nature portfolio

Peer Review File

A metagenomic dark matter enzyme catalyzes oxidative cellulose conversion

Corresponding Author: Professor Mario Murakami

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
In this paper, the authors performed metagenomic analysis on soil samples stocked with sugarcane bagasse for decades and isolated a gene encoding a protein with a new type of β-jerry roll fold. Heterologous expression of the gene gave a recombinant protein that adsorbed on cellulose, giving products only from Avicel (cellulose hydrolysate) and phosphate swollen cellulose (PASC), and X-ray crystallography revealed that it was a homodimer and had a copper ion in the active center. Like other copper enzymes such as lytic polysaccharide monooxygenase (LPMO), it gave cellobionic acid as a product only when ascorbic acid was added, leading to the conclusion that it is an enzyme that oxidatively degrades cellulose in an exo-type from the end of the cellulose chain. The volume of experiments is enormous, and the fact that each experiment is very thorough is a good impression, but on the other hand, there are few biochemical, especially quantitative, experiments and no appropriate control experiments, and the reviewer felt that it was too early to conclude that "this enzyme is a new oxidative cellulase". The reasons for this are given below.

What the reviewer finds most critical is that cellobiose is being made from cellulose substrates such as Avicel and PASC simply by adding ascorbic acid without the enzyme. It is not clear what the reason for this is, but if cellobiose is released from the above cellulose substrates and the enzyme just oxidizes the reducing end of it, like cellobiose dehydrogenase, the same results are obtained in all experiments. Although the authors have confirmed no degradation activity against cellooligosaccharides with DP=3-6, they have not confirmed the oxidative activity of cellobiose, so the conclusion that this is a "new type of cellobiose dehydrogenase" cannot be ruled out.

In addition, although the adsorption of the new protein on cellulose is observed, many hydrophobic proteins should be adsorbed in such a qualitative experiment, and it should not be said that "this protein has adsorption ability on cellulose" with this result. For example, adsorption of cellulase on cellulose can occur even with the use of blocking agents such as BSA, but what about this protein?

I believe that it is essential to quantitatively follow the changes over time to demonstrate the activity of a new enzyme. For example, the authors incubate the enzyme for 16 hours at 37°C. What is the time profile during this period? 1 microM of the enzyme is used, but how many microM of product is produced in how many hours and what is the specific activity? When claiming a new activity, we must also be particularly careful about contamination, and to rule out this possibility, we need to examine the enzyme over time with different preparations of the enzyme.

The fact that this enzyme is a homodimer is another reason for skepticism. In Figure 3, the authors speculate that hydrogen peroxide is supplied by one monomer and oxidatively cuts the glucoside bond at the other, but in this case, what happens to the activity in the second round? In addition, the authors draw a picture as if cellulose is degraded from the non-reducing end, but could the same picture be drawn from the reducing end?

As described above, if this enzyme improves the degradation activity of cellulase in real-time, it is a very useful discovery for the enzymatic degradation of cellulosic biomass, but on the other hand, the paper is composed of too many speculations, so at this stage, I cannot propose acceptance for publication in Nature.

P.S. There are some noticeable deficiencies in the references, such as not italicizing the scientific names and not using abbreviations for the names of the papers.

Referee #2

(Remarks to the Author)
General Comments
The manuscript on “A metalloenzyme discovered from microbial dark matter promotes oxidative cellulose oxidation” by Murakami and colls. describes a highly interesting study starting with the metabolomic isolation and identification of an unknown gene, continuing with its expression and biochemical investigation and ending with its elucidated structure. The key results of this study is a new bacterial enzyme involved in cellulose depolymerization that is of potential interest for the biobased economy, especially biorefineries. The described enzyme is similar, but different from the well-investigated lytic polysaccharide monooxygenases (LPMOs). It is interesting to see such a small copper enzyme with such a high substrate specificity. Even smaller than most LPMOs it features besides a flat, substrate binding interface a substrate binding pocket for the non-reducing, terminal cellobiose unit of cellulose. The copper center ligation is quite different from LPMO, but seems to perform a similar reaction. Unfortunately, the authors do not report the specific activity or turnover numbers of the enzyme, but demonstrate the activity by the acceleration of a complex enzymatic mixture or rater qualitative conversion experiments. To increase the interest in this work to the level adequate of this journal, a minimal kinetic characterization of CellOx is a must. Nevertheless, the presented enzyme is a new enzyme of significant interest, the manuscripts reports original research, the instrumental and computational methods are valid and the statistical methods appropriate. The interpretation of results is clear and valid and discussed in combination with reliable references.

Specific Comments and requested improvements
Title: An important bit of information would be the bacterial origin of the new enzyme. “Microbial” is ambiguous and leave the reader with the question: fungal or bacterial?
Line 41: “Here” is not a good start of the sentence.
Line 44: The term “cellulose-compatible catalytic interface” is quite original, but considering that an active site consists of a catalytic site and a binding site, the phrasing should use the term “specificity” instead of “compatible” and “binding site” instead of “catalytic interface”.
Line 49: While the term “microbial” used above in the abstract is fine, here it is not, because bacteria and fungi differ in their secreted oxidoreductases and produced reaction products and intermediates. It is also suggested to specify the term “redox systems”, e.g. “”bacterial enzymatic redox systems”.
Line 76: Why is cellulose “despite being a homopolymer” recalcitrant? This should be specified, e.g. by the microfibrillar, highly ordered structure. The reason why heteropolysaccharides, especially highly decorated ones, are recalicitrant to enzymatic hydrolysis has a different reason.
Line 94: “carbohydrate enzymology”, “fourth generation synchrotron methods” – use more specific terms
Line 112: “was further investigated due to its uncharted lignocellulolytic potential” – two points: please describe more specifically the markers that were used to identify the potential of the MAGs and “ligno” is not correct in this case when only cellulolytic/hemicellulolytic enzymes are targeted by the study.
Lines 121-123: Sure, it’s a screening, but please indicate that the boosting effect depends on the presence/absence of many enzymes in T. reesei and it is not straightforward that a bacterial enzyme boosts a fungal enzyme system.
Line 127: use instead: “via a redox mechanism”
Line 133: In Figure 1, also xyloglucan and arabinoxylan are given as substrates for metabolic pathwas in SBS.bin.55. These are also on the list of substrates, but not shown here or in fig. S5. If already indicated in Fig.1, the reader expects to hae these substrates tested.
Lines 138-143: The reported methods showed a strong adsorption, but why was the Kd not measured? This would be more important that three methods just qualitatively indicating binding.
Line 145: The name “CellOx” is ill chosen. “Ox” indicated an oxidase, which the enzyme certainly is not. The authors later state that they thik it is a similar mechanism as in LPMO, a peroxygenase reaction. Then the name should reflect this and follow the guidelines given by the enzyme commission.
Line 158: In this context “oxidoreductase” would be more suitable.
Line 159: rephrase “a distinguished mode of action”, maybe a “unique activity”.
Line 164: rephrase “long-term soil”
Figure 2B: In the conversion reactions analyzed by HPAEC-PAD, CellOx, KdgF and BagB produce also cellobiose (the DP2 peak). This needs discussion since the peaks are quite prominent compared to the cellobionic acid peak of CellOx. Is this an artefact? Are the other enzymes also active on cellulose? Ist CellOx not only producing cellobionic acid, but also cellobiose? Please comment on the reaction products seen at DP2.
Line 209: Use “copper atom” instead of “copper metal” since a metal is a material with unique properties based on the atomic orbitals of its constituting element(s).
Line 227: “Supporting the function of copper for catalysis”
Line 248 and elsewhere: The term “oxidative activity” is not well chosen for an peroxygenase. Also “redox chemistry” is very vague for the intended meaning “redox mechanism”.
Line 256: “a comparative time-course analysis” is what? A kinetic analysis?
Figure 4C: There are no “activity rates”. Activity as a kinetic term would here be already far stretched, since no units, rates are given. Just the relative production of cellobionic acid during the experiment for which one has to search carefully in the material and methods section to find the time of the experiments (16 h). Please calculate e.g. turnover numbers from this experiment, it would be so much more useful to compare the performance of CellOx to other enzymes.
Line 296: A “mutant” is typically a modified organism. Here “mutant enzyme” or “enzyme variant” should be used.
Line 309: It would be so much better to say “carbohydrate active oxidoreductases” then “carbohydrate oxidative enzymes”.
Line 326: It would be very good for the comparison and interpretation of the results to have the measured/estimated concentration/percentage of CellOx in the secretome.

Figure 5: Relative activities are awful, please report the formed product concentration, even as concentration of reducing ends.
Line 370: again “carbohydrate enzymology”
Line 373: replace “microbial” by “bacterial”
Supplementary Materials:
Description of anaerobic experiments: The experimental time is not given.
Fig. S5: Where are the measurements of xyloglucan and arabinoxylan?
Fig. S17: Here again a product below the DP2 standard is visible beside the cellobionic acid. It is difficult to quantify, but might be around 5% of the main product. Is this a side activity pf”CellOx”?
Fig. S19: This figure as a bar chart is difficult to interpret. A diagram with two lines connecting the datapoints of the two datasets and a continuously scaled X-axis would allow the reader to evaluated the reaction in regard to steady-state conditions, enzyme deactivation, etc. Also the Y-axis should show the concentration of cellobionic acid, so that one can calculate turnover numbers. This would be important to evaluate the argument that oxygen is a similar or even better cosubstrate than hydrogen peroxide for “CellOx”. Under the assumption that after 16 h the full dosage of 100 µM hydrogen peroxide is consumed, the consumption after 4 h when steady-state conditions can be still assumed is about 35 µM. With the mentioned 1 µM enzyme concentration and an experimental time of 16 h, a turnover number of 0.0024 s-1 can be calculated. This is very low, even compared to LPMO using oxygen to generate hydrogen peroxide in situ. If the estimated turnover numbers are correct, CellOx is either (i) a very slow enzyme with a low activity, or (ii) the physiological substrate/co-substrate has not been identified yet.
Fig S20: Please report the activity or turnover number, but not relative ativities. The comparison of cellobionic acid production rates versus hydrogen peroxide production rates would be absolutely interesting! What is the rate limiting step in the reaction? The substrate conversion step, or the production of the co-substrate?
Fig. S26: The protein titer is marginally affected by the expression of CellOx. Please indicate the concentration of the expressed CellOx.
The crystal structures are of sufficient quality to merit the interpretation.
Please harmonize the use of “minutes” and “min” in the supplementary information.

Referee #3

(Remarks to the Author)
The paper from Murakami describes a new oxidative enzyme that conducts exo-cleavage of cellulose to produce cellobionic acid. The enzyme was discovered from a metagenomics study of sugarcane bagasse in Brazil. Overall, this paper is very well-written and the authors have done an extremely thorough job on this work. I think that this discovery is extremely exciting and, as the authors note, it shows that oxidative cellulose depolymerization is yet even more complicated than the current paradigms My comments overall are quite minor and follow below in no particular order.

In the first paragraph, the note about cellobiohydrolases being exo-acting only is oversimplified – well-characterized CBHs have been shown to conduct both exo- and endo-cleavage. See Ståhlberg et al. Biochim. Biopsy’s. Acta 1993 and Kurašin et al. J. Biol. Chem. 2011.

I have a minor quibble with the comment about “pilot scale” experiments in the third paragraph of the paper. How “pilot scale” is defined is not clear. I would consider just stating what scale the cocktail enzymatic hydrolysis experiments were conducted at.

I’m not sure what to take away from Figure 1C.

Figure 2B – this is much too small to read and interpret. I would very much consider changing this to make it clearer to read.

Out of curiosity in the structural section, did the authors try AlphaFold modeling, and how did that do relative to the structure?

In Figure 3, I would consider showing the tunnel in more detail. There are programs that can do space-filling of voids in proteins, and that could be interesting to include here to show that this enzyme has a tunnel (assuming I am interpreting this correctly).

In Figure 3, why not show F33 considering this is the amino acid that is mutated later?

Also, the order of data presentation is a bit odd. The authors present the structure in Figures 3A,B, then talk about Figure 4, then they go back to Figure 3. This is very minor, but perhaps consider rearranging the figures to go in order of presentation in the text?

The structure shown in Figure 3D looks quite unrealistic in terms of the angles of the substrate. I would encourage the authors to more carefully check this.

Likely my most important comments are on Figure 5:

- first, how much cellobionic acid is produced in the cocktail context?
- how much of the CellOx enzyme is present in the T. reesei secretome? Did the authors do quantitative proteomics? It might be nice to figure out how much enzyme is present in the cocktail relative to Cel7A, Cel7B, Cel6A, etc.
- What enzyme loading is used in Figure 5 in mg enzyme/g enzyme? What solids loading is used? Put this in the caption.
- I'm surprised that the authors only show a single timepoint. Why not show a time course reaction for these reactions instead of a single timepoint? Usually these types of experiments are measured by "time to a conversion target" and a time course profile would be nice to see.
- Is there a difference in selectivity of products with and without CellOx? In other words, can the authors please show the full product profile, including cellobionic acid, in Figure 5?
- Does beta-glucosidase cleave cellobionic acid to monomers? This would be nice to study.

The authors make several claims about the industrial relevance of this enzyme as well. In the absence of a process model, and in this case, in understanding the difference in product selectivity, I would suggest that the authors tone this language down. In no way does this diminish the exciting discovery of the work here, but I think it is important to not over-promise process relevance.

On a similar note, what would cellobionic acid do in the presence of an ethanologen? Are these substrates able to be utilized and converted to EtOH?

I would suggest that the authors consider changing the name of the enzyme to be more descriptive of what it is doing. CellOx is not very descriptive. Perhaps exocellulo-peroxygenase? I am not sure what the right name should be, but it might be nice, given that the authors have so much mechanistic information already, to name the enzyme more appropriately.

Overall, this is excellent and extremely comprehensive work. I hope that these comments are useful for the authors to further improve their paper.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
First of all, I commend you for the many experiments you conducted in response to the points I raised in my previous comments. After reading the revised manuscript, I think many of my raised points have been answered. On the other hand, there are some points in the newly added data that I do not understand, so I would like you to answer them properly.

Why is the adsorption behavior sigmoidal in Fig. 2c? I think you should give the Hill coefficient and other values for this.

Reducing or non-reducing is explained in Supplementary Fig. 34, although only cellobiose is produced in the first cleavage, cellobionic acid is produced after the second cleavage. In other words, unless the enzyme is an endo-type, the yield of cellobiose from cellulose with a degree of polymerization of 200 is only 1%, so it is unlikely to be detected as a product. Therefore, it is impossible to determine the activity direction from these experiments. In particular, the substrate-bound structure has not been obtained, and I think it is hasty to determine the direction of activity based on the docking of sugars alone.

Furthermore, in the experiment (Fig. 5a) that showed that there was a synergistic effect with endo-type enzymes but not with CBH, I think that the synergy plot is usually carried out by varying the mixing ratio of the two (e.g. 1/9, 2/8, etc.). Just adding them together is an "additive effect", so it is not clear whether it is a "synergistic effect" or not.

Basically, I am quite positive to accept this manuscript, so please address the above questions.

Referee #2

(Remarks to the Author)
The revised version of "A metalloenzyme discivered from metagenomic dark matter promotes oxidative cellulose breakdown" has been improved according the the reviewer's suggestions. The questions have been answered and supported by additional experiments. The additional experiments verify the previous conclusions. This includes: affinity measurements for cellobiose with ITC and cellulose binding isotherms as well as a competitive binding assay; additional HPAEC-PAD experiments to rule out cellobiose as a substrate and better cellobionic acid quantitation; calculated turnover numbers and substrate docking. The accumulated evidence support the previous data and show a more complete picture of the enzyme. The changed enzyme name is much better than the initially used one. Considering all changes made, I support the publication of this manuscript.

Referee #3

(Remarks to the Author)
It is clear from the response letter and revised document that the authors took all of the feedback from the reviewers very seriously. This was already a tour de force in terms of the vast amount of work in the paper, and the new added experiments have made this work even better. This paper is excellent, and it will spark a lot of follow-up mechanistic studies. I have no additional comments.

Revision and resubmission of manuscript 2024-06-11459

Dear Editor and Reviewers,

We sincerely thank you and the reviewers for your time and thoughtful feedback on our manuscript. We appreciate the positive comments regarding the potential interest of our work and have carefully considered all points raised. We have conducted additional experiments and analyses to address these concerns and believe the revised manuscript now provides a more complete and compelling picture of our work and its potential applications.

Please see below a summary of the major points addressed in the revision and also a detailed, point-by-point response to each reviewers' comments:

- **HPAEC-PAD analyses:** We have repeated all HPAEC-PAD analyses using an optimized protocol, clearly demonstrating that no cellobiose is present in the control reactions (Figs. 2b and Supplementary Figs. 6, 23, 24, 28, 35, 40 and 45), a major concern raised by Reviewer #1. Multiple controls have been included to support the results. Moreover, we showed that the enzyme did not bind cellobiose (ITC data, Supplementary Fig. 7) and no consumption of cellobiose was observed by the enzyme in the presence of ascorbic acid after 16 hours of incubation, and only residual presence of oxidized cellobiose was observed (Supplementary Fig. 8).
- **Enzyme assays:** Enzyme assays were performed using three independent enzyme preparations, starting from new transformations. Each preparation utilized new purification columns to ensure there was no contamination or other unexplained phenomena affecting the results.
- **Product quantification and turnover numbers:** the product, cellobionic acid, was quantified in all reactions and turnover number was calculated for the enzyme and compared to the literature.
- **Binding Assays with Cellulose:** Binding isotherms with microcrystalline and amorphous cellulose were obtained for the enzyme in the absence or presence of reductant and dissociation constants (K_d) and maximum binding capacities (B_{max}) were calculated (Fig. 2c,d). Competitive binding assays were also conducted using BSA as blocking agent (Supplementary Fig. 9).
- **Copper Binding Measurements:** Copper binding to the enzyme was measured using isothermal titration calorimetry (ITC, Fig. 3d), resulting in a low micromolar dissociation constant (K_d = 1.14 ± 0.11 µM), further supporting our findings.
- **Biotechnological applications:** To strengthen the biotechnological relevance of the newly discovered enzyme, CelOCE (Cellulose Oxidative Cleaving Enzyme, please see below the comments regarding the proposed name following the Reviewers' suggestions), we compared glucose release from pretreated lignocellulosic materials (sugarcane bagasse and eucalyptus) using two enzyme cocktails produced under industrially-relevant conditions (pilot plant scale and high solids loading of 15%) and benchmarked them against the commercial Cellic CTec2 cocktail (Fig. 5c). One cocktail produced by a *Trichoderma* strain expressing CelOCE, while the other expressing the well-known AA9 LPMO from *Lentinus similis* (*Ls*AA9A). Our results demonstrate that the CelOCE-containing cocktail achieved approximately 10% higher glucose release than the *Ls*AA9A-containing cocktail, also surpassing the

glucose yields of Cellic CTec2 by about 10%. This highlights the CelOCE potential for synergistic action with *Trichoderma* hydrolases, offering a promising avenue for enhancing lignocellulose breakdown in industrial bioprocesses.

- **Scaling up production in pilot-plant bioreactors:** To further emphasize the biotechnological potential, we have scaled up the production of the enzyme cocktail by the strain expressing CelOCE in 65-L and 300-L pilot plant bioreactors (Supplementary Fig. 37). Four independent cultivations of 7 days were carried out in 65-L bioreactors, resulting statistically equivalent protein titers and yields, highlighting the scalability and reproducibility from lab to pilot plant scale. For the 300-L bioreactor experiments, two independent experiments were conducted, reinforcing the scaling up of the cultivation. Biomass saccharification results were updated with the enzyme cocktail produced in the 65-L pilot-plant bioreactors, and a time-course analysis was conducted for the saccharification assays as requested.

Thank you again for your thoughtful feedback and continued consideration of our manuscript.

Yours sincerely,

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Response to referees' comments

Referee #1 (Remarks to the Author):

In this paper, the authors performed metagenomic analysis on soil samples stocked with sugarcane bagasse for decades and isolated a gene encoding a protein with a new type of β-jerry roll fold. Heterologous expression of the gene gave a recombinant protein that adsorbed on cellulose, giving products only from Avicel (cellulose hydrolysate) and phosphate swollen cellulose (PASC), and X-ray crystallography revealed that it was a homodimer and had a copper ion in the active center. Like other copper enzymes such as lytic polysaccharide monooxygenase (LPMO), it gave cellobionic acid as a product only when ascorbic acid was added, leading to the conclusion that it is an enzyme that oxidatively degrades cellulose in an exo-type from the end of the cellulose chain. The volume of experiments is enormous, and the fact that each experiment is very thorough is a good impression, but on the other hand, there are few biochemical, especially quantitative, experiments and no appropriate control experiments, and the reviewer felt that it was too early to conclude that "this enzyme is a new oxidative cellulase". The reasons for this are given below.

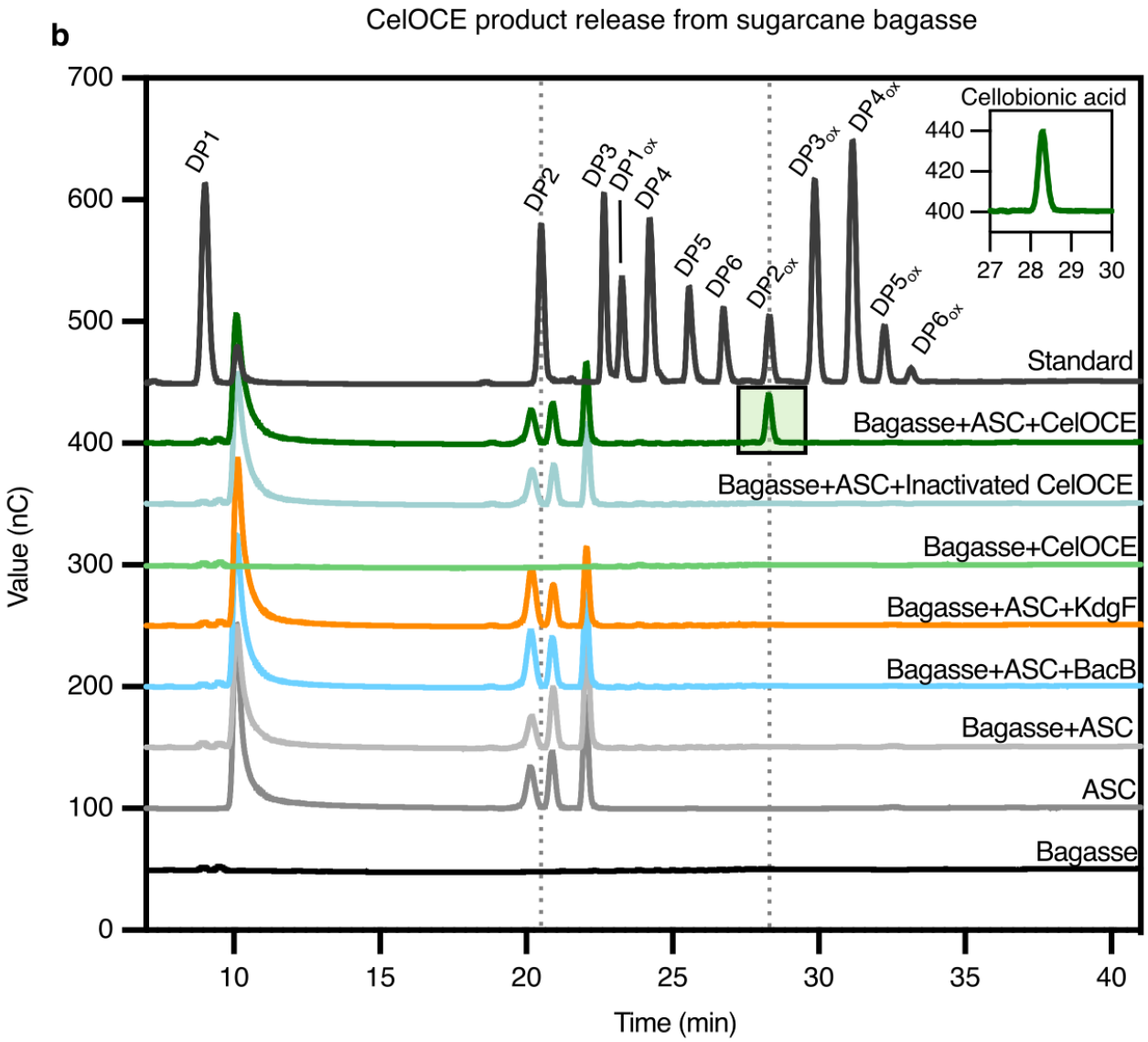
What the reviewer finds most critical is that cellobiose is being made from cellulose substrates such as Avicel and PASC simply by adding ascorbic acid without the enzyme. It is not clear what the reason for this is, but if cellobiose is released from the above cellulose substrates and the enzyme just oxidizes the reducing end of it, like cellobiose dehydrogenase, the same results are obtained in all experiments. Although the authors have confirmed no degradation activity against cellooligosaccharides with DP=3-6, they have not confirmed the oxidative activity of cellobiose, so the conclusion that this is a "new type of cellobiose dehydrogenase" cannot be ruled out.

R: We acknowledge the concern of the Reviewer #1 regarding the release of cellobiose from cellulose in the presence of ascorbic acid alone. To address this issue, we optimized the HPAEC-PAD protocol, performed additional control experiments and repeated all experiments involving this technique, including quantification of the product cellobionic acid. As shown in revised Fig. 2b and also Supplementary Figs. 6, 22, 23, 27, 34, 39 and 45, ascorbic acid generates four different peaks, and using the previous protocol, one of these peaks was co-eluting with cellobiose. With the optimized protocol, this issue has been resolved, and **it is now clear that cellobiose is not present in the controls or in cellulose reactions.**

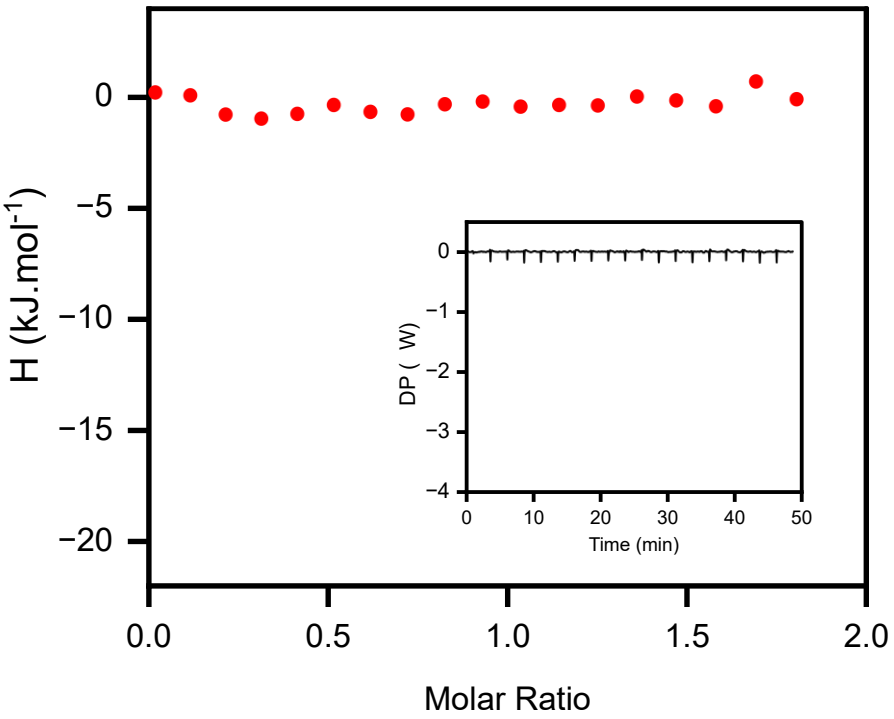
With this improved protocol, we successfully separated both non-oxidized and oxidized cellooligosaccharides in the same chromatographic run. Please see standards from Fig. 2b, for instance.

Based on these results, we have demonstrated that the cellobionic acid, generated in the reaction with the discovered enzyme and sugarcane bagasse or Avicel in the presence of ascorbic acid, is the product of the reaction. Thus, this enzyme acts on cellulose chains with exo-type activity. It does not display any "cellobiose dehydrogenase" activity on cellobiose. Moreover, as demonstrated in the New Supplementary Fig. 7, our **ITC measurements indicate that the enzyme does not exhibit any affinity for cellobiose under tested conditions**. Additionally, when conducting reactions with cellobiose in the presence or absence of ascorbic acid, **we observed no relevant cellobiose consumption even after 16 hours and only residual presence of cellobionic acid under the tested conditions** (New Supplementary Fig. 8).

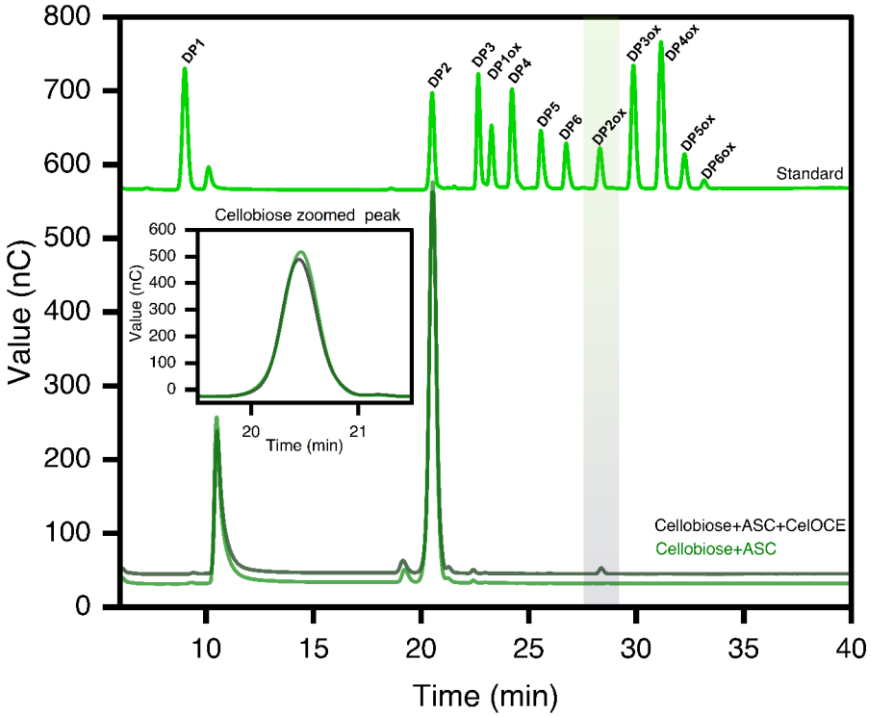
In summary, the presented data and methodological refinements provide compelling evidence against cellobiose dehydrogenase-like activity, further solidifying our conclusion that the novel enzyme acts directly on cellulose chains.



New Fig. 2b. HPAEC-PAD chromatograms of control and experimental reactions with CelOCE and sugarcane bagasse. Standards of non-oxidized and oxidized cello-oligosaccharides are shown for reference. These data highlight absence of cellobiose in all reactions.



New Supplementary Fig. 7. Isothermal titration calorimetry analysis of cellobiose binding to CelOCE. The ITC thermogram and binding isotherm demonstrate a lack of detectable heat change upon cellobiose titration into CelOCE, indicating no significant binding affinity under the tested conditions.

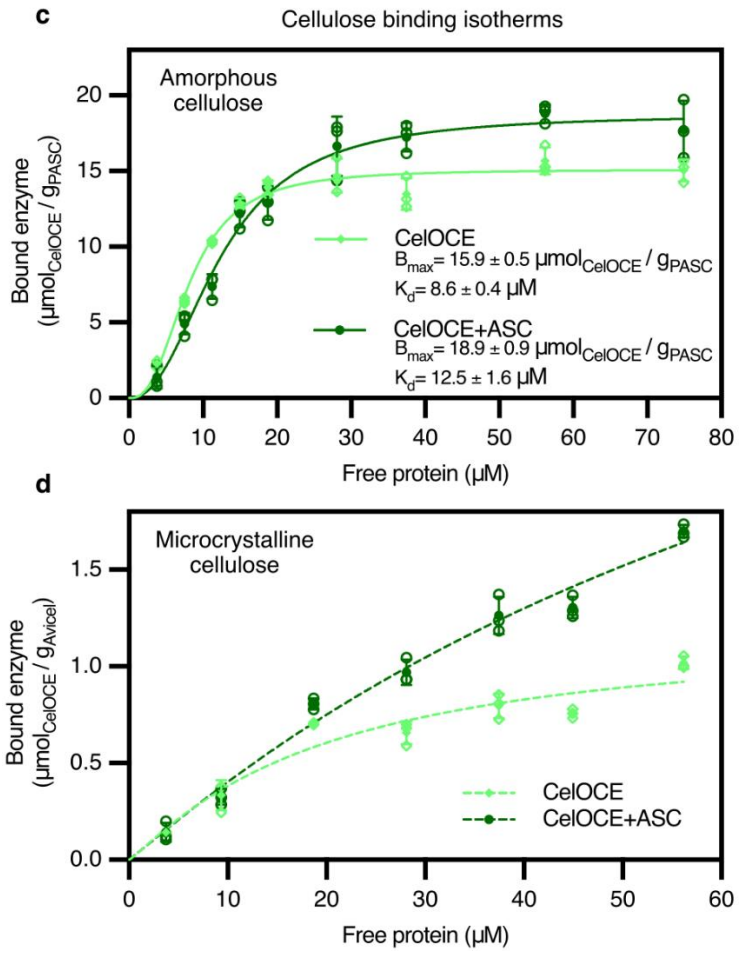


New Supplementary Fig. 8. HPAEC-PAD chromatograms of control and experimental reactions with CelOCE and 50 µM cellobiose as a substrate. Standards of non-oxidized and oxidized cello-oligosaccharides are shown for reference. These data highlight no relevant cellobiose consumption after 16 hours reaction time. Only residual presence of cellobionic acid was observed under the tested conditions.

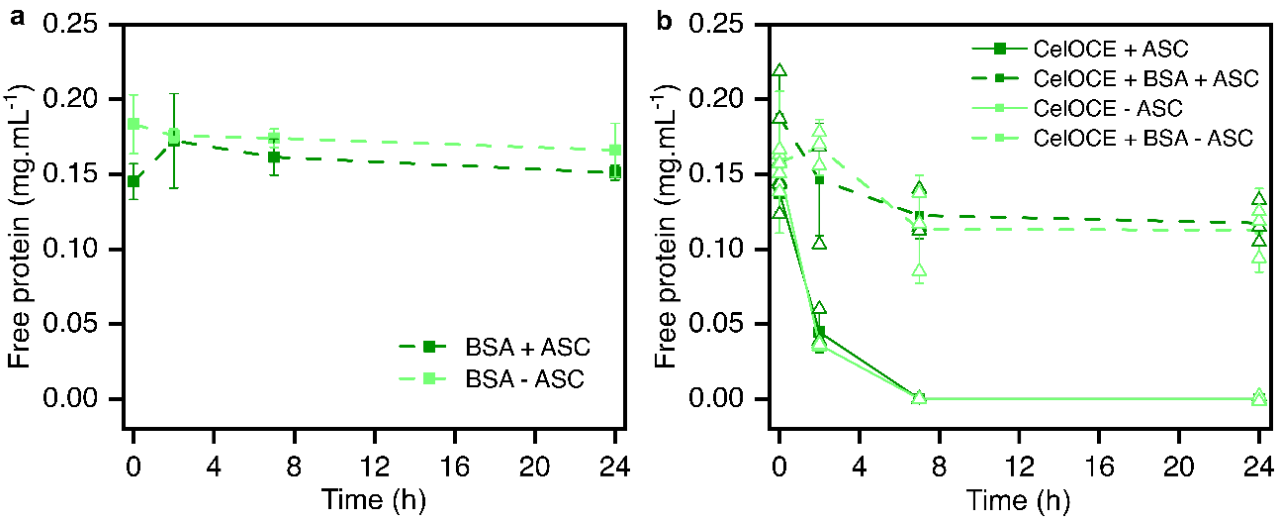
In addition, although the adsorption of the new protein on cellulose is observed, many hydrophobic proteins should be adsorbed in such a qualitative experiment, and it should not be said that "this protein has adsorption ability on cellulose" with this result. For example, adsorption of cellulase on cellulose can occur even with the use of blocking agents such as BSA, but what about this protein?

R: We thank the Reviewer for highlighting this point and we agree that additional experiments would strengthen our conclusions as also pointed by Reviewer #2, principally providing quantitative data.

We have therefore performed binding isotherm experiments to determine the dissociation constant (K_d) and maximum binding capacity (B_{max}) for both oxidized and reduced enzyme forms on Avicel and PASC (New Fig. 2c-d). As with cellulose-active LPMOs, we observed low micromolar K_d values for PASC, while saturation was not reached for Avicel. Interestingly, the absence or presence of reductant (ascorbic acid) did not affect the binding as much as observed for LPMOs (Kracher et al., 2018) (New Fig. 2c-d). This difference likely reflects the buried nature of the catalytic copper in our enzyme compared to the exposed copper in LPMOs. Moreover, even in the presence of BSA as blocking agent (BSA competitive binding assay), the enzyme retained its adsorption ability to cellulose in levels similar to that observed for cellulases (New Supplementary Fig. 12) (Yang et al., 2006).



New Fig. 2c-d. Binding isotherms of CelOCE on amorphous (PASC) and microcrystalline (Avicel) cellulose. Binding isotherms revealed dissociation constants (K_d) in the low micromolar range for amorphous cellulose (PASC), while saturation was not reached for microcrystalline cellulose (Avicel), similar to what has been observed for cellulose-active LPMOs.

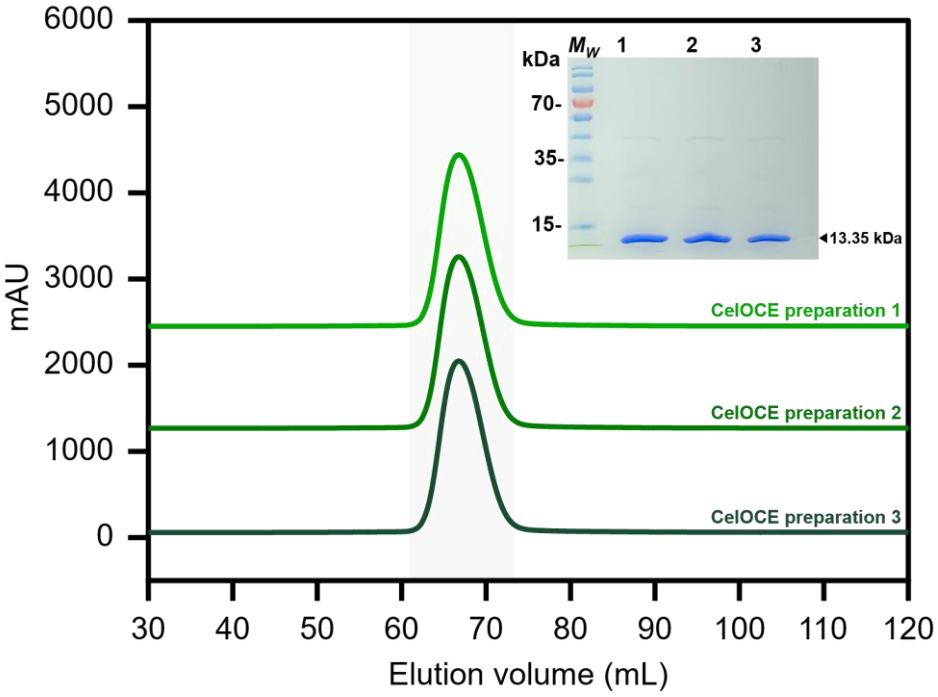


New Supplementary Fig. 12. CelOCE competitive binding assay. Binding capacity of CelOCE to 5% (*w/v*) Avicel in the presence of bovine serum albumin (BSA) as blocking agent. (a) Control experiments were conducted to assess the individual binding of BSA to Avicel. These experiments involved incubating BSA alone with Avicel, ensuring that BSA did not bind to Avicel. (b) Comparison of the binding capacity of CelOCE, with or without reductant (ascorbic acid), to Avicel, in the presence and absence of BSA. All reactions were performed at the same mass concentration of CelOCE (0.17 mg/mL). To visualize the specific binding of CelOCE in the presence of BSA, the BSA-only curves were subtracted from the corresponding "CelOCE + BSA" curves. Results are expressed as mean ± standard deviation from three independent experiments ($n = 3$).

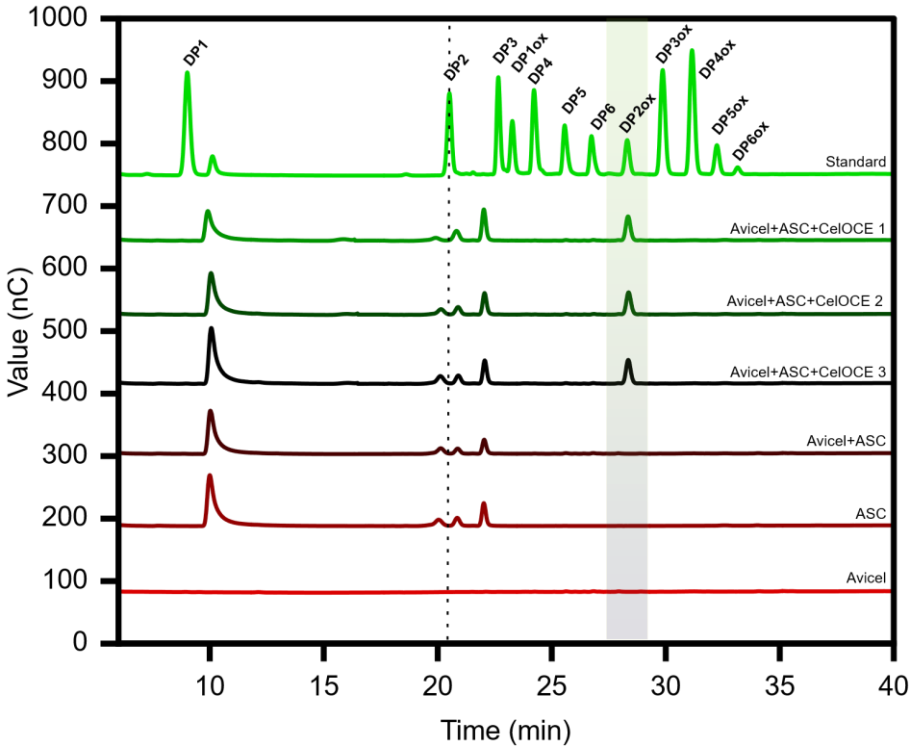
I believe that it is essential to quantitatively follow the changes over time to demonstrate the activity of a new enzyme. For example, the authors incubate the enzyme for 16 hours at 37°C. What is the time profile during this period? 1 microM of the enzyme is used, but how many microM of product is produced in how many hours and what is the specific activity? When claiming a new activity, we must also be particularly careful about contamination, and to rule out this possibility, we need to examine the enzyme over time with different preparations of the enzyme.

R: We thank the Reviewer for bringing up this important point for the robustness of our conclusions. To address this, we performed three independent enzyme preparations, each starting from new transformations and utilizing brand-new purification columns. This approach ensures that the results are free from contamination or any other factors that could affect the enzyme assays (see revised Supplementary Fig. 44).

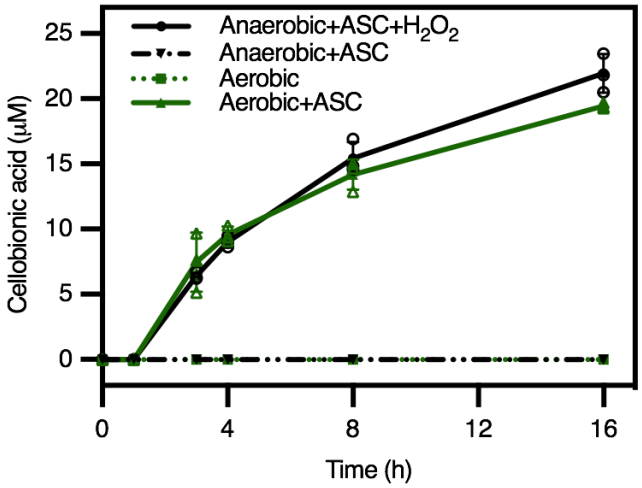
As depicted in the New Supplementary Fig. 45, all three independent enzyme preparations consistently produced cellobionic acid as the primary product. Furthermore, we monitored product release over time, demonstrating clear cellobionic acid accumulation as sole product from the very beginning of the reaction, supporting its exo mode of action (New Fig. 3f). The products were quantitatively measured, and the data were expressed as specific activity, providing a robust and reproducible characterization of the enzyme activity. Using the linear range of the time course data, we calculated the turnover rate of 5.3×10^{-2} min^{-1} on Avicel, which is comparable with values obtained for some cellulose-active LPMOs without the addition of exogenous hydrogen peroxide (New Supplementary Table 9). It is worth noting that CelOCE is a non-processive (pocket-like active site topology) and exo-acting enzyme, which is typically slower than endo-enzymes. Moreover, without the presence of an endo-acting enzyme generating novel ends, its activity is limited by the natural occurrence of non-reducing ends in the cellulosic substrate.



New Supplementary Fig. 44. Quality control of CelOCE enzyme preparations. Size-exclusion chromatograms and SDS-PAGE analyses of three independent CelOCE enzyme preparations obtained from new transformants and purified using previously unused columns. These data confirm the purity and integrity of the enzyme preparations used in subsequent experiments.



New Supplementary Fig. 45. HPAEC-PAD chromatograms for three independent CelOCE enzyme preparations. The consistent production of cellobionic acid as the sole product across all three preparations highlights the reproducibility and specificity of CelOCE catalytic activity.



New Fig. 3f. Time-dependent analysis of cellobionic acid production by CelOCE under aerobic and anaerobic conditions. Anaerobic reactions were conducted within a Whitley DG250 Anaerobic Workstation and supplemented with 100 µM H_2O_2 added initially. Data points represent the mean ± standard deviation of three independent experiments (*n* = 3).

New Supplementary Table 9. Comparison of turnover rates of CelOCE and LPMOs. The turnover rate observed for CelOCE falls within the range seen for some LPMOs acting on Avicel under aerobic conditions without the addition of exogenous hydrogen peroxide.

Enzyme (conc.)	Avicel ($g.L^{-1}$)	Oxygen source	Turnover (min^{-1})	Reference
CelOCE (1 µM)	1	O_2	0.05	This study
SscLPMO10B (0.5 µM)	10	O_2	0.12	Stepnov et al., 2022a
TbAA10-CBM2 (1 µM)	0.3	O_2	0.10	Crouch et al., 2016
ScLPMO10C (2 µM)	10	O_2	0.05	Stepnov et al., 2022b
AA10_07 (1 µM)	10	O_2	0.06	Stepnov et al., 2022a

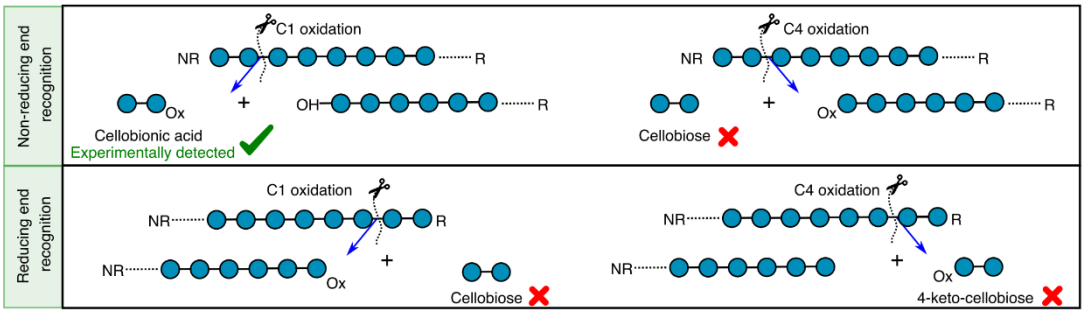
All the reactions, the reductant used was ascorbic acid at 1 mM.

The fact that this enzyme is a homodimer is another reason for skepticism. In Figure 3, the authors speculate that hydrogen peroxide is supplied by one monomer and oxidatively cuts the glucoside bond at the other, but in this case, what happens to the activity in the second round? In addition, the authors draw a picture as if cellulose is degraded from the non-reducing end, but could the same picture be drawn from the reducing end?

R: We appreciate the reviewer raising these insightful questions, particularly concerning the homodimeric nature of the enzyme and its proposed mechanism. The role of hydrogen peroxide has been extensively investigated as a co-substrate in LPMO-catalyzed reactions as reviewed in Munzone et al. (2024) and recent studies have demonstrated that hydrogen peroxide can be generated *in situ* either by macromolecular partners or by LPMO molecules not engaged with the cellulose fiber. For instance, it has been shown that Cellobiose Dehydrogenases (CDHs), have the dual capacity to both reduce the LPMO and generate hydrogen peroxide to fuel the LPMOs peroxygenase activity (Kracher et al. 2020).

Our model is also consistent with recent findings by Tamburrini et al. (2024), where it was proposed that LPMOs with a disordered C-terminal region dimerize, positively affecting both their binding affinity and catalytic activity on cellulose. Similar to our findings, the authors propose that one subunit of the dimeric enzyme interacts with cellulose while the other subunit remains free to generate hydrogen peroxide. This configuration increases the efficiency of hydrogen peroxide utilization by the adjacent subunit. Due to its non-processive nature (pocket-like active site), CelOCE likely releases and re-attaches to cellulose at new locations after each catalytic cycle. The three-dimensional architecture of the dimer suggests that both active sites are unlikely to interact simultaneously, allowing one active site to generate hydrogen peroxide for the other during each catalytic cycle. Under laboratory conditions, the reductant restores the functional copper redox state, while under industrial conditions, components of the feedstock, such as lignin, are expected to serve as electron donors.

Regarding the question of whether the enzyme could attack the reducing ends of cellulose chains, our inference is based on the pocket-like topology of the active site and the strict C1 regioselectivity observed with the detection of cellobionic acid as the sole product. Docking studies with a tetrasaccharide suggest that recognition of the reducing end, as opposed to the non-reducing end, would be unfavorable for the reaction stereochemistry involving the catalytic copper. If the enzyme could recognize both ends, we would expect to observe C4 oxidation and cellobiose as well; however, these molecules were not detected in our experiments. In the New Supplementary Fig. 34, it is represented a scheme indicating putative products according to enzyme regioselectivity and mode of substrate recognition. The detection of cellobionic acid as the sole product along with the structural data indicate only one possible mode of action, which involves the non-reducing end recognition with C1 sugar regioselectivity.



Supplementary Fig. 34. Schematic representation of putative modes of substrate recognition and oxidative cleavage by CelOCE.

As described above, if this enzyme improves the degradation activity of cellulase in real-time, it is a very useful discovery for the enzymatic degradation of cellulosic biomass, but on the other hand, the paper is composed of too many speculations, so at this stage, I cannot propose acceptance for publication in Nature.

R: We recognize the Reviewer concern regarding the need for additional experiments to support our discovery. In response, we have conducted the following experiments to strengthen our findings. Specifically:

- We have repeated all HPAEC-PAD experiments, including comprehensive controls, to clarify that the enzyme indeed generates cellobionic acid from cellulose, and we resolved any ambiguity regarding the presence of cellobiose in the controls.
- We quantitatively measured the over the time product release by the enzyme and calculated the turnover rates, under both aerobic and anerobic conditions.
- Binding isotherms were determined, and the dissociation constants for microcrystalline and amorphous cellulose binding were calculated. We also performed BSA competitive binding assays showing the enzyme retaining its capacity to interact with cellulose.
- Copper binding affinity was measured using isothermal titration calorimetry (ITC), resulting in a dissociation constant in the low micromolar range, underscoring the enzyme binding to the catalytic copper.
- To eliminate the possibility of contamination, we performed three independent enzyme preparations using new purification columns. All preparations consistently produced cellobionic acid, confirming the enzyme activity.
- The depolymerization of cellulosic biomass was demonstrated using an enzyme cocktail containing CelOCE, produced by an industrially relevant *Trichoderma* strain under typical operational conditions, ie, high total solids (15%) and 10 mg enzyme/g biomass at 50ºC and pH 5.0, 48-72 hours.
- Additionally, we scaled up the strain expressing CelOCE in 65-L and 300-L pilot-plant bioreactors, successfully performing six independent cultivations (4 in 65-L and 2 in 300-L), demonstrating the scalability, robustness and industrial applicability of our findings.
- To further emphasize the biotechnological relevance of this enzyme, we engineered the same *Trichoderma* strain to express a well-studied LPMO from *Lentinus similis* (*Ls*AA9A), known for its thermotolerance under industrial conditions. Remarkably, the enzyme cocktail containing CelOCE outperformed the one containing *Ls*AA9A and the commercial cocktail Cellic CTec2, highlighting the potential of CelOCE for lignocellulose biorefinery applications.

Taken together, these additional experiments, conducted at both bench and pilot plant scales, substantially increase the relevance and potential impact of our discovery. We believe that these efforts address the major concerns.

P.S. There are some noticeable deficiencies in the references, such as not italicizing the scientific names and not using abbreviations for the names of the papers.

R: We apologize for the oversight in the formatting of the references. We have carefully revised and double-checked all citations in the manuscript to ensure that scientific names are appropriately italicized, and journal abbreviations adhere to the journal style guidelines.

Referee #2 (Remarks to the Author):

General Comments

The manuscript on “A metalloenzyme discovered from microbial dark matter promotes oxidative cellulose oxidation” by Murakami and colls. describes a highly interesting study starting with the metabolomic isolation and identification of an unknown gene, continuing with its expression and biochemical investigation and ending with its elucidated structure. The key results of this study is a new bacterial enzyme involved in cellulose depolymerization that is of potential interest for the biobased economy, especially biorefineries. The described enzyme is similar, but different from the well-investigated lytic polysaccharide monooxygenases (LPMOs). It is interesting to see such a small copper enzyme with such a high substrate specificity. Even smaller than most LPMOs it features besides a flat, substrate binding interface a substrate binding pocket for the non-reducing, terminal cellobiose unit of cellulose. The copper center ligation is quite different from LPMO, but seems to perform a similar reaction. Unfortunately, the authors do not report the specific activity or turnover numbers of the enzyme, but demonstrate the activity by the acceleration of a complex enzymatic mixture or rater qualitative conversion experiments. To increase the interest in this work to the level adequate of this journal, a minimal kinetic characterization of CellOx is a must. Nevertheless, the presented enzyme is a new enzyme of significant interest, the manuscripts reports original research, the instrumental and computational methods are valid and the statistical methods appropriate. The interpretation of results is clear and valid and discussed in combination with reliable references.

R: We sincerely appreciate the positive assessment of our work and the recognition of its potential significance for a bio-based economy. We agree that the lack of turnover rates is a limitation that needs to be addressed to enhance the impact of this study. In response to this valuable feedback, we have conducted additional experiments to characterize the enzyme activity including the cellobionic acid quantification in all reactions. The turnover rates were determined as discussed above and in the specific comments below. These new data provide a more comprehensive understanding of the enzyme catalytic capabilities.

Specific Comments and requested improvements

Title: An important bit of information would be the bacterial origin of the new enzyme. “Microbial” is ambiguous and leave the reader with the question: fungal or bacterial?

R: We acknowledge that the term "microbial" might not be the most precise way to describe the origin of this enzyme. As the enzyme was discovered within a metagenome-assembled genome (MAG) obtained from a metagenomic study and belongs to an uncultured bacterial phylum 4 (UBP4), we have replaced "microbial" with "metagenomic" in the title. This change better reflects the approach used in this study. Additionally, we have observed orthologous sequences in archaea, further supporting the use of the broader term "metagenomic" rather than "bacterial" to describe the enzyme origin.

Line 41: “Here” is not a good start of the sentence.

R: Thanks for pointing this out. The word “here” was avoided, and the sentence now reads “By mining the metagenomic dark matter of a microbial community specialized in lignocellulose digestion, we discovered a metalloenzyme that oxidatively cleaves cellulose with C1 regioselectivity via an exo-acting mechanism.”

Line 44: The term “cellulose-compatible catalytic interface” is quite original, but considering that an active site consists of a catalytic site and a binding site, the phrasing should use the term “specificity” instead of “compatible” and “binding site” instead of “catalytic interface”.

R: The text has been changed accordingly and now reads “The structure reveals a catalytic copper embedded in a compact jelly-roll scaffold that features a cellulose binding site.”

Line 49: While the term “microbial” used above in the abstract is fine, here it is not, because bacteria and fungi differ in their secreted oxidoreductases and produced reaction products and intermediates. It is also suggested to specify the term “redox systems”, e.g. “bacterial enzymatic redox systems”.

R: The term 'redox systems' has been updated to 'bacterial redox enzymatic systems' to emphasize the redox nature of this enzymatic system in bacteria."

Line 76: Why is cellulose “despite being a homopolymer” recalcitrant? This should be specified, e.g. by the microfibrillar, highly ordered structure. The reason why heteropolysaccharides, especially highly decorated ones, are recalicitrant to enzymatic hydrolysis has a different reason.

R: We appreciate the reviewer's attention to this part of the text. We have revised the sentence to provide a more specific explanation:

“Cellulose, the most abundant renewable polymer on Earth, poses a significant challenge for biological depolymerization. Although composed entirely of glucose units, its crystalline microfibrillar structure, along with its association with lignin and hemicellulose in plant cell walls, makes it highly recalcitrant. As a result, its breakdown in nature is slow and involves complex multi-component enzymatic systems.”

This revision now highlights the key structural features and interactions that contribute to cellulose's resistance to enzymatic breakdown.

Line 94: “carbohydrate enzymology”, “fourth generation synchrotron methods” – use more specific terms

R: The terms have been expanded to provide more specific descriptions of the techniques used.

“carbohydrate enzymology” has been replaced with “carbohydrate enzymology by anion-exchange chromatographic, colorimetric and mass spectrometric methods”

“fourth generation synchrotron methods” is now “Fourth-generation synchrotron-based X-ray diffraction, fluorescence, and absorption spectroscopies”

Line 112: “was further investigated due to its uncharted lignocellulolytic potential” – two points: please describe more specifically the markers that were used to identify the potential of the MAGs and “ligno” is not correct in this case when only cellulolytic/hemicellulolytic enzymes are targeted by the study.

R: Thank you for highlighting this point. We have revised the text accordingly as shown below.

“Among the recovered high-quality MAGs (Fig. 1c and Supplementary table 1 and Dataset 1), one member from a recently proposed and uncharacterized uncultured bacterial phylum 4 (UBP4) was further investigated

due to its uncharted potential for plant cell wall breakdown, evidenced by multiple genes encoding glycosyl hydrolases, such as those from families GH3, GH5, GH9, GH39, GH43, GH44, GH74 and GH148 (Fig. 1c,d)."

Lines 121-123: Sure, it's a screening, but please indicate that the boosting effect depends on the presence/absence of many enzymes in T. reesei and it is not straightforward that a bacterial enzyme boosts a fungal enzyme system.

R: The text was revised to provide additional details about the *T. reesei* enzyme cocktail composition and to emphasize the remarkable boosting effect of a bacterial enzyme in a comprehensive fungal CAZyme system, as shown below:

"Remarkably, one of these proteins showed the capacity to boost the depolymerization of lignocellulose (~20% improvement) by a cellulolytic enzyme cocktail produced by an industrially competitive *Trichoderma reesei* strain (Fig. 2a). This fungal cocktail comprises key enzymatic activities for the efficient depolymerization of cellulose and heteroxylans, including cellobiohydrolases (CBH1 and CBH2), endo-β-glucanases (EGL1, EGL2 and EGL5), LPMO (LPMO9A), β-glucosidase (heterologous ReCEL3A), endo-xylanases (XYN1, XYN2 and XYN4), β-xylosidase (BXL1), and other accessory enzymes (Supplementary Table 4 and Dataset 2). The fact that a bacterial enzyme can enhance the performance of an optimized fungal CAZyme cocktail is notable and highlights its potential for plant biomass degradation applications."

Line 127: use instead: "via a redox mechanism"

R: It was modified accordingly.

Line 133: In Figure 1, also xyloglucan and arabinoxylan are given as substrates for metabolic pathways in SBS.bin.55. These are also on the list of substrates, but not shown here or in fig. S5. If already indicated in Fig.1, the reader expects to see these substrates tested.

R: Thank you for pointing this out. We have now included the results for xyloglucan and arabinoxylan in Supplementary Fig. 5, highlighting that the enzyme did not release any oxidized sugars from these substrates.

Lines 138-143: The reported methods showed a strong adsorption, but why was the Kd not measured? This would be more important that three methods just qualitatively indicating binding.
R: We acknowledge the importance of quantitative binding data. Please see the response to the Reviewer #1, in which we discuss the binding isotherms (New Fig. 2c,d) and the experiment using BSA as blocking agent (New supplementary Fig. 12).

Line 145: The name "CellOx" is ill chosen. "Ox" indicated an oxidase, which the enzyme certainly is not. The authors later state that they thik it is a similar mechanism as in LPMO, a peroxygenase reaction. Then the name should reflect this and follow the guidelines given by the enzyme commission.

R: Thank you for bringing this to our attention. We agree that "CellOx" could be misleading, as it might imply oxidase activity, which does not accurately describe the enzyme's primary function. We recognize the challenges of naming redox enzymes, as seen with LPMOs, which may need revision following recent insights into the peroxygenase mechanism of action.

To avoid similar issues, we propose naming **this enzyme as "Cellulose Oxidative Cleaving Enzyme" (CelOCE)**. This name is both descriptive and concise, accurately reflecting the enzyme role in the oxidative

cleavage of cellulose. It also adheres to enzyme nomenclature conventions and provides flexibility for broader substrate specificity or future discoveries of **other enzymes from the same class**. The abbreviation "CelOCE" is clear and easy to recall.

Line 158: In this context “oxidoreductase” would be more suitable.

Line 159: rephrase “a distinguished mode of action”, maybe a “unique activity”.

R: Based on these comments and other suggestions, this sentence now reads:

“Our results support the discovery of a Cellulose Oxidative Cleaving Enzyme (CelOCE) from an uncharted phylum associated with plant cell wall breakdown. CelOCE cleaves cellulose via an unprecedented exo-acting mechanism, releasing cellobionic acid as the sole product.”

Line 164: rephrase “long-term soil”

R: The “long-term soil” was rephrased to “long-term sugarcane bagasse-covered soil”. The figure title was revised and now reads:

“Unprecedented bacterial taxa associated with plant cell wall breakdown revealed through metagenomic analysis of long-term sugarcane bagasse-covered soil.”

Figure 2B: In the conversion reactions analyzed by HPAEC-PAD, CellOx, KdgF and BagB produce also cellobiose (the DP2 peak). This needs discussion since the peaks are quite prominent compared to the cellobionic acid peak of CellOx. Is this an artefact? Are the other enzymes also active on cellulose? Ist CellOx not only producing cellobionic acid, but also cellobiose? Please comment on the reaction products seen at DP2.

R: We sincerely appreciate your comment on this point that also was a concern of the Reviewer #1. As mentioned in the response to Reviewer #1, the peak that was superposing with cellobiose was actually something derived from ascorbic acid. Note that in the original version of the manuscript, this artifact only appeared in the presence of ascorbic acid. For instance, this peak is not present in the experiment with sugarcane bagasse in which ascorbic acid was not added. However, to avoid this, the HPAEC-PAD protocol was optimized, and all peaks are now resolved without any superposition (New Fig. 2b).

Line 209: Use “copper atom” instead of “copper metal” since a metal is a material with unique properties based on the atomic orbitals of its constituting element(s).

R: R: We thank the Reviewer for pointing this out. We have modified the text to "copper atom" instead of "copper metal."

Line 227: “Supporting the function of copper for catalysis”

R: Thank you for the suggestion. The text was revised accordingly.

Line 248 and elsewhere: The term “oxidative activity” is not well chosen for an peroxygenase. Also “redox chemistry” is very vague for the intended meaning “redox mechanism”.

R: Given the potential impact of this comment on our revision, we sought further clarification from the Reviewer through the Editor, who kindly provided the following response:

“The authors can use 'oxidation' as a general, generic term for the reaction they observe, which is fine. However, if they already know it is a peroxygenase, why not use 'peroxygenase activity' instead of 'oxidative activity' or 'oxidation activity,' which are unspecific and might lead to the wrong connotation for readers. The term 'oxidase activity' is definitely not a good choice. A lot of the confusion in LPMO reaction mechanisms originates from the unfortunately wrong, and not yet changed name 'lytic polysaccharide monooxygenase.' In this regard, the name the authors chose for the enzyme also concerns me, as I foresee similar issues as with LPMO naming.”

In light of this clarification, we have revised the manuscript to avoid using the term “oxidative activity” when referring to the primary activity of the enzyme (cellulose cleavage), as it is unspecific and could lead to reader misinterpretation. As suggested, we have replaced "oxidative activity" with "peroxygenase activity" where appropriate. The terms "oxidation", “oxidative” and "oxidatively" have been retained only in general contexts, such as in the title and abstract, where we believe their use is clear and appropriate. Additionally, we have replaced the term "redox chemistry" with the more precise term "redox mechanism" throughout the manuscript.

Line 256: “a comparative time-course analysis” is what? A kinetic analysis?

R: The generation of cellobionic acid (the only product of the enzyme) was monitored over the time from 1 to 16 hours. In the original version of the paper the production was followed by the area from HPAEC-PAD chromatograms; however, in the revised version we quantified the cellobionic acid and the turnover rates were calculated based on the linear range of the enzyme. We did not call it kinetic analysis because one would expect a typical substrate saturation curve, which is not feasible for cellulosic substrates such as PASC or Avicel. Therefore, instead of, we referred it as a comparative product release analysis over the time, providing now the turnover rates for the enzyme as discussed above.

Figure 4C: There are no “activity rates”. Activity as a kinetic term would here be already far stretched, since no units, rates are given. Just the relative production of cellobionic acid during the experiment for which one has to search carefully in the material and methods section to find the time of the experiments (16 h). Please calculate e.g. turnover numbers from this experiment, it would be so much more useful to compare the performance of CellOx to other enzymes.

R: We agree that presenting turnover rates will offer a more accurate and informative assessment of enzyme activity, enabling meaningful comparisons with other enzymes reported in the literature. As mentioned earlier, cellobionic acid production was quantified and the turnover rates was provided in the text.

Line 296: A “mutant” is typically a modified organism. Here “mutant enzyme” or “enzyme variant” should be used.

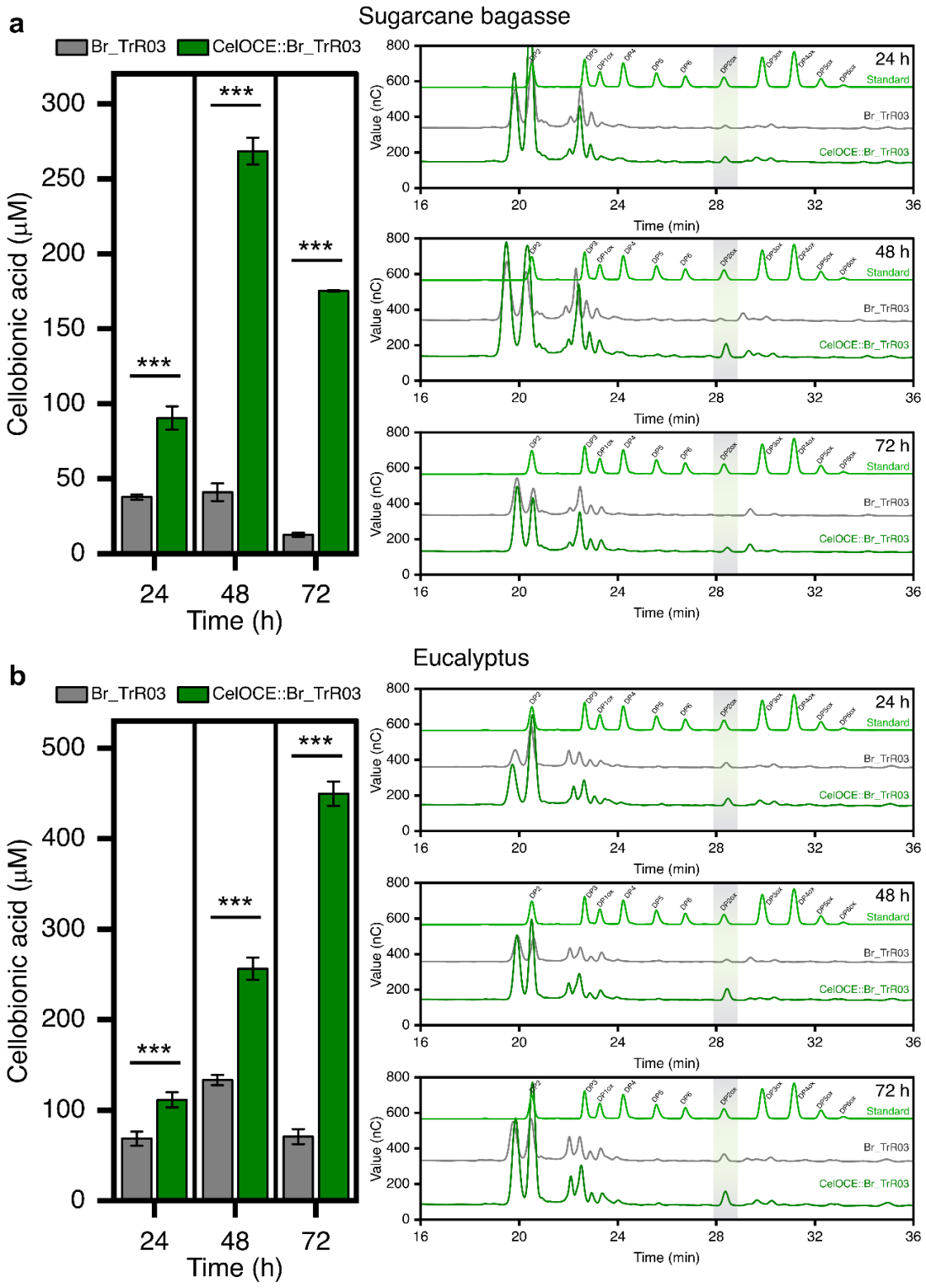
R: We thank the Reviewer for pointing this out. We have replaced the term "mutant" with the more appropriate "enzyme variant" in the manuscript to avoid any confusion.

Line 309: It would be so much better to say “carbohydrate active oxidoreductases” then “carbohydrate oxidative enzymes”.

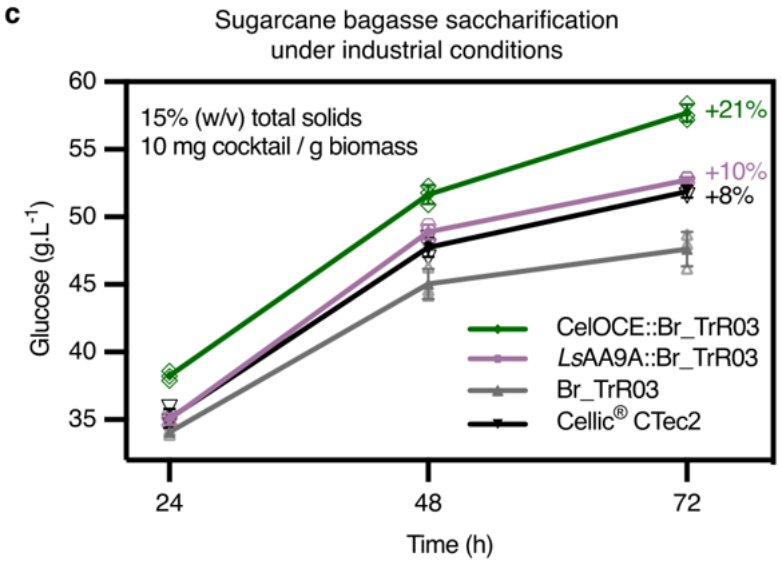
R: We appreciate the suggestion for improved terminology. We have replaced "carbohydrate oxidative enzymes" with the more precise and informative term "carbohydrate-active oxidoreductases" in the revised manuscript.

Line 326: It would be very good for the comparison and interpretation of the results to have the measured/estimated concentration/percentage of CellOx in the secretome.

R: This information is available in Supplementary Table 10 and Dataset 3. According to semi-quantitative LC-MS proteomic analysis, CelOCE was identified in the secretome, with its estimated concentration based on fingerprint peptide analysis at approximately 0.1%. For comparison, the most abundant CAZyme, cellobiohydrolase Cel7A, constitutes ~15% of the secreted CAZymes. Despite the low relative abundance, the amounts of cellobionic acid produced increased in approximately 10-fold in the CelOCE-containing cocktail (New Supplementary Fig. 40) and the synergism was superior to that observed for an LPMO-containing cocktail (New panel 5c), where the relative abundance of *Ls*AA9A is ~1.2%, about 12 times higher than CelOCE. These results highlight the remarkable synergistic effect of this enzyme with the *Trichoderma* secretome in enhancing biomass saccharification.



New Supplementary Fig. 40: Cellobionic acid quantification in the enzymatic hydrolysates of pretreated sugarcane bagasse and eucalyptus by the secretome of the parental (Br_TrR03) and engineered (CelOCE::Br_TrR03) *T. reesei* strains. Cellobionic acid was quantified by HPAEC-PAD analysis using a standard curve. Increased release of cellobionic acid was observed in the saccharification assays performed with the secretome of the strain co- expressing CelOCE. The data has been recorded at 24, 48 and 72 h of hydrolysis. Data consists of three independent experiments and are expressed as mean ± SD ($n = 3$). Tukey post hoc tests were performed ($P < 0.05$) and asterisk bars present significant statistical differences.



New Panel 5c: **Saccharification efficiency of enzyme cocktails produced by the engineered strains (CelOCE or *Ls*AA9A expressing strains) under industrially relevant conditions, using pretreated sugarcane bagasse as the substrate.** Results are expressed as mean ± standard deviation from three independent experiments ($n = 3$).

Figure 5: Relative activities are awful, please report the formed product concentration, even as concentration of reducing ends.

R: We appreciate the feedback regarding the presentation of activity data in Fig. 5. We have updated this figure to report the absolute concentration of reducing or oxidized sugars, providing a more informative and comparable measure of enzyme activity. All relevant data in the text and tables have also been updated accordingly.

Line 370: again “carbohydrate enzymology”

R: We acknowledge the previous feedback regarding the term "carbohydrate enzymology" and have revisited its usage throughout the manuscript. This paragraph now reads:

“This discovery opens new avenues in redox biochemistry for plant polysaccharide depolymerization by revealing that copper-catalyzed peroxygenase reactions, hitherto restricted to LPMOs, have evolved in other biocatalysts. Furthermore, by shedding new light on the bacterial redox systems involved in carbohydrate breakdown and metabolism, this study impacts our understanding of the mechanisms underlying the global carbon cycle and creates new opportunities for the bioconversion of agro-industrial residues into value-added bioproducts.”

Line 373: replace “microbial” by “bacterial”

R: We acknowledge the Reviewer comment and have replaced "microbial" with the more specific term "bacterial".

Supplementary Materials:

Description of anaerobic experiments: The experimental time is not given.

R: We recognize the lack of this information for the anaerobic assays. We have now included this information in the relevant section of the experimental procedures.

Fig. S5: Where are the measurements of xyloglucan and arabinoxylan?

R: The results for xyloglucan and arabinoxylan have been added to the New Supplementary Fig. 6, confirming that no oxidized products were generated from these polysaccharides.

Fig. S17: Here again a product below the DP2 standard is visible beside the cellobionic acid. It is difficult to quantify, but might be around 5% of the main product. Is this a side activity pf"CellOx"?

R: We appreciate the observation regarding this peak in the old Fig. S17. As discussed above, we have shown that this peak derives from the ascorbic acid degradation, and it was resolved with the optimized HPAEC-PAD protocol.

Fig. S19: This figure as a bar chart is difficult to interpret. A diagram with two lines connecting the datapoints of the two datasets and a continuously scaled X-axis would allow the reader to evaluated the reaction in regard to steady-state conditions, enzyme deactivation, etc. Also the Y-axis should show the concentration of cellobionic acid, so that one can calculate turnover numbers. This would be important to evaluate the argument that oxygen is a similar or even better cosubstrate than hydrogen peroxide for "CellOx". Under the assumption that after 16 h the full dosage of 100 µM hydrogen peroxide is consumed, the consumption after 4 h when steady-state conditions can be still assumed is about 35 µM. With the mentioned 1 µM enzyme concentration and an experimental time of 16 h, a turnover number of 0.0024 s-1 can be calculated. This is very low, even compared to LPMO using oxygen to generate hydrogen peroxide in situ. If the estimated turnover numbers are correct, CellOx is either (i) a very slow enzyme with a low activity, or (ii) the physiological substrate/co-substrate has not been identified yet.

R: We appreciate the feedback on the presentation of data in previous Supplementary Fig. 20. We agree that a line graph with a continuous X-axis would provide a clearer visualization of the reaction progress and allow for better assessment of steady-state conditions and potential enzyme deactivation. Considering that following the reactions over the time (under aerobic or anaerobic conditions) is more relevant than a single point, this figure is now panel 3f (the previous 4c), following the recommendations for data presentation. We also included inset in this panel the turnover rates calculated for the linear range.

We appreciate the observation concerning the relatively low turnover number estimated for CelOCE. However, we would like to point out that this value falls within the range reported for some LPMOs acting on Avicel under aerobic conditions in the absence of exogenous hydrogen peroxide (New Supplementary Table 9). Additionally, this observation is consistent with CelOCE non-processive (pocket-like active site) and exo-acting mechanism. When acting alone on Avicel, without an endo-acting cellulase, its activity is inherently constrained by the limited availability of naturally occurring non-reducing ends on the cellulose chains, which serve as its primary catalytic targets.

Fig S20: Please report the activity or turnover number, but not relative activities. The comparison of cellobionic acid production rates versus hydrogen peroxide production rates would be absolutely interesting! What is the rate limiting step in the reaction? The substrate conversion step, or the production of the co-substrate?

R: We appreciate the suggestion for reporting absolute activity or turnover numbers instead of relative activities in the old Fig. S20 (New Supplementary Fig. 29). In our perspective, the presentation of the data as relative scale is instrumental for the comparative analysis under different Avicel concentrations as each enzyme or variant has distinct capacity for hydrogen peroxide generation. Then, the turnover numbers for each enzyme or variant are provided in the figure to indicate their capacity to generate hydrogen peroxide. It is worth noting that all figures are now presenting absolute values, except this one by the reason aforementioned.

Regarding the rate-limiting step for CelOCE, the equivalent product formation was observed for the enzyme under different times in both aerobic and anaerobic (with exogenous hydrogen peroxide) conditions. It indicates that the hydrogen peroxide generation under aerobic conditions is not a factor limiting the enzyme activity. Moreover, we need to consider that CelOCE is a non-processive and exo-acting enzyme with specificity for non-reducing ends and C1 regioselectivity. It implicates that the enzyme alone is intrinsically limited by the natural abundance of available non-reducing ends in cellulose for its activity. Under anaerobic conditions with exogenous H_2O_2 supply (5 µM) incubated with 0.1 % Avicel resulted in the production of approximately 4.3 µM cellobionic acid (New Supplementary Fig. 27), supporting a 1:1 stoichiometric ratio for co-substrate and production formation, consistent with data for LPMOs (Muller et al., 2018). Taking into account that the turnover number for oxidase activity (H_2O_2 generation) of CelOCE (0.23 ± 0.05 min^{-1}, New Supplementary Fig. 26) is higher than the turnover number for cellobionic acid production (0.053 ± 0.003 min^{-1}) and the 1:1 stoichiometry, it indicates that hydrogen peroxide generation is not a rate-limiting factor.

However, in our understanding, the fully mechanistic elucidation that includes hydrogen peroxide supply/generation, rate-limiting factors, side activities, oxygen transfer reaction mechanism and reactive intermediates constitute another long-term and complex study, as extensively studied and debated for LPMOs.

Fig. S26: The protein titer is marginally affected by the expression of CellOx. Please indicate the concentration of the expressed CellOx.

R: It was addressed in the response to the comment to “Line 326” and is provided in the New Supplementary Table 10. We would also like to mention that the low relative abundance of CelOCE is therefore not expected to impact the overall protein titers, as observed.

The crystal structures are of sufficient quality to merit the interpretation.

Please harmonize the use of “minutes” and “min” in the supplementary information.

R: We have carefully reviewed the text, figures, and tables, and standardized the usage of "minutes" and "min".

Referee #3 (Remarks to the Author):

The paper from Murakami describes a new oxidative enzyme that conducts exo-cleavage of cellulose to produce cellobionic acid. The enzyme was discovered from a metagenomics study of sugarcane bagasse in Brazil. Overall, this paper is very well-written and the authors have done an extremely thorough job on this work. I think that this discovery is extremely exciting and, as the authors note, it shows that oxidative cellulose depolymerization is yet even more complicated than the current paradigms My comments overall are quite minor and follow below in no particular order.

R: We appreciate the positive assessment of our work, and we have carefully considered all the comments and addressed each one in detail below. We are confident that the revisions made in response to these comments will further strengthen the manuscript. Thank you again for your valuable feedback.

In the first paragraph, the note about cellobiohydrolases being exo-acting only is oversimplified – well-characterized CBHs have been shown to conduct both exo- and endo-cleavage. See Ståhlberg et al. Biochim. Biopsy's. Acta 1993 and Kurašin et al. J. Biol. Chem. 2011.

R: We recognize that the statement regarding cellobiohydrolases (CBHs) being solely exo-acting was an oversimplification. We appreciate the references provided, which clearly demonstrate the capacity of well-characterized CBHs to exhibit both exo- and endo-cleavage activities. We have revised the relevant section in the first paragraph to accurately reflect the current understanding of CBH functionality:

" This process can be carried out by a plethora of microorganisms through distinct biochemical routes and the canonical model, based on extensive research on filamentous fungi and bacteria, comprises at least three major hydrolytic activities: endo-glucanase, cellobiohydrolase, and β-glucosidase. In this model, endo-glucanases cleave cellulose chains internally, while cellobiohydrolases primarily act on the ends of cellulose chains, although they have also been shown to exhibit some endo-cleavage activity. Both enzymes release cello-oligosaccharides, which are then converted into glucose by β-glucosidases."

I have a minor quibble with the comment about "pilot scale" experiments in the third paragraph of the paper. How "pilot scale" is defined is not clear. I would consider just stating what scale the cocktail enzymatic hydrolysis experiments were conducted at.

R: We acknowledge that the definition of "pilot scale" can vary and might not have been clear in the original context. In this work, the pilot-plant experiments encompassed both biomass pretreatment and the scaling up of strain cultivation in both 65-L and 300-L bioreactors (in total six independent cultivations, Supplementary Fig. 37). We focused on these two aspects at pilot-plant scale because it is well-established that working with biomass pretreated in reactors mimicking industrial conditions is crucial, which is not properly attainable at the bench scale. Moreover, scaling up filamentous fungal strains presents a significant challenge that remains underexplored in the literature. Here, we demonstrate the scalability and reproducibility of the engineered strain expressing CelOCE using an industrially relevant cultivation medium based on the byproduct molasses as a carbon source.

To clarify this in the fourth paragraph of the introduction, we have revised the text to specify the focus on strain cultivation at pilot-plant scale:

"In this work, by exploring the genomic dark matter of microbial communities specialized in plant biomass breakdown and employing a multidisciplinary approach including metagenomics, proteomics, carbohydrate

enzymology by chromatographic, colorimetric and mass spectrometric methods, fourth-generation synchrotron-based X-ray diffraction, fluorescence, and absorption spectroscopies, site-directed mutagenesis, CRISPR-Cas9 fungal genetic engineering and 65-L and 300-L pilot-plant bioreactors experiments, we unveiled a metalloenzyme that enhances cellulose breakdown through an unprecedented mechanism of substrate binding and oxidative cleavage. This discovery establishes a new frontier in redox biochemistry for plant biomass depolymerization, one of the most important bioreactions in nature with far-reaching implications for biotechnology."

I'm not sure what to take away from Figure 1C.

R: The primary goal of this figure is to illustrate the overall bacterial community composition at the phylum level, emphasizing the presence of several novel taxa associated with plant biomass breakdown. We acknowledge that the original MAG names might not have been informative. In the revised figure, we have renamed the MAGs based on their taxonomic classification to enhance clarity and interpretation, and the figure was enlarged. We hope this revision makes the figure more accessible and informative.

Figure 2B – this is much too small to read and interpret. I would very much consider changing this to make it clearer to read.

R: We acknowledge the feedback regarding the readability of Fig. 2b. We have redesigned Fig. 2 to improve clarity. Specifically, we have:

- **Relocated the binding gels:** The binding gels have been moved to a supplementary figure to make space for a larger and more legible HPAEC-PAD chromatogram.
- **Enlarged panel 2b:** The HPAEC-PAD chromatogram in panel 2b has been significantly enlarged to enhance readability and facilitate interpretation of the data.
- **Added binding isotherms:** Panels 2c and 2d now present the binding isotherms, replacing the binding gels.

We believe these changes significantly improve the clarity and overall presentation of Fig. 2.

Out of curiosity in the structural section, did the authors try AlphaFold modeling, and how did that do relative to the structure?

R: AlphaFold was instrumental for solving the crystallographic structure of CelOCE. Initial attempts using structural coordinates of homologs did not provide a clear solution for molecular replacement. Whereas the AlphaFold-generated model resulted a viable starting point, with crystallographic residuals (Rfactor and Rfree) in the 30-35% range, indicating a correct initial phases estimation, but yet requiring substantial model refinement, particularly in the loops, copper-binding site, and rotamers of residues on the surface. In summary, while AlphaFold was indispensable in the experimental structure determination, its model lacked the precision needed to accurately resolve the copper-binding site and to capture the specific loop conformations critical for enzyme function.

In Figure 3, I would consider showing the tunnel in more detail. There are programs that can do space-filling of voids in proteins, and that could be interesting to include here to show that this enzyme has a tunnel (assuming I am interpreting this correctly).

R: We appreciate the suggestion to highlight the substrate-binding region in more detail. While the enzyme active site is a pocket-like architecture specifically accommodating two glucosyl units, enabling C1 regioselectivity, we acknowledge that Fig. 3 might not have conveyed this clearly. Therefore, we have modified panel 3a to visually indicate the substrate binding pocket, emphasizing its pocket-like topology. This aspect of the active-site architecture is also highlighted in the novel Fig. 4, describing the proposed action mechanism of the enzyme. We believe these changes will enhance the clarity of the figure and provide a better understanding of the active-site architecture associated with the sole release of cellobionic acid as product from cellulose.

In Figure 3, why not show F33 considering this is the amino acid that is mutated later?

R: We appreciate the suggestion to highlight F33 in Fig. 3. In the revised figure, we have now explicitly showed and labeled F33 to emphasize its relevance to the subsequent mutagenesis experiments. F33 is also shown now in the New Fig. 4, specifically panel 4a.

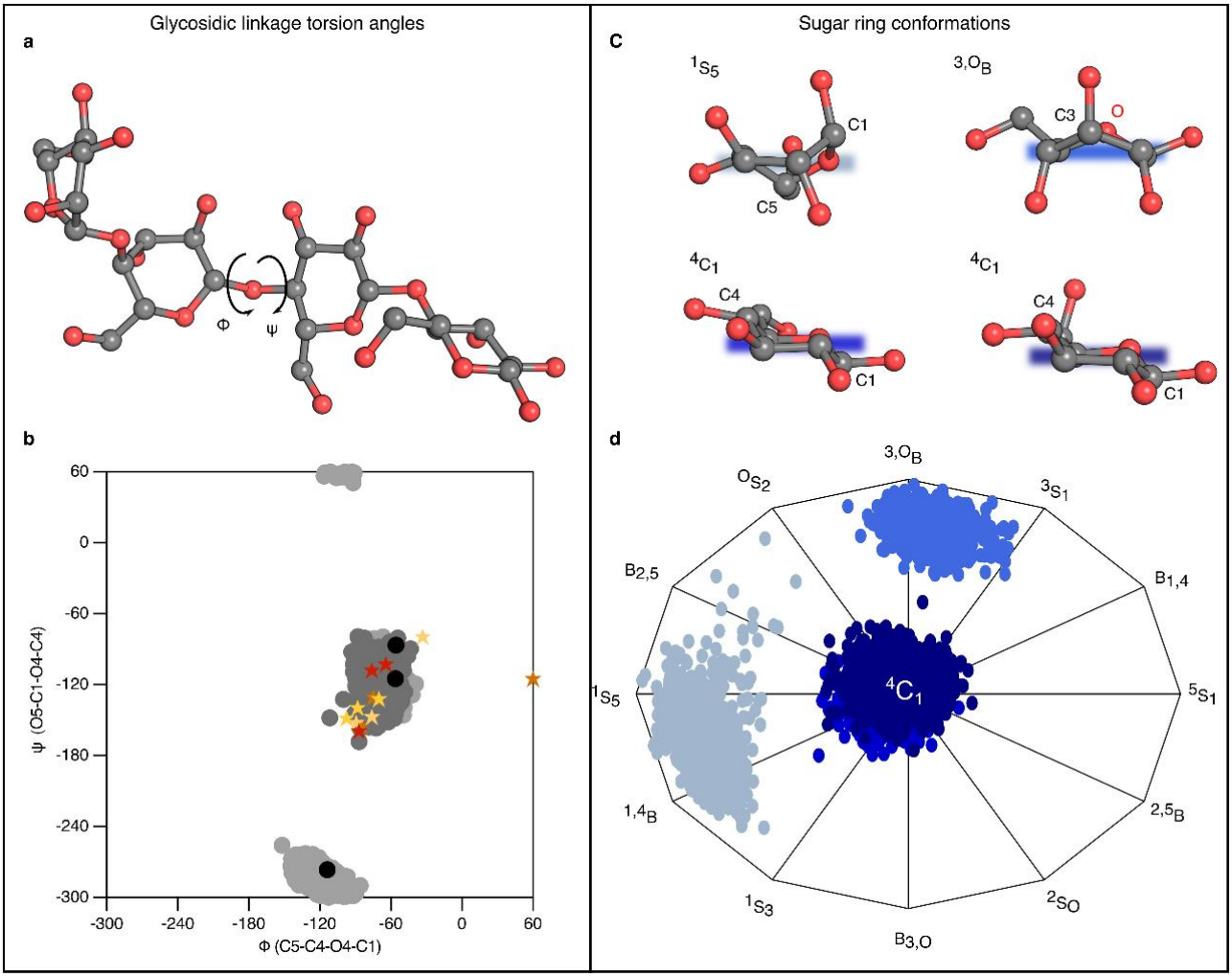
Also, the order of data presentation is a bit odd. The authors present the structure in Figures 3A,B, then talk about Figure 4, then they go back to Figure 3. This is very minor, but perhaps consider rearranging the figures to go in order of presentation in the text?

R: We appreciate the feedback regarding the order of data presentation. We have carefully reviewed the flow of information in the manuscript and have rearranged the figures to ensure they align with the order of their discussion in the text. Specifically, we have combined panels from Figs. 3 and 4, ensuring a smoother and more logical progression of the data presentation. While the New Fig. 3 encompasses the overall structural characteristics and copper binding properties, the new Fig. 4 describes the proposed action mode of the enzyme.

The structure shown in Figure 3D looks quite unrealistic in terms of the angles of the substrate. I would encourage the authors to more carefully check this.

R: Thank you for pointing this out. We carefully checked the substrate structure and performed additional molecular dynamics simulations with an extended equilibration time to ensure accuracy. The New Fig. 4 now reflects a representative frame from this new simulation. It is worth noting that we employed the GLYCAM06 force field (Kirschner et al., 2008), which has been shown to accurately reproduce glycosidic torsion angles observed in experimental data (Salisbury et al., 2009).

The glycosidic linkages in our simulations remained within local and global energetic minima (New Supplementary Fig. 33), consistent with DFT calculations on cellobiose (French et al., 2021). Furthermore, the calculated puckering coordinates indicate that the glucose rings adopt either distorted ($^{1}S_{5}$, $^{3,O}B$) or chair ($^{4}C_{1}$) conformations (New Supplementary Fig. 33), all of which are energetically feasible and commonly observed (Biarnés et al., 2007). We believe this revised figure and the supporting information provide a more accurate representation of the substrate conformational flexibility and energetic landscape within the enzyme binding pocket.



New Supplementary Fig. 33: Glycosidic linkage torsion angles and sugar ring conformations for the saccharide docked in CelOCE. **(a)** Representative frame from the equilibrium procedure, highlighting the torsion angles (O5-C1-O4-C4) and (C5-C4-O4-C1). **(b)** Torsion angles explored during the molecular dynamics trajectory post-docking, considering the three linkages in the saccharide. Stars indicate experimental data retrieved from PDB structures, and the black circles represent the angles present in the frame shown in (a). **(c)** Sugar conformations of each subunit in the saccharide, as represented in (a). **(d)** Stoddart diagram depicting the sugar conformations explored during the same trajectory as (b).

Likely my most important comments are on Figure 5:

- first, how much cellobionic acid is produced in the cocktail context?

R: The production of cellobionic acid was quantified and is detailed in Supplementary Figure 40. In the context of the saccharification assays, the presence of CelOCE led to a remarkable increase in the amount of cellobionic acid, highlighting the CelOCE activity under tested conditions. However, it is worth noting that this increase did not compromise the overall glucose yield since the cellobionic acid concentration is in the $mg.L^{-1}$ scale compared to $g.L^{-1}$ of released glucose.

- how much of the CellOx enzyme is present in the T. reesei secretome? Did the authors do quantitative proteomics? It might be nice to figure out how much enzyme is present in the cocktail relative to Cel7A, Cel7B, Cel6A, etc.

R: This point was addressed in the responses to “old Line 326” and “old Fig. S26” from Reviewer #2. As previously mentioned, CelOCