Ramachandran plot.

Response: Thank you for the helpful comments. We apologize for the confusing descriptions. We would like to indicate that YASARA was used only for visualization, not for modelling. The revised description is shown below. In addition, we have now provided the standard quality metrics of AlphaFold2 model in the revised supplementary materials and here as well (below).

Lines 449-452: "First, since the crystal structure of *Af*HPPD was not yet available, we used AlphaFold2 for modelling. The reliability of the model was demonstrated by standard quality metrics and a Ramachandran plot (Supplementary Fig. 5)."

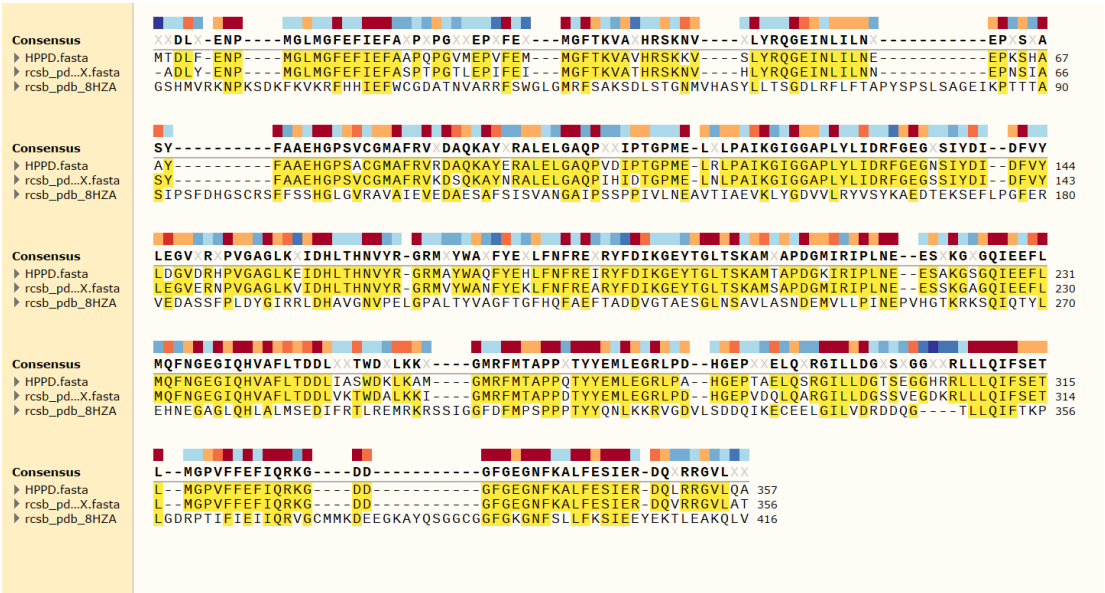


Supplementary Fig. 5. Reliability analysis of AlphaFold2 modelling. a Standard quality metrics of AlphaFold2 modelling based on atom-plddts. pTM = 0.93. RMSD = 0.325. b Ramachandran plot of the structure of *Af*HPPD based on AlphaFold2, showing that in 3-dimensional/1-dimensional analysis, at least 80% of the amino acid scores are equal to or higher than 0.1, and more than 90% of the amino acids are detected in appropriate positions.

Lines 417-418: Crystal structure of *Pf*HPPD was used as the template for modeling, yet the manuscript offers no justification for this choice. A sequence-alignment comparison of *Af*HPPD against all HPPD with available crystal structures should be presented to demonstrate that *Pf*HPPD is the most appropriate template (highest identity, best coverage, conserved active-site geometry). Likewise, the final downstream analyses must specify which *Af*HPPD model (YASARA, MODELLER, or AlphaFold2) was adopted and provide the selection rationale.

Response: Thank you for the helpful comments. As suggested, we have now revised the manuscript (below) accordingly. In addition, we have added an additional figure (Supplementary Fig. 6) in the revised supplementary materials to demonstrate that *Pf*HPPD is the most appropriate template.

Lines 452-460: "To investigate whether the model generated by AlphaFold2 can be used for further modification, we used Modeller for homology modelling. A template search using SWISS-MODEL identified only two HPPD crystal structures that satisfy the sequence similarity and coverage criteria for *Af*HPPD modelling: *Pf*HPPD (*Pseudomonas fluorescens*, PDB: 1cjx) and *At*HPPD (*Arabidopsis thaliana*, PDB: 8hza). The sequence alignment results revealed that compared with *At*HPPD, *Pf*HPPD is more suitable as a template (Supplementary Fig. 6). The model established by Modeller is similar to the model generated by AlphaFold2. Therefore, the model established by AlphaFold2 was used for semirational design."



Comparison with *Af*HPPD	Coverage (%)	Identify (%)	Similarity (%)
*Pf*HPPD	99	84.27	91.29
*At*HPPD	97	28.10	45.10

HPPD structure	Binding site with metal ion
*Af*HPPD	H162-H241-E323
*Pf*HPPD	H161-H240-E322
*At*HPPD	H226-H308-E394

Supplementary Fig. 6. Sequence alignment of *Pf*HPPD, *At*HPPD, and *Af*HPPD. Compared to *At*HPPD, *Pf*HPPD has a higher identify and similarity with *Af*HPPD. For the active center, the important metal binding domains are analyzed. *Pf*HPPD and *At*HPPD bind Fe^{2+}, while *Af*HPPD bind Cu^{2+}. They are all composed of H-H-E motifs.

Lines 462-464: The authors attribute the improved HGA production to a 0.03-nm decrease in the F338–4-HPPA distance in the variant. This displacement is within the coordinate error of a homology model and is indistinguishable from thermal noise; it is therefore insufficient grounds for claiming enhanced catalysis. In addition, the manuscript states that S224G acts as a "structural bridge," but never defines what is being bridged or how glycine—lacking a side chain—could fulfill such a role. Provide (i) ensemble docking or MD-derived distance distributions to show the 0.03-nm shift is statistically significant, and (ii) a clear mechanistic explanation of the proposed S224G bridging function, supported by hydrogen-bond maps, etc.

Response: Thank you for the helpful comments. We have now conducted a significance test on the distance distribution exported by MD. The results showed that the change in distance distribution of mutant F338-4-HPPA was not significant. Therefore, we deleted the relevant descriptions in the revised manuscript. The modified figure is also shown below. In addition, we apologize for the inappropriate description of the S224G acting as a "structural bridge." We have now revised relevant description (below).

Lines 496-500: “The molecular dynamics simulation results showed that the root mean square fluctuation (RMSF) of the S224G and E314Y enzymes was greater than that of the WT enzyme (Fig. 4g). This revealed an increase in flexibility near the mutant, which may be related to the increase in the enzymatic activity.”

Lines 467-472: The authors assert that a higher RMSD does not imply greater instability for the variant, yet lines 398–401 equate lower RMSD with enhanced structural stability. This is internally inconsistent. Clarify whether RMSD is being used as a proxy for stability in this study; if not, remove the stability claim. If it is, provide evidence—e.g., radius-of-gyration trends, hydrogen-bond counts, or free-energy calculations—to demonstrate that the variant’s increased RMSD does not compromise thermostability.

Response: Thank you for the helpful comments. We are sorry for the inappropriate description. Yes, RMSD is being used as a proxy for stability in this study. However, this is conflicting with the results demonstrated in *in vitro* stability experiments. To further investigate the cause of this discrepency, we supplemented molecular dynamics simulations at temperature of 350 K. The revised manuscript and figure are shown below. In addition, we demonstrated the radius-of-gyration trends of E314Y/S224G and WT enzyme at 300 K and 350 K as evidence of improved thermal stability (Response Figure 1).

Lines 500-506: “In the analysis of the mechanism underlying the improvement in thermal stability, the RMSD of E314Y/S224G was greater than that of the WT enzyme at 300 K (Fig. 4h), which is contrary to the results of the in vitro stability experiments. To further investigate this, we performed molecular dynamics simulations at 350 K, and the results revealed that the RMSD of the E314Y/S224G enzyme were smaller than those of the WT enzyme (Fig. 4i). This explains why E314Y/S224G has better thermal stability than the WT enzyme does.”

