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Peer Review File

Biocatalytic enantioselective formation and ring-opening of oxetanes

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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 significantly improved and extended the manuscript based on the reviewer comments. Moreover, all reviewer comments were satisfactorily addressed.

Meanwhile, however, a similar paper starting from a different halohydrin dehalogenase has been published by another group in Angewandte Chemie (https://doi.org/10.1002/anie.202411326). Hence, the authors' statement in the Conclusion section that their "findings constitute the inaugural biocatalytic platform capable of the enantioselective formation as well as ring-opening of oxetans" is actually not true anymore. I highly recommend the authors to reference this other paper and to compare their results to the ones reported in that (earlier) paper.

Moreover, based on the current revision, I have a few more (new) comments that should be addressed as well in a new revision:
1. If mutant M4 turned out to be better (higher conversion) than mutant M7 in the ring-opening of oxetan 1b, could that simply be due to a higher expression of M4 in E. coli compared to M7? Since both mutants cannot be purified to determine kinetic parameters, an impact of the slightly different mutation at position R127 on the kinetic parameters cannot be studied. But maybe the authors can compare expression levels of both mutants (inlcuding respective data also in the SI)?
2. Based on the kinetic parameters of mutant M3 in comparison to WT, not only substrate binding in the mutant is improved but also the kcat value is almost 8-fold higher. Thus, the speculation in lines 246-248 that the improvements in catalytic activity of the mutant are based on increased substrate affinity does not make sense. Indeed, the mutant is improved in terms of substrate binding (not only substrate affinity), as evidenced by the performed MD simulations, and maximal reaction rate (activity). The latter could be explained by QM/MM calculations, however, these calculations are not straightforward and easily performed by unexperienced users. Therefore, I am not expecting the authors to perform such calculations.
3. If mutant M3 is already less thermostable than WT (lines 248-250), it is very likely that mutant M4 is even less stable, explaining its precipitation during purification. This in turn means that mutant M4 can/should only be applied as whole-cell biocatalyst. I think the authors should state that more explicitly in their manuscript.
4. Figures 3+4: The authors should state more explicitly in the figures which reactions have been performed with which mutant. At the moment, some of this information is given in the main text and some information in the SI (tables 6 and 7), but not in the figures 3 and 4 or their corresponding figure captions.
5. The authors performed additional rounds of mutation to invert the enantioselectivity of mutant M4. It is very interesting to see that they managed to find a respective mutant that exhibits sufficient enantioselectivity at least for some substrates. This sixtuple mutant includes two additional positions, that where not mentioned based on their docking study to be important for substrate binding. So, I am wondering how the authors selected those positions for mutagenesis and where those two residues are located in the enzyme active site. This information is currently not given anywhere in the manuscript or SI.
6. Minor comment, line 222: It is misleading what "these mutated residues" is really referring to as in the lines before the mutants at the catalytic residues of HheD8 are discussed, while the authors start discussing the mutations of mutant M3 in this new paragraph. Hence, line 222 should be adjusted for clarity.

Reviewer #2

(Remarks to the Author)
This reviewer thinks that the authors have made satisfactory edits to the manuscript, and all previous issues have been addressed. One comment is that recently a paper has been published in Angew Chem for the same reaction: doi.org/10.1002/anie.202411326. It is advised that the authors quote the above paper and add a few lines to discuss the difference for the two work (for example, difference enzymes were used). Since this work is no longer the first reported enzymatic synthesis of oxetanes by HHDHs, it is better to remove relevant comments from the text (for example, the comments at Line 95-96). Additionally, ISM was used in the paper, and the following paper should also be quoted: DOI: 10.1038/nprot.2007.72.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed all reviewer comments satisfactorily and, in my opinion, the revised manuscript is now ready to be accepted for publication.

Response to Reviewers

Reviewer #1 (Remarks to the Author):

Comments: The authors significantly improved and extended the manuscript based on the reviewer comments. Moreover, all reviewer comments were satisfactorily addressed.

Answer: We are very grateful to your comments for the manuscript and have carefully considered each of your comments.

Question 1: Meanwhile, however, a similar paper starting from a different halohydrin dehalogenase has been published by another group in Angewandte Chemie (https://doi.org/10.1002/anie.202411326). Hence, the authors' statement in the Conclusion section that their "findings constitute the inaugural biocatalytic platform capable of the enantioselective formation as well as ring-opening of oxetans" is actually not true anymore. I highly recommend the authors to reference this other paper and to compare their results to the ones reported in that (earlier) paper.

Answer: We appreciate your insightful suggestion.

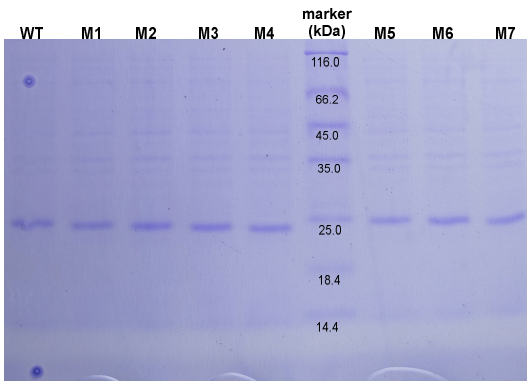
According to your suggestion, we have changed the statement in the Conclusion section as "In summary, we developed a biocatalytic platform capable of the enantioselective formation as well as ring-opening of oxetanes."

Actually, our work was published as a preprint on ResearchSquare on May 15, 2024 (https://www.researchsquare.com/article/rs-4316588/v1), which is earlier than the recent paper (https://doi.org/10.1002/anie.202411326). According to your suggestion, we have referenced and discussed the paper in the revised manuscript (line 431-433).

Question 2: If mutant M4 turned out to be better (higher conversion) than mutant M7 in the ring-opening of oxetan 1b, could that simply be due to a higher expression of M4 in E. coli compared to M7? Since both mutants cannot be purified to determine kinetic parameters, an impact of the slightly different mutation at position R127 on the kinetic parameters cannot be studied. But maybe the authors can compare expression levels of both mutants (inlcuding respective data also in the SI)?

Answer: We appreciate your insightful suggestion.

According to your suggestion, the expression levels of HheD8-WT, M1, M2, M3, M4, M5, M6, and M7 were analyzed and compared by SDS-PAGE. The results indicated all these mutants exhibit very similar expression levels (line 241-244, please see Supplementary Fig. 6a in the revised Supporting Information).



Question 3: Based on the kinetic parameters of mutant M3 in comparison to WT, not only substrate binding in the mutant is improved but also the kcat value is almost 8-fold higher. Thus, the speculation in lines 246-248 that the improvements in catalytic activity of the mutant are based on increased substrate affinity does not make sense. Indeed, the mutant is improved in terms of substrate binding (not only substrate affinity), as evidenced by the performed MD simulations, and maximal reaction rate (activity). The latter could be explained by QM/MM calculations, however, these calculations are not straightforward and easily performed by unexperienced users. Therefore, I am not expecting the authors to perform such calculations.

Answer: We appreciate your insightful suggestion.

According to your suggestion, we have changed the speculation as "We therefore speculate that the increased substrate affinity might be one of the factors contributing to the enhanced activity and enantioselectivity." (line 247-249)

Question 4: If mutant M3 is already less thermostable than WT (lines 248-250), it is very likely that mutant M4 is even less stable, explaining its precipitation during purification. This in turn means that mutant M4 can/should only be applied as whole-cell biocatalyst. I think the authors should state that more explicitly in their manuscript.

Answer: We appreciate your insightful suggestion.

According to your suggestion, we have state it more explicitly in their manuscript.

Line 253-255: "This also indicates that the easily precipitated mutant HheD8-M4 is best used as a whole-cell catalyst to ensure its stability."

Question 5: Figures 3+4: The authors should state more explicitly in the figures which reactions have been performed with which mutant. At the moment, some of this information is given in the main text and some information in the SI (tables 6 and 7), but not in the figures 3 and 4 or their corresponding figure captions.

Answer: We appreciate your insightful suggestion.

According to your suggestion, we have stated more explicitly in the figures which reactions have been performed with which mutant. Please find these changes in the revised manuscript (Fig. 3, **16a**, HheD8-M3; Fig. 4, **16b**, HheD8-M3, **17b**, HheD8-M7).

Question 6: The authors performed additional rounds of mutation to invert the enantioselectivity of mutant M4. It is very interesting to see that they managed to find a respective mutant that exhibits sufficient enantioselectivity at least for some substrates. This sixtuple mutant includes two additional positions, that where not mentioned based on their docking study to be important for substrate binding. So, I am wondering how the authors selected those positions for mutagenesis and where those two residues are located in the enzyme active site. This information is currently not given anywhere in the manuscript or SI.

Answer: We appreciate your insightful suggestion.

According to your suggestion, we have indicated the selected six mutated residues in the Supplementary Fig. 10 in the resivsed Supporting Information. In addition, we have included the statement in the revised manuscript (line 374-379).

Question 7: Minor comment, line 222: It is misleading what "these mutated residues" is really referring to as in the lines before the mutants at the catalytic residues of HheD8 are discussed, while the authors start discussing the mutations of mutant M3 in this new paragraph. Hence, line 222 should be adjusted for clarity.

Answer: We appreciate your insightful suggestion.

According to your suggestion, we have revised the statement to "Through detailed comparison of the three mutated residues (A69F, M124P, and R127G) in HheD8-M3…" for better understanding (line 222-223).

Reviewer #2 (Remarks to the Author):

Comments: This reviewer thinks that the authors have made satisfactory edits to the manuscript, and all previous issues have been addressed. One comment is that recently a paper has been published in Angew Chem for the same reaction: doi.org/10.1002/anie.202411326. It is advised that the authors quote the above paper and add a few lines to discuss the difference for the two work (for example, difference enzymes were used). Since this work is no longer the first reported enzymatic synthesis of oxetanes by HHDHs, it is better to remove relevant comments from the text (for example, the comments at Line 95-96). Additionally, ISM was used in the paper, and the following paper should also be quoted: DOI: 10.1038/nprot.2007.72.

Answer: We are very grateful to your comments for the manuscript and have carefully considered each of your comments.

Actually, our work was published as a preprint on ResearchSquare on May 15, 2024 (https://www.researchsquare.com/article/rs-4316588/v1), which is earlier than the recent paper (https://doi.org/10.1002/anie.202411326). According to your suggestion, we have referenced and discussed the paper in the revised manuscript (line 431-433).

In addition, we have quoted the paper (10.1038/nprot.2007.72) in the manuscript for the ISM methodology (line 161). Please find these changes in the Reference section (ref. 67 and 78).