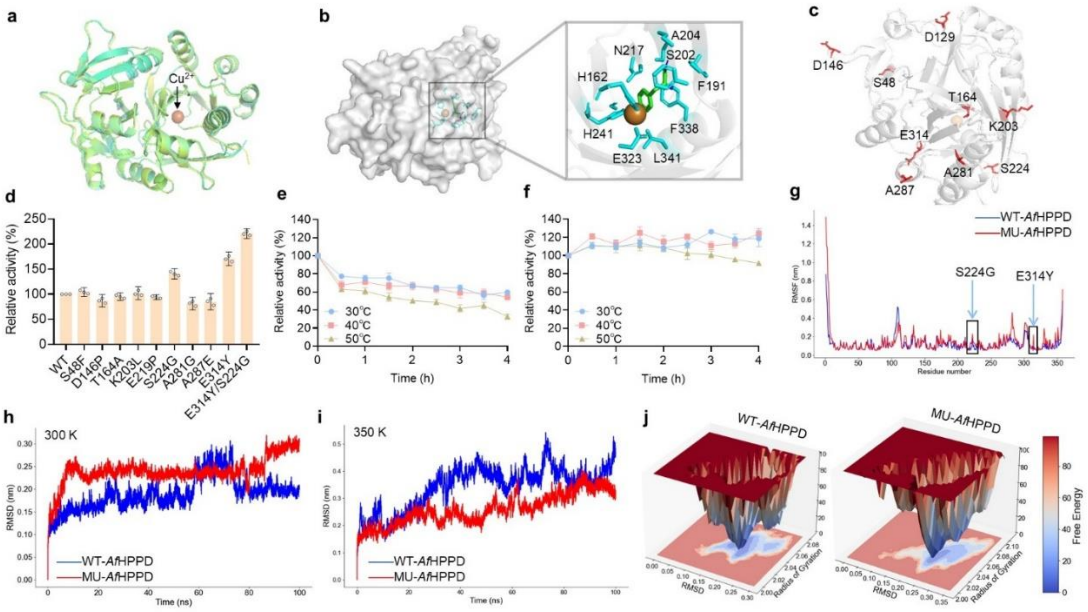
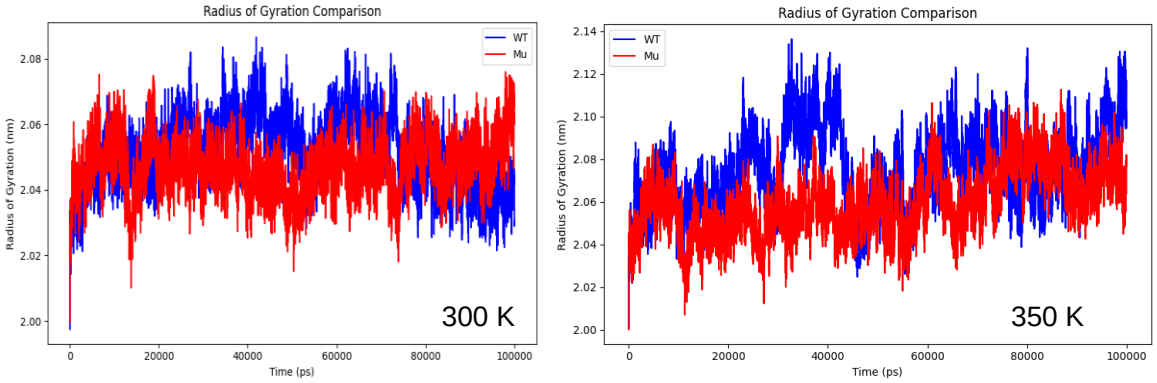


Fig. 4 Semi rational design and modification of *Af*HPPD enzyme. a Structural modeling of *Af*HPPD. The HPPD of *Pseudomonas fluorescens* is represented in green, which serves as the template chain (PDB: 1CJX); the *Af*HPPD structure predicted by AlphaFold 2 is shown in yellow; and the *Af*HPPD structure obtained from homology modeling was shown in blue. b Complex of *Af*HPPD and substrate 4-HPPA based on molecular docking. c Schematic diagrams of 9 different mutation sites obtained under two semirational design strategies. d Enzyme activity assay of different variants. e and f Thermal stability assay of the wild type (WT) and variant E314Y/S224G at three different temperatures. g. RMSF analyses of variant E314Y/S224G at 300 K. The WT and the variant E314Y/S224G are represented in blue and red, respectively. h RMSD analyses of variant E314Y/S224G at 300 K. The WT and the variant E314Y/S224G are represented in blue and red, respectively. i RMSD analyses of variant E314Y/S224G at 350 K. The WT and the variant E314Y/S224G are represented in blue and red, respectively. j 3D free energy landscape (FEL) diagram. Data are means of 3 biological replicates with error bars representing standard deviations.



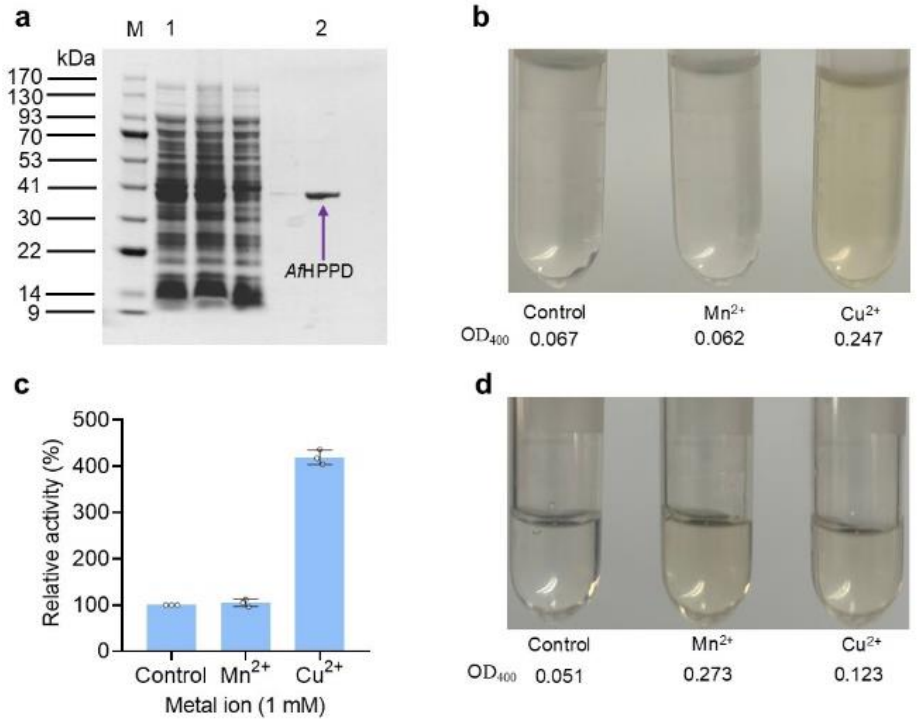
Response Figure 1. Molecular dynamics simulations for *Af*HPPD and *Af*HPPD$^{E314Y/S224G}$, showing the radius of gyration (Rg) at 300 K (left) and 350 K (right).

Figure 2f: Did the authors test the effect of Mn^{2+}? It would be better if the result of Mn^{2+} was added.

Response: Thank you for the helpful comments. No, we did not test the effectiveness of Mn^{2+}. As suggested, we have now tested the effect of Mn^{2+} on the catalytic activity of *Af*HPPD enzyme. The experimental details are as follows:

*Af*HPPD enzyme was purified again and established two standard enzyme reaction systems containing Cu^{2+} and Mn^{2+} (Response Figure 2a). A reaction system without metal ion served as the control, and the amount of HGA produced after completion was used as the benchmark for relative enzyme activity. The results showed that only the reaction system containing Cu^{2+} exhibited a noticeable color change (Response Figure 2b), indicating higher HGA production, whereas Mn^{2+} had little to no effect on *Af*HPPD activity (Response Figure 2c). However, it was suspected that the above response may not fully solve this problem. It has been reported that the addition of Mn^{2+} enhances the production of pyomelanin. For example, Zhang et al. demonstrated that the addition of Mn^{2+} increased the yield of pyomelanin (J. Biosci. Bioeng., 2023, 135, 93-101). Therefore, we further conducted additional experiments to solve this problem. The experimental details are as follows: HGA standard powder (Aladdin, Shanghai, China) was prepared into a water solution with equal amounts of Mn^{2+} and Cu^{2+} added. After shaking for 24 h, the solution with added Mn^{2+} and Cu^{2+} showed a color change, appearing brownish yellow, with an increase in OD_{400} compared to the control

(Response Figure 2d). These results indicated that Mn^{2+} and Cu^{2+} could promote nonenzymatic oxidation of HGA, and the efficiency of Mn^{2+} is higher than that of Cu^{2+}. In summary, Cu^{2+} have a dual function of promoting nonenzymatic oxidation of HGA and enhancing the catalytic activity of *Af*HPPD enzyme, while Mn^{2+} can only promote nonenzymatic oxidation of HGA.



Response Figure 2 Comparison of the effects of Mn^{2+} and Cu^{2+} on *Af*HPPD enzyme activity and HGA oxidative polymerization. a SDS-PAGE analysis of *Af*HPPD protein. Lane M, the protein marker; lane 1, whole cell protein supernatant; lane 2, the purified *Af*HPPD protein. b Enzyme reaction system containing Cu^{2+} exhibits the darkest color after the reaction is completed. c Effect of Mn^{2+} and Cu^{2+} on *Af*HPPD enzyme activity. d Effect of Mn^{2+} and Cu^{2+} on promoting nonenzymatic oxidation of HGA.

Other minor issues:

Response: Thanks so much for reminding us of these issues. We have now carefully checked the whole manuscript and corrected these issues accordingly.

1. 'de novo' should be italic. Check this thoroughly.

Response: Revised as suggested in our manuscript.

2. Line27, further synthesis of pyomelanin.

Response: As suggested, the "systhesis" has been corrected to "synthesis" in our revised manuscript.

3. Line81: constructed

Response: As suggested, the "constucted" has been corrected to "constructed" in our revised manuscript.

4. The legend of supplementary fig.6, the nine mutated enzymes were not from *Komagataella phaffii*.

Response: We apologize for this careless error, which is now corrected from "*Komagataella phaffii*" to "*Alcaligenes faealis*".

Responses to Reviewer 2

Reviewer #2 (Remarks to the Author):

What are the noteworthy results?

This study engineered *Komagataella phaffii* for de novo pyomelanin production, achieving a titer of 70.5 g/L. Key advances include modular pathway optimization, establishment of a color-based screening system, and use of directed evolution and semi-rational design for enzyme improvement.

Response: Thank you for the positive comments on our manuscript. We have now provided a point-by-point response to address your concerns. We apologize for our confusing description on experimental details, and we have now carefully revised these descriptions in the revised manuscript.

Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.

This paper reports a titer of 70.5 g/L, representing a significant improvement in pyomelanin production than previously published values. Larroude et al. (doi: 10.3390/microorganisms9040838) reported a pyomelanin titer of 4.5 g/L, Lorquin et al.

(https://doi.org/10.1038/s41598-021-87328-2) reported 0.55 g of pyomelanin per liter of medium, and Koch et al. (https://doi.org/10.3389/fmicb.2023.1233740) reported 1.48 g of pyomelanin per liter of medium. The later paper also comparing titer/yield of pyomelanin production in different organisms by different approaches. Thus, this work demonstrates a significant improvement in terms of pyomelanin titers. However, important information on the process are missing to obtain a complete picture of the fermentation process. First of all the description of the fermentation L254 ff. (methods) and L567 ff. (results) is not well described. How was glycerol and methanol fed and which feeding profile was used? How much methanol and glycerol was actually consumed?

Response: Thank you for the helpful comments. We appreciate very much for providing us these valuable references. As suggested, we have now revised the manuscript (below) as follows:

Lines 272-274: "After the glycerol was exhausted, the fed-batch phase (Phase II) was initiated by feeding limited amounts of glycerol at a speed of 15−30 mL/h to promote further cell growth."

Lines 279-282: "Methanol was first added at a speed of 5 mL/h, with an increase of 1–2 mL/h every 5–8 h until the flow rate reached 20–25 mL/h. If no methanol accumulated, this flow rate was used throughout the entire remaining fermentation process. However, if necessary, this flow rate was adjusted appropriately according to the dissolved oxygen level."

Lines 612-613: "During the entire fermentation process, a total of 340.92 ± 14.51 g of glycerol and 1.84 ± 0.17 kg of methanol were consumed."

Does the work support the conclusions and claims, or is additional evidence needed? Additional evidence is needed.

Response: We apologize for not providing additional evidence to support the conclusions and claims. As suggested, we have now provided detailed explanations.

The title states “de novo biosynthesis of pyomelanin from methanol,” but the engineered *K. phaffii* strains actually produce homogentisic acid (HGA) as the final metabolic product. Pyomelanin formation occurs non-enzymatically via oxidation/polymerization of HGA or enzymatically after laccase addition, rather than through an intracellular biosynthetic pathway.

Response: Yes, you are absolutely right! We have now revised the title of the manuscript (below).

Revised title: Biosynthesis of pyomelanin from methanol with engineered *Komagataella phaffii* and its characterizations.

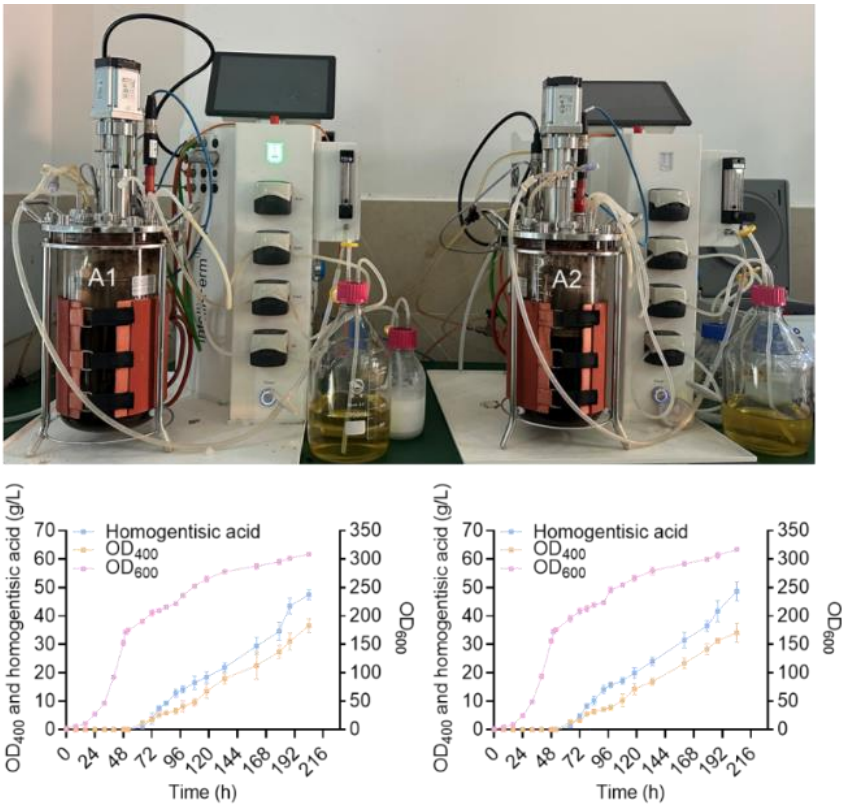
The reported value of 70.5 g/L refers to the titer, not the yield (lines 33 and 95). Titer represents the final concentration of product in the culture medium (often as grams per liter), while yield describes the efficiency of conversion in reference to a given substrate (here: yield on methanol or total carbon source would be appropriate). Additional data on carbon input, substrate consumption, and productivity are needed to support the claims. How reproducible is this fermentation in the 5L setup?

Response: Thanks for your detailed explanations. We used two 5-L fermenters for high-density fermentation again (Response Figure 3). Based on the combined data from the 5 L fermenter in our study, we averaged the fermentation results of the three 5-L fermenters. In summary, the fermentation results demonstrated high reproducibility. As suggested, we have now revised the manuscript (below).

Lines 32-34: “After high-density fermentation in a 5-L fermenter for 204 h, strain Pyo29 achieve the highest titer (70.5 ± 0.7 g/L).”

Lines 102-104: “achieving a pyomelanin titer of 70.5 ± 0.7 g/L with a specific pyomelanin yield of 0.63 ± 0.04 g/g DCW and a productivity of 0.37 ± 0.05 g/L/h; the carbon conversion ratio is 8.83%.”

During the entire fermentation process, a total of 340.92 ± 14.51 g of glycerol and 1.84 ± 0.17 kg of methanol were consumed.



Response Figure 3 Fed-batch culture of strain Pyo29 in two 5-L fermenters.

Purity of the reported pyomelanin is also not certain. Should pyomelanin contain N in its structure? Nitrogen detected in the dry weight suggests possible contamination with secreted proteins, meaning not all measured mass is pure pigment.

Response: Thanks for your suggestive comments. We apologize for the lack of corresponding evidence and adequate discussion. We have now performed additional relevant experiments to address your concerns. The source data are shown below (Response Figure 4). We have now revised the manuscript accordingly (below).

Lines 637-652: "However, that HGA does not contain nitrogen in its structure while pyomelanin does is confusing. Therefore, the nitrogen detected in the dry weight suggested that pyomelanin was possibly contaminated with secreted proteins. However, owing to the lack of a pyomelanin standard, its purity could not be determined. Interestingly, Lorquin et al. conducted an elemental analysis on the pyomelanin obtained by laccase-catalysed HGA-lactone and pyomelanin synthesized by *Halomonas titanicae*. The results also demonstrated the presence of nitrogen in

pyomelanin, possibly because of the abundance of laccase extract or the components of the *H. titanicae* culture medium [22]. To further clarify this issue, we conducted oxidative polymerization of HGA standards using laccase and alkali. Two types of dry pyomelanin powders were subsequently prepared for elemental analysis, and the results revealed that the pyomelanin obtained after laccase treatment contained nitrogen, whereas nitrogen was not detected in the pyomelanin obtained after alkali treatment. These findings indicated that pyomelanin does not contain nitrogen in its structure and that the nitrogen detected in this study was inevitably introduced during yeast culture medium or laccase treatment."

analytic functional testing
UNICUBE
serial number: 0400.191023
HGA-NaOH

Samples

	Weight [mg]	Name	Method	N Area	C Area	H Area	S Area	N [%]	C [%]	H [%]	S [%]
15	1.120	44221	2mg Standard	0	11 137	2 888	71	0.0000	39.48	3.2562	0.5162
16	2.323	44221	2mg Standard	8	22 984	6 137	63	0.0509	39.38	3.4161	0.2137

analytic functional testing
UNICUBE
serial number: 0400.191023
HGA-Laccase

Samples

	Weight [mg]	Name	Method	N Area	C Area	H Area	S Area	N [%]	C [%]	H [%]	S [%]
21	1.241	43810	2mg Standard	1 218	16 244	3 664	49	2.8657	52.19	3.7434	0.2945
22	1.050	43810	2mg Standard	1 027	13 803	3 095	47	2.8702	52.38	3.7128	0.3301

Response Figure 4 Elemental analysis of pyomelanin prepared by alkaline or laccase oxidative polymerization of HGA standard.

Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?

Yes, this paper needs revision. It lacks sufficient quantitative and methodological detail to fully support its conclusions.

Response: We appreciate the opportunity you have given us to make revisions. We apologize for the confusing description about quantitative and methodological detail. As suggested, we have now revised the manuscript.

The authors should clarify how 70 g/L was achieved, meaning what carbon sources were used, feeding rates of methanol and glycerol, and the carbon conversion ratio.

Response: Thanks for your suggestive comments. We apologize for the unclear descriptions. We would like to indicate that throughout the fermentation process, we used two types of carbon sources, namely glycerol and methanol. The revises information is as follows:

Lines 272-274: "After the glycerol was exhausted, the fed-batch phase (Phase II) was initiated by feeding limited amounts of glycerol at a speed of 15−30 mL/h to promote further cell growth."

Lines 279-282: "Methanol was first added at a speed of 5 mL/h, with an increase of 1–2 mL/h every 5–8 h until the flow rate reached 20–25 mL/h. If no methanol accumulated, this flow rate was used throughout the entire remaining fermentation process. However, if necessary, this flow rate was adjusted appropriately according to the dissolved oxygen level."

The carbon conversion ratio reached 8.83%.

In summary, we have now provided these results in the revised manuscript (below).

Lines 101-104: "Next, we then conduct high-density fermentation of the top-producing strain (Pyo29) in a 5-L fermenter, achieving a pyomelanin titer of 70.5 ± 0.7 g/L with a specific pyomelanin yield of 0.63 ± 0.04 g/g DCW and a productivity of 0.37 ± 0.05 g/L/h; the carbon conversion ratio is 8.83%. [23]."

Productivity (g L^{-1} h^{-1}) and specific yield (for example grams of pyomelanin per grams of cell dry weight) are missing and should be compared with literature values.

Response: Thanks for your suggestive comments. We apologize for not providing these results, i.e., "A specific pyomelanin yield of 0.63 ± 0.04 g/g DCW and a productivity of 0.37 ± 0.05 g/L/h." As suggested, we have now compared the ability of different strains to produce pyomelanin in bioreactor (Response Table 1). We have also added a comparison with the literature in the revised manuscript.

Response Table 1. Comparative analysis of the ability of *Komagataella phaffii* and others producers to produce pyomelanin in bioreactor. Symbol "/" indicates no data available.

Strain	Titer (g/L)	Yield (g/g DCW)	Productivity (g/L/h)	References
Komagataella phaffii	70.5 ± 0.7	0.63 ± 0.04	0.37 ± 0.05	This study
Flavobacterium kingsejongi	6.07 ± 0.32	/	0.03	Lee H. S. et al.
Escherichia coli	3.76 ± 0.30	/	0.04	Lee H. S. et al.

Lee H. S. et al. Melanin biopolymer synthesis using a new melanogenic strain of *Flavobacterium kingsejongi* and a recombinant strain of *Escherichia coli* expressing 4-hydroxyphenylpyruvate dioxygenase from *F. kingsejongi*. *Microb. Cell Fact*. **21**:75 (2022).

Some results (for example the reported 6.3 % increase in HGA, lines 548 and 549) don't have evidence of statistical significance.

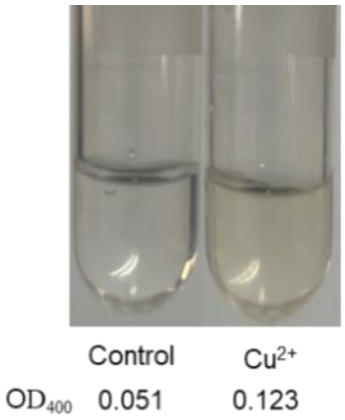
Response: Thanks for reminding us of lacking evidence of statistical significance. We have now carefully checked the entire manuscript and corrected all results.

OD_{400} readings are presented without justification that the signal detected is only pyomelanin.

Response: We apologize for the lacking this justification. We agree with you that OD_{400} readings does not fully represent pyomelanin. In our study, we only treated the OD_{400} readings as the relative value of changes in pyomelanin titer among different strains. In addition, we measured the OD_{400} of BMMY fermentation medium and the reading remained stable in the range of 0.47–0.51. In fact, each OD_{400} reading in our manuscript has subtracted this value to enhance the correspondence between OD_{400} and pyomelanin.

Figure 2 and related text imply inconsistent effects of the addition of copper ions, which may both enhance HPPD activity and promote non-enzymatic oxidation of HGA. This needs clarification.

Response: Thanks for your suggestions. You are definitely right. HGA standard powder was prepared into a water solution with Cu^{2+} added. After shaking for 24 h, the solution with Cu^{2+} showed a color change, appearing brownish yellow, with an increase in OD_{400} readings compared to the control (Response Figure 5). This indicates that Cu^{2+} can also promote nonenzymatic oxidation of HGA. In summary, Cu^{2+} has shown a dual function of promoting non-enzymatic oxidation of HGA and enhancing the catalytic activity of *Af*HPPD enzyme. We have now revised our manuscript as follows: Lines 387-395: "Interestingly, Seo D. et al. demonstrated that Cu^{2+} ions do not increase the catalytic activity of the HPPD enzyme of *Ralstonia pickettii*. However, the addition of Cu^{2+} to the culture medium of *R. pickettii* can increase the production of pyomelanin [8]. Therefore, we speculate that Cu^{2+} may both increase HPPD activity and promote the nonenzymatic oxidation of HGA. To verify this hypothesis, HGA standard powder was prepared as a solution, and Cu^{2+} was added to the solution. After shaking for 24 h, compared with the control solution, the solution with Cu^{2+} added showed a colour change, appearing brownish yellow, with an increase in the OD_{400} (Supplementary Fig. 3). This proves that Cu^{2+} can also promote the nonenzymatic oxidation of HGA."



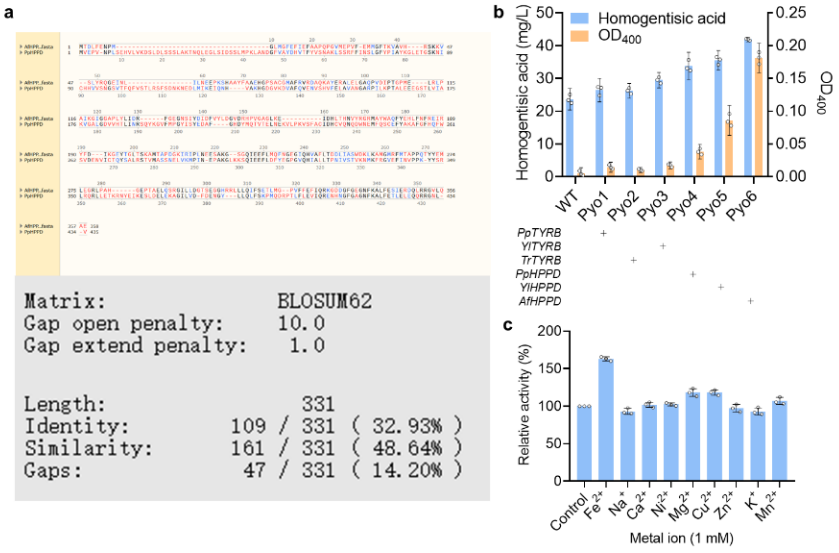
Response Figure 5 Effect of Cu^{2+} on promoting nonenzymatic oxidation of HGA.

It would be interesting to see the comparison of the native HPPD enzyme from *Komagataella phaffii* to the engineered one from this paper.

Response: Thanks for your suggestive comment. Yes, we also think it will be very interesting. We will compare *Pp*HPPD enzyme (*Komagataella phaffii*) and *Af*HPPD (*Alcaligenes faecalis*) from two perspectives (below).

On the one hand, the sequence alignment of *Pp*HPPD and *Af*HPPD showed that the two enzymes have 32.93% identical residues and 48.64% similar residues (including conservative substitutions) within the alignment region. Although there are 14.2% gaps, reflecting insertion or deletion during evolution, the core functional region that binds to metal ions is conserved (Response Figure 6a).

On the other hand, previous manuscript explored *HPPD* genes from various sources and identified *AfHPPD* significantly enhanced HGA production than *PpHPPD* (Response Figure 6b). Then, we have added new experiments to further compare *Pp*HPPD and *Af*HPPD. According to the standard of "2.8 In vitro *Af*HPPD enzyme assays" in the manuscript's Materials and methods, we tested which metal ions could enhance the catalytic activity of *Pp*HPPD enzyme. The results showed that Fe^{2+} could slightly enhance the activity of *Pp*HPPD enzyme. And the results showed that the enhancement effect of Fe^{2+} on *Pp*HPPD enzyme was inferior to that of Cu^{2+} on *Af*HPPD enzyme (Response Figure 6c). Therefore, thank you again for your question, which has strengthened our determination to choose the *Af*HPPD enzyme for this study.



Response Figure 6 Comparison of *Af*HPPD enzyme and *Pp*HPPD enzyme. a Sequence alignment of *Af*HPPD enzyme and *Pp*HPPD enzyme. b,c In vitro and in vivo comparison of *Af*HPPD enzyme and *Pp*HPPD enzyme.

The English in general needs more revision to be clearer and gramatically correct.

Response: Thanks for reminding us! As suggested, the language has been thoroughly revised with the Nature Research Editing Service provided by *Nature Communications* affiliates (Order#: 1P6M32SJ).

Given these issues, the work needs significant revision before publication.

Response: Thank you again for giving us the opportunity to revise and improve our manuscript.

Is the methodology sound? Does the work meet the expected standards in your field?

Not fully. The experimental concept is interesting and the modular metabolic engineering strategy is promising, but methodological details are missing.

Response: We appreciate your positive evaluation on our work. As suggested, we provided a detailed description of the methodological details in the revised manuscript and supplementary materials.

There is no clear genetic characterization of strains in this work. Number of integrated copies of some genes is mentioned (lines 338, 499, 501, 547, 700), but there is no information on the way that was achieved.

Response: Sorry for the confusing description. In order to clearly describe the genetic characterization of strains, the plasmids and strains information table in the supplementary materials have been modified. The details of the gene copy number integration method have also been revised in the revised manuscript (below).

Lines 361-362: "Strain Pyo9, which carried three *Af*HPPD copies integrated into the genome at different loci (*HIS4*, *INT1*, and *INT18*)."

Lines 531-533: "A second copy of *TKL1* subsequently added to the *INT18* locus of the

genome of strain Pyo15 to obtain strain Pyo22."

Lines 533-534: "The results showed that strain Pyo22, carrying the *TKL1* gene integrated into two different loci (*INT1* and *INT18*)."

Lines 578-579: "suggesting that an extra copy of *AfHPPD* (integrated into the *INT*21 locus) might be needed to channel carbon flux effectively for into HGA synthesis.

Lines 802-804: "In this study, the highest pyomelanin-producing strain, Pyo29, carried four copies of the *Af*HPPD enzyme (integrated into *HIS4*, *INT1*, *INT18* and *INT21*)."

No plasmid sequences are provided in the supplementary material.

Response: Thanks for reminding us! We have included the plasmids sequences and integration sites used in this study in the supplementary materials (Table S5).

The lack of OD measurements during inoculation (lines 238 and 240), the vague description of adding copper "as needed" (lines 262 and 263) and "pyomelanin solutions of varying concentrations" (lines 291 and 292) make replication and data interpretation difficult.

Response: Sorry for the confusing description. We corrected these descriptions in the revised manuscript (below).

Lines 254-257: "Afterwards, approximately 4% of the seed culture (OD_{600}=1.0) was transferred into a 500 mL Erlenmeyer flask containing 100 mL of YPD medium and cultured under the same conditions for approximately 18 h."

Lines 282-283: "Next, 10 mL of 200 mM Cu^{2+} was supplemented every 18 h during the induction process."

Lines 310-312: "After incubation, the culture medium was removed from each well, and different concentrations (0, 100, 500 and 1000 μg/mL) of pyomelanin solutions dissolved in DMSO were added."

Is there enough detail provided in the methods for the work to be reproduced?

No. A lot of experimental details are missing or too vague to reproduce, as mentioned above.

Response: Thanks for your suggestive comment. As mentioned above, we revised the manuscript and supplementary materials carefully.

Responses to Reviewer 4

Reviewer #4 (Remarks to the Author):

The manuscript "De novo biosynthesis of pyomelanin from methanol with engineered *Komagataella phaffii* and its characterizations" presents several interesting aspects, such as the production of pyomelanin in a yeast strain, the directed evolution of enzymes to improve biosynthetic processes, the metabolic push-pull strategy and manipulation of *K. phaffii* for pyomelanin production.

Response: We appreciate your positive evaluation on our work!

However, we identified several major issues that need to be addressed:

Response: Thanks for your suggestive comments. We here provide a point-by-point reply to address all your concerns (below).

The main observation that I have is that the pigment being produced, pyomelanin, is widely distributed among bacterial species, like those of the genus *Pseudomonas*, among others. It is not clear what the advantage would be of producing pyomelanin in a eukaryotic organism. On one hand, achieving high pigment production requires a rather complex fed-batch fermentation scheme, which is not inexpensive. Although methanol is a low-cost substrate, bacteria can utilize waste compounds from agro-industrial or fuel industries, for example. If the advantage lies in cost, this point has not been thoroughly discussed.

Response: Thank you for the helpful comments. Yes, you are completely right. Almost all reports of pyomelanin are widely distributed among bacterial species. Especially, pyomelanin from *Pseudomonas aeruginosa* ΔhmgA, which has been reported and well characterized by many research groups. In this study, we choose *K.phaffii* to produce pyomelanin. On the one hand, the genetic background of *K.phaffii* is clear, and gene editing tools are constantly improving. On the other hand, many studies use *K.phaffii*

as a cell factory for synthesizing various high-value compounds. This provides the possibility for *K.phaffii* to produce pyomelanin. Therefore, we have added more discussion to introducte *K.phaffii* to you in the revised manuscript (below).

Lines 57-69: "The methylotrophic yeast *Komagataella phaffii* is classified as a "Generally Recognized as Safe" (GRAS) organism by the U.S. Food and Drug Administration (FDA) [15,16]. Many studies have demonstrated its potential as an effective host for the production of L-tyrosine-derived compounds, including resveratrol, naringenin, and norcoclaurine [17,18]. Compared with bacteria, *K. phaffii* has advantages in terms of efficient and high-level expression of heterologous enzymes, and its Crabtree-negative phenotype further enhances its capacity for high-density fermentation in minimal salt media when methanol is used as the sole carbon source [19]. In terms of cost, although bacteria can ferment waste compounds from the agro-industrial or fuel industries, methanol is also a low-cost carbon source. In addition, methanol, as a renewable and carbon–neutral single-carbon (C1) feedstock, has emerged as a promising alternative carbon source for green biomanufacturing because of its noncompetition with food resources and scalability for industrial production [20,21]. Consequently, engineered *K. phaffii* is expected to offer a sustainable platform for the production of pyomelanin, a downstream derivative of L-tyrosine."

Moreover, the fermentation lasts five days (example in line 205), which is quite long, and no comparison is made to yields obtained in other studies using fast-growing bacterial producers.

Response: Thanks for your suggestive comment. In order to better compare the differences in the levels of pyomelanin production between *K. phaffii* and bacterial producers, we have listed a table to better present this data of titer, yield, and productivity to you (below).

Response Table 2. Comparison of the ability of *K. phaffii* and bacterial producers to produce pyomelanin in shaking flask.

Strain	Titer	Productivity	Reference
Komagataella phaffii	5.11 ± 0.13 g/L	42.6 mg/L/h	This study
Escherichia coli	315.5 ± 12.9 mg/L	13.1 mg/L/h	Seo et al.
Escherichia coli	804.4 ± 5.9 mg/L	16.8 mg/L/h	Zhang et al.
Escherichia coli	213 mg/L	1.48 mg/L/h	Bolognese et al.
Halomonas titanicae	0.55 g/L	11.5 mg/L/h	Lorquin et al.
Aspergillus niger	1.48 g/L	10.3 mg/L/h	Koch et al.
Pseudomonas aeruginosa	1.79 ± 0.18 g/L	12.8 mg/L/h	Urbaniak et al.
Yarrowia lipolytica	4.5 g/L	13.3 mg/L/h	Larroude et al.

Seo, D. et al. Heterologous production of pyomelanin biopolymer using 4-hydroxyphenylpyruvate dioxygenase isolated from *Ralstonia pickettii* in *Escherichia coli*. *Biochem. Eng. J.* **157,** 107548 (2020).

Zhang, Q. H. et al. Pyomelanin production via heterologous expression of 4-hydroxyphenylpyruvate dioxygenase (HPPD) and construction of HPPD inhibitor scr eening model. *J. Biosci. Bioeng*. **135**, 93-101 (2023).

Bolognese, F. et al. Bacterial melanin production by heterologous expression of 4 hydroxyphenylpyruvate dioxygenase from *Pseudomonas aeruginosa*. *Int. J. Biol. Macromol*. **133**, 1072-1080 (2019).

Lorquin, F. et al. Production and properties of non-cytotoxic pyomelanin by laccase and comparison to bacterial and synthetic pigments. *Sci. Rep.* **11**, 1–16 (2021).

Koch, S. et al. *Aspergillus niger* as a cell factory for the production of pyomelanin, a molecule with UVC radiation shielding activity. *Front. Microbiol*. **14**, 1233740 (2023).

Urbaniak, M. et al. In Vitro and In Vivo Biocompatibility of Natural and Synthetic *Pseudomonas aeruginosa* Pyomelanin for Potential Biomedical Applications. *Int. J. Mol. Sci*. **24**,7846 (2023).

In the Results section (line 320), no measurement uncertainty or deviation is indicated, which prevents assessing reproducibility.

Response: Thanks for reminding us of this missing information. We have now carefully checked the whole manuscript and provided missing information in results.

Considering the importance of genetic stability in organisms intended for biotechnological use, it would be highly relevant to discuss the stability of this yeast strain, which carries multiple genetic modifications, even the use of Crispr-Cas9 can lead to off-targets editions that should be analyzed.

Response: Thanks for your helpful suggestions. We have added a description and discussion on the genetic stability of *K. phaffii* in the revised manuscript (below).

Lines 121-124: "Considering the importance of genetic stability, the pZACH plasmid was utilized for gene overexpression integration. The *Cre/loxP* Zeo^R marker recycling method [24] ensured the stability of every integration. The pENO1-Cas9 plasmid was used for gene knockout to avoid off-target effects."

Lines 816-820: "In addition, *K. phaffii* usually expresses exogenous genes through chromosome integration rather than through plasmid amplification. Its genetic stability is high in microbial expression systems, which ensures that strain Pyo29 is suitable for long-term and large-scale fermentation production of pyomelanin [46,47]."

More importantly, while the reported yield of 1.65 g/L is high, productivity (yield per unit time) is not expressed, so it is unclear whether this microorganism is actually a better producer than bacterial systems, either natural producers or recombinant, fast growing strains. For example, line 573 states that fermentation was completed after 168 hours (seven days), yet this aspect is not discussed at all.

Response: We apologize for the incomplete description. We have now provided appropriate description in the revised manuscript (below).

Line 340-342: "After various remoulding strategies were implemented, we obtained a high-yield HGA-producing *K. phaffii* strain, Pyo29, with a titer of 1.65 ± 0.04 g/L, and a productivity of 13.8 mg/L/h."

We apologize for any misunderstanding caused our inadequate discusson. In this study, we divided the synthesis of pyomelanin into two distinct processes. First, we achieved

efficient production of HGA from methanol. Second, we explored the oxidative polymerization of HGA into pyomelanin. The final strain Pyo29 can produce 1.65 ± 0.04 g/L HGA at the shake flask level, which can then be oxidized and polymerized into 5.11 g/L pyomelanin. However, we have not seen any reports on HGA titer by bacterial systems. The comparative analyis of the production capacity of pyomelanin in shaking flask is shown in the Response Table 2 (above). Then, as you mentioned, the high-density fermentation time in a 5-L fermentor was a total of 204 hours, and the induction time was 168 hours. The comparison of the production capacity of pyomelanin in bioreactor is shown in the Response Table 1 (above). In summary, regardless of shaking flask or bioreactor, the *K. phaffii* used in this study exhibited higher pyomelanin titer and productivity (mg/L/h) than bacterial producers. In fact, we are very grateful to all the research teams involved in the production of pyomelanin by bacteria. These reports have provided excellent guidance for our study!

Regarding the chemical characterization, we found significant issues:

Response: Thanks for your suggestive comments. We here now provide a point-by-point response to address all your concerns (below).

First, in the M-M section (line 210), the purification method described appears to yield poorly purified material, potentially containing impurities such as proteins and polysaccharides. These contaminants could have influenced some of the reported scavenging results. Moreover, the reported production values might be highly overestimated due to this purification protocol.

Response: Thanks for your suggestive comments. We aologize for the concern caused by lacking sufficient discussion and experimental evidence. We would like to address your concerns in the following three aspects (below).

Firstly, the purification method used in this study is acid precipitation, which is consistent with most previous reports. Especially, we noticed that the publications that you provided us also used the same method for the purification of pyomelanin.

Secondly, we have been trying to determine the purity of pyomelanin. However, due to

the lack of standard and the fact that pyomelanin is a polymer, the detection of its purity is technically challenging. As you mentioned that our purified pyomelanin may contain proteins or polysaccharides. We speculated that your concerns are based on the elemental analysis of pyomelanin in our study. In fact, another reviewer also expressed the same concerns. We would like to indicate that we obtained HGA standard to use in oxidative polymerization treatment based on alkali or laccase. We conduct elemental analysis after obtaining two types of pyomelanin. Our conclusion is that the structure of pyomelanin definitely does not contain nitrogen. The reason why pyomelanin produced by *K. phaffi* contains nitrogen may come from the rich extract of laccase or the components of the culture medium. Therefore, we agree with this reviewer on that the purified pyomelanin in our study may contain a small amount of protein. Interestingly, our results are consistent with Lorquin's report, which seems to be a common issue.

Finally, we agree with this reviewer on the concern that the incorporation of nitrogen can lead to an overestimation of the titer and yield of pyomelanin. However, considering that the nitrogen content of pyomelanin in this study is less than 5%, the protein content is relatively low. Again, we would like to express our sincere gratitude to your constructive advice on the purity of pyomelanin. Indeed, the entire pyomelanin industry should pay attention to the problem of purity, which is crucial for large-scale industrial production and product applications. We now look forward to working together with other research groups involved in the production of pyomelanin by bacteria to establish a standardized purification process for pyomelanin.

On the other hand, all experiments should have included, at least as a control, the best-characterized pyomelanin from *Pseudomonas aeruginosa* ΔhmgA. While it is true that commercial melanin from Sigma (derived from alkaline treatment of tyrosine) is available, the authors could have used *P. aeruginosa* ΔhmgA pyomelanin as reference, which has been well characterized by several research groups over time.

Response: Yes, you are definitely right! We apologize for overlooking the reports by several research groups on the use of *P. aeruginosa* ΔhmgA to produce pyomelanin.

Firstly, through our research and the reference you provided of pyomelanin based on *Pseudomonas aeruginosa* ΔhmgA, we agree that many researchers have conducted detailed characterization of pyomelanin produced by *P. aeruginosa* ΔhmgA. As you mentioned, this microbial-derived pyomelanin can serve as the standard for the entire pyomelanin industry. Therefore, we agree with this reviewer on the argument that, as one of the few cases of fungal production of pyomelanin, it is necessary to compare our results with this standard pyomelanin. Secondly, as mentioned in our response to your question below, we have now provided comparison and discussion of *P. aeruginosa* ΔhmgA in the revised manuscript regarding the characterization experiments of pyomelanin, in order to draw readers' attention to the pyomelanin produced by *P. aeruginosa* ΔhmgA. Finally, we would like to express our gratitude to all the research groups studying the production and characterization of pyomelanin by *P. aeruginosa* ΔhmgA for their contributions to the development of the pyomelanin industry. It is strongly believed that these studies have significantly guided and improved our manuscript. Most importantly, we would like to thank you for your suggestions and tolerance of our inappropriate descriptions in this study, as well as for taking your time to provide us with valuable references on the production and characterization of pyomelanin by *P. aeruginosa* ΔhmgA. We believe that our manuscript is now significantly improved.

Furthermore, in the M-M (lines 269 and 290), in the antioxidant and cell assays, it is not stated how the pyomelanin solutions were prepared, given that the natural pyomelanin is insoluble in water, buffer, or ethanol therefore is crucial to understand how the experiments were made.

Response: We apologize for the confusing description. As you suggested, we have now added more details in the revised manuscript (below).

Lines 293-294: "DPPH and pyomelanin dry powder were dissolved in ethanol and DMSO, respectively."

Lines 310-312: “After incubation, the culture medium was removed from each well, and different concentrations (0, 100, 500 and 1000 μg/mL) of pyomelanin solutions dissolved in DMSO were added.”

Additionally, there is a lack of controls with pyomelanins from bacterial. Antioxidant, cosmetic, and UV-protective properties of pyomelanin have already been extensively described in the literature, so many of the authors’ statements overlook the substantial existing body of work (lines 623–627).

Response: Thanks so much for your critical comments. We have provided relevant comparison and discussion in the revised manuscript (below).

Lines 704–719: “Notably, pyomelanin from bacteria has been widely reported. In particular, pyomelanin from *Pseudomonas aeruginosa* ΔhmgA has been well studied and characterized by several research groups [32-35]. Therefore, comparing the antioxidant and antiradiation properties of pyomelanin produced by *K. phaffii* with those from bacterial sources is necessary. Interestingly, Diaz Appella et al. demonstrated that the composition and physiological role of pyomelanin produced by *P. aeruginosa* from diverse lifestyles also differ [33]. A similar study also demonstrated significant differences in functional applications between water-soluble and water-insoluble pyomelanin from the same source [34]. These findings are similar to our results. Furthermore, almost all the microbial sources of pyomelanin exhibit excellent antioxidant and radiation resistance properties. However, the diversity of experimental cell models and evaluation methods make comparisons difficult. In summary, this study demonstrated that compared with pyomelanin produced by bacterial producers, the pyomelanin produced by *K. phaffii* has similar antioxidant and radiation resistance, which can supplement the biotechnology applications of pyomelanin.”

The characterization relies mainly on IR spectroscopy, which provides limited structural information for polymers as complex and heterogeneous as melanins. Even for pyomelanin, key structural differences have been revealed primarily by NMR

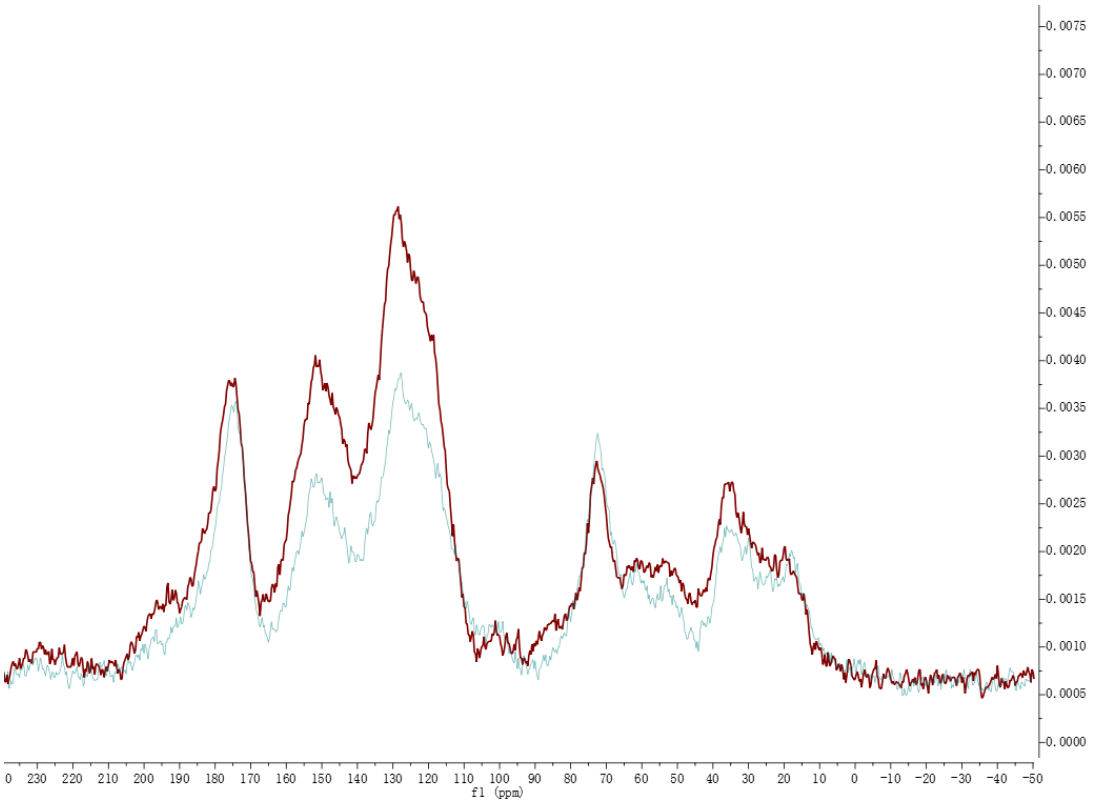
analysis. Therefore, the claims made in lines 607–609 cannot supported by IR data alone.

Response: Thanks so much for your suggestive comments. We have now conducted ^{13}C CP-MAS solid-state nuclear magnetic resonance analysis (NMR) on two pyomelanin samples, Pyo-NaOH and Pyo-Lac. Then, we have provided the explanations of NMR methods and results in the revised manuscript (below).

Lines 229-232: "The CP-MAS method for ^{13}C CP-MAS solid-state nuclear magnetic resonance (NMR) analysis was used. The instrument used was a JNM-ECZL600G from Japan Electronics, with a ^{1}H resonance frequency of 599.57 MHz, a 3.2 mm solid rotor sample, and an HX dual resonance probe."

Lines 657-685: "To further analyse the chemical structural characteristics of pyomelanin, ^{13}C CP-MAS solid-state nuclear magnetic resonance (NMR) analysis was performed on Pyo-NaOH and Pyo-Lac. The results revealed that the overall profile and main signal distribution of Pyo-NaOH and Pyo-Lac were highly similar, which is consistent with the spectral characteristics of amorphous polymer melanin substances (Supplementary Fig. 12) and highly consistent with the spectra of pyomelanin reported in the literature [22]. At δ 170–180 ppm, a clear wide blunt signal was observed in both samples, which can be attributed to the unpotonated carbonyl carbon (–COOH) in the carboxyl group, indicating that a certain proportion of the carboxyl structural units were still retained in the polymer structure. The aromatic carbon region (δ 110–160 ppm) was the most concentrated part of the spectrum, and the overall signal of Pyo-Lac in the aromatic region was greater than that of Pyo-NaOH. A strong broad peak appeared at approximately δ 120–130 ppm, corresponding to the C–H and C=C carbon signals in the aromatic ring, whereas the secondary signal in the δ 145–150 ppm region was attributed to the aromatic carbon connected by phenolic hydroxyl groups. This spectral characteristic indicates that the main chain skeleton of the sample is composed mainly of aromatic structural units. Notably, no clear independent signal peak was observed in the δ 160–165 ppm region, indicating that the samples do not contain aromatic ether bond (Car–O–Car) structures and that the polymerization process may be achieved mainly through aromatic carbon–carbon bond (Car–CAr) coupling, which is highly

consistent with the reported mechanism of pyomelanin polymerization in the literature. At δ 30–80 ppm, weak but distinguishable signals were detected in both samples, with peaks near δ 30–35 ppm attributed to aliphatic–CH_2–carbon, and at δ 50–80 ppm, broad peak signals were observed in Pyo-Lac and Pyo-NaOH, which are typically attributed to aliphatic carbon (C–O type carbon) connected to oxygen atoms. In summary, Pyo-Lac and Pyo-NaOH both exhibit polymerization characteristics of melanin with aromatic carbon–carbon bond connections as the main and highly disordered structures according to solid-state nuclear magnetic resonance spectra, and their overall structures are highly similar."



Supplementary Fig. 12 ^{13}C Cross-Polarization Magic Angle Spinning (CP-MAS) solid-state NMR spectra of Pyo-NaOH and Pyo-Lac. The red and blue lines represent Pyo-Lac and Pyo-NaOH, respectively.

In line 629, the authors discuss the use of pyomelanin for electrode fabrication in sodium-ion batteries. However, it is unclear what the advantage would be of using such an expensive compound for this purpose. Without a clear rationale, this part of the study

lacks relevance. There are numerous inexpensive precursors for synthesizing hard carbon anodes for SIBs, including agricultural biomass/waste (lignin included), industrial pitch, and waste plastics (e.g., PET), all of which have demonstrated competitive performance (DOI: 10.1016/j.jechem.2018.01.025; DOI: 10.1038/s41467-023-39637-5; DOI: 10.1039/C5TA08601A).

Response: Thank you for the helpful comments. We appreciate very much your providing us the three publications on sodium ion batteries. We have provided additional comparative analysis of the application of pyomelanin as a sodium ion battery in the revised manuscript (below). We have now cited and discussed these references you provided in our revised manuscript.

Lines 742-753: "Although the performance of pyomelanin hard carbon as a negative electrode for sodium-ion batteries is similar to that of some materials reported in the literature, these inexpensive biomass hard carbon materials (industrial pitch or rice husk) seem to have lower costs [36,37]. In particular, Tang et al. used waste wood as a hard carbon negative electrode and reported a high reversible capacity of 430 mAh/g [38]. Compared with these methods, the advantage of this study lies in the direct carbonization of pyomelanin to prepare hard carbon negative electrode materials, which is a simpler preparation process. Of course, applying pyomelanin to sodium-ion batteries is meaningful, and in the future, the preparation process of pyomelanin can be further regulated to obtain structurally unique precursors to enhance the performance of hard carbon negative electrodes in sodium-ion batteries."

Finally, the Discussion contains numerous claims about the advantages and novelty of the study while overlooking prior research that had already addressed many of these points. For example, the comparison between laccase-synthesized and chemically synthesized pyomelanins (under alkaline conditions) has already been thoroughly studied, including NMR analysis, as reported in Lorquin et al., Scientific Reports (2021): 10.1038/s41598-021-87328-2. Given that this work is even cited by the authors themselves in the present manuscript, the claimed novelty is practically null and

contradictory in this regard. Lorquin's study provides a complete structural analysis with NMR data.

Response: Thanks for reminding us of this issue. We agree with this reviewer on these arguments. We have now revised relevant description in the revised manuscript (below). In addition, we have supplemented the NMR data to compare with the NMR results by Lorquin's study (above). Additionally, we greatly appreciate the research published by Lorquin et al. for providing guidance to our work.

Lines 766-768: "Afterwards, we characterized the pyomelanin produced by *K. phaffii* and tested its performance in cosmetics. In addition, we first demonstrated its potential for applications in emerging energy storage."

Lines 824-827: "Interestingly, the comparison between laccase catalysis and chemically synthesized pyomelanins (under alkaline conditions) has already been thoroughly studied [22]. Therefore, this study referred to previous work to explore the structural and functional differences in the application of pyomelanin obtained through these two methods."

Lines 841-842: "Furthermore, this study explored the potential of pyomelanin in the new energy storage field."

Lastly, the statement that this melanin could serve as a reference pyomelanin standard is not well justified, since naturally producing bacterial strains already exist and are routinely used as reference sources in various studies.

Response: Thanks for your kind clarification. Yes, you are definitely right! We have now removed the description in the revised manuscript (below).

"In addition, it is expected that this study of pyomelanin would be used as a guidance to further explore the functional application values of other types of melanin."

More importantly, we are truly grateful for all previous reports on bacterial production of pyomelanin, which have provided strong guidance for our study.

Some important publications:

Rodríguez-Rojas A, Mena A, Martín S, Borrell N, Oliver A, Blázquez J. Inactivation of the hmgA gene of *Pseudomonas aeruginosa* leads to pyomelanin hyperproduction,

stress resistance and increased persistence in chronic lung infection. Microbiology (Reading). 2009 Apr;155(Pt 4):1050-1057. doi: 10.1099/mic.0.024745-0. PMID: 19332807.

Lorquin, F., Ziarelli, F., Amouric, A. et al. Production and properties of non-cytotoxic pyomelanin by laccase and comparison to bacterial and synthetic pigments. Sci Rep 11, 8538 (2021). https://doi.org/10.1038/s41598-021-87328-2

Lorquin F, Piccerelle P, Orneto C, Robin M, Lorquin J. New insights and advances on pyomelanin production: from microbial synthesis to applications. J Ind Microbiol Biotechnol. 2022 Jul 30;49(4):kuac013. doi: 10.1093/jimb/kuac013. PMID: 35482661; PMCID: PMC9338888.

Diaz Appella MN, Kolender A, Oppezzo OJ, López NI, Tribelli PM. The structural complexity of pyomelanin impacts UV shielding in *Pseudomonas species* with different lifestyles. FEBS Lett. 2024 Nov;598(21):2702-2716. doi: 10.1002/1873-3468.15000. Epub 2024 Aug 16. PMID: 39152523.

Urbaniak MM, Gazińska M, Rudnicka K, Płociński P, Nowak M, Chmiela M. In Vitro and In Vivo Biocompatibility of Natural and Synthetic *Pseudomonas aeruginosa* Pyomelanin for Potential Biomedical Applications. Int J Mol Sci. 2023 Apr 25;24(9):7846. doi: 10.3390/ijms24097846. PMID: 37175552; PMCID: PMC10178424.

Ben Tahar I, Kus-Liśkiewicz M, Lara Y, Javaux E, Fickers P. Characterization of a nontoxic pyomelanin pigment produced by the yeast *Yarrowia lipolytica*. Biotechnol Prog. 2020 Mar;36(2):e2912. doi: 10.1002/btpr.2912. Epub 2019 Nov 4. PMID: 31525285.

Al Khatib M, Costa J, Spinelli D, Capecchi E, Saladino R, Baratto MC, Pogni R. Homogentisic Acid and Gentisic Acid Biosynthesized Pyomelanin Mimics: Structural Characterization and Antioxidant Activity. Int J Mol Sci. 2021 Feb 9;22(4):1739. doi: 10.3390/ijms22041739. PMID: 33572316; PMCID: PMC7916096.

Bayram S. Production, purification, and characterization of *Streptomyces* sp. strain MPPS2 extracellular pyomelanin pigment. Arch Microbiol. 2021 Sep;203(7):4419-4426. doi: 10.1007/s00203-021-02437-w. Epub 2021 Jun 14. PMID: 34128104.

Urbaniak MM, Gazińska M, Rudnicka K, Płociński P, Nowak M, Chmiela M. In Vitro and In Vivo Biocompatibility of Natural and Synthetic *Pseudomonas aeruginosa* Pyomelanin for Potential Biomedical Applications. Int J Mol Sci. 2023 Apr 25;24(9):7846. doi: 10.3390/ijms24097846. PMID: 37175552; PMCID: PMC10178424.

Response: We appreciate very much your time and providing these important publications. We have now cited and discussed these references in our revised manuscript. These reports provide valuable references for us to better understand bacterial production of pyomelanin. Finally, we are grateful for your guidance in this study.

Responses to Reviewer 5

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We thank the reviewer for the time and helpful comments.

Responses to four reviewers' comments (NCOMMS-25-76159B)

Dear Reviewers:

We appreciate very much all your constructive comments and suggestions, which definitely gave us helpful guidance to improve our manuscript. We have now revised our manuscript accordingly. For your reference, the changes in the revised manuscript are highlighted in red. We have also provided a point-by-point response (below) with detailed explanations. We are willing to make further changes upon your request.

Responses to Reviewer 1

Reviewer #1 (Remarks to the Author):

The authors have almost addressed all the issues.

Response: Thank you for the positive comments, especially your guidance in enzyme engineering and analysis.

One remaining major issue: In response figure 2d, Mn^{2+} promoted nonenzymatic oxidation of HGA. Why in response figure 2b, no nonenzymatic oxidation phenonmenon was observed in the presence of Mn^{2+}.

Response: Thank you for these helpful comments. We are sorry for any confusion caused due to a lack of detailed explanation. As suggested, we have now provided additional explanations (below) to clarify this issue.

In response to figure 2b, our aim is to test whether Mn^{2+} can enhance the catalytic activity of *Af*HPPD enzyme. The substrate in the system is 4-hydroxyphenylpyruvic acid (4-HPP), and HGA is the product after the reaction is completed. The results showed that the amount of generated HGA is trivial in the presence of Mn^{2+} and cannot be nonenzymatically oxidized into pyomelanin; therefore, no color phenomenon can be observed.

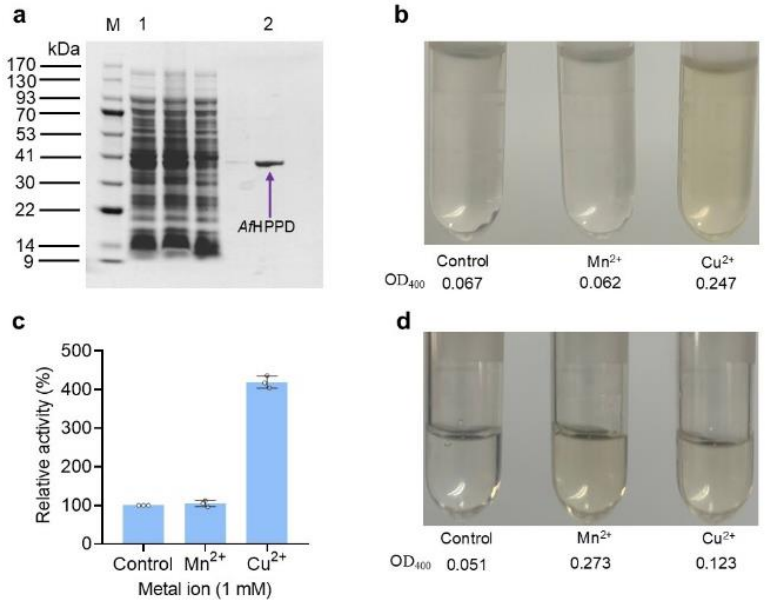
In response to figure 2d, our aim is to investigate whether Mn^{2+} can promote nonenzymatic oxidative polymerization of HGA. We prepared a low concentration

HGA standard, which was shaken for 24 h. We observed a color change in the HGA water solution with added Mn^{2+}, indicating that Mn^{2+} can promote nonenzymatic oxidative polymerization of HGA.

The in vitro *Af*HPPD enzyme assay method described in the manuscript is also provided here for your reference:

Lines 210-215: "An *Af*HPPD enzyme activity assay was conducted by measuring HGA production. The reaction mixture (500 μL) contained 50 mM phosphate buffer (pH 7.4), 1 mM $CuSO_4$, 4 mM ascorbic acid, 1 mM 4-hydroxyphenylpyruvic acid (4-HPP), and 50 μg of *Af*HPPD enzyme. After mixing, the reaction was incubated at 30 °C for 10 min and then quenched with 100 μL of 10% trifluoroacetic acid. HGA levels were quantified by HPLC analysis."

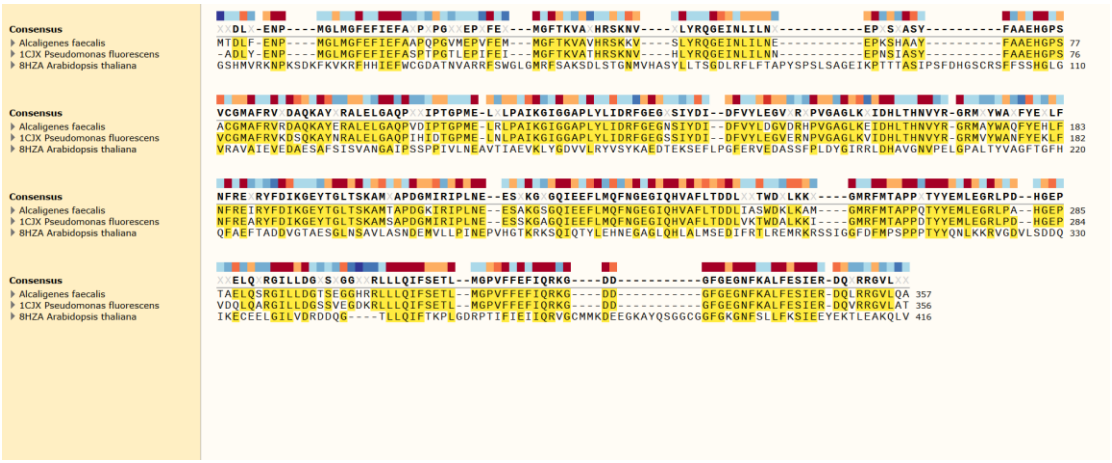
Overall, our results indicated that Mn^{2+} can promote the nonenzymatic oxidative polymerization of HGA into pyomelanin but cannot enhance the production of HGA based on the substrate 4-HPP catalyzed by *Af*HPPD enzyme.



Response Figure 2 Comparison of the effects of Mn^{2+} and Cu^{2+} on *Af*HPPD enzyme activity and HGA oxidative polymerization. a SDS-PAGE analysis of *Af*HPPD protein. Lane M, the protein marker; lane 1, whole cell protein supernatant; lane 2, the purified *Af*HPPD protein. b Enzyme reaction system containing Cu^{2+} exhibits the darkest color after the reaction is completed. c Effect of Mn^{2+} and Cu^{2+} on *Af*HPPD enzyme activity. d Effect of Mn^{2+} and Cu^{2+} on promoting nonenzymatic oxidation of HGA.

Another minor issue in supplementary figure 6: for the sequence alignment, the name for each sequence should be clear. In the present version, I don't know which sequence they used.

Response: Thank you for the helpful comments. We apologize for the unclear descriptions. As suggested, we have now modified supplementary figure 6 in the revised supplementary materials (below).



Comparison with *Af*HPPD	Coverage (%)	Identify (%)	Similarity (%)
*Pf*HPPD	99	84.27	91.29
*At*HPPD	97	28.10	45.10

HPPD structure	Binding site with metal ion
*Af*HPPD	H162-H241-E323
*Pf*HPPD	H161-H240-E322
*At*HPPD	H226-H308-E394

Supplementary Fig. 6. Sequence alignment of *Pf*HPPD, *At*HPPD, and *Af*HPPD. Compared to *At*HPPD, *Pf*HPPD has a higher identify and similarity with *Af*HPPD. For the active center, the important metal binding domains are analyzed. *Pf*HPPD and *At*HPPD bind Fe^{2+}, while *Af*HPPD bind Cu^{2+}. They are all composed of H-H-E motifs.

Responses to Reviewer 2

Reviewer #2 (Remarks to the Author):

The authors carefully revised the manuscript, clarified the methodology, improved the representation of their data and corrected some inconsistencies. Thus, the manuscript appears ready for publication.

Response: Thank you for your positive comments. We appreciate very much this reviewer for the recognition of our manuscript.

Responses to Reviewer 4

Reviewer #4 (Remarks to the Author):

The manuscript has been substantially improved and is now supported by a much stronger scientific framework. The addition of key structural experiments significantly strengthens the main conclusions, and the discussion is more rigorous and better integrated with previously published data from multiple independent groups.

The purification of the material, however, remains inherently complex: although the nitrogen content is reported to be approximately 5%, the nitrogen-containing components may differ in molecular mass, and their contribution to the final purified melanin preparation is therefore not necessarily linear. However, this limitation reflects a broader methodological challenge in the field rather than a specific weakness of this study. Overall, and within my area of expertise all our questions were addressed.

Response: Thank you for your positive comments on our revised manuscript, especially the suggestions on characterization and purification of pyomelanin. Indeed, the purification of melanin is a technical challenge faced by the entire industry. We definitely should spend more time and effort in our future research on melanin to try to optimize the purification scheme. In addition, as suggested, we have added addtional discussion in our revised manuscript to stimulate readers' interest and attract the attention of other melanin research groups (below).

Lines 832-833: “In fact, the purification of pyomelanin remains a technical challenge in the entire field.”

Lines 836-837: “and (3) the strategies to obtain purer pyomelanin for application research.”

Responses to Reviewer 5

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We thank this reviewer for the time and helpful comments during the review of our manuscript.

Responses to four reviewers' comments (NCOMMS-25-76159C)

Dear Reviewers:

We appreciate very much all your constructive comments and suggestions, which definitely gave us helpful guidance to improve our manuscript. We have now revised our manuscript accordingly. For your reference, the changes in the revised manuscript are highlighted in red. We have also provided a point-by-point response (below) with detailed explanations. We are willing to make further changes upon your request.

Responses to Reviewer 1

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns.

Response: Thank you for the positive comments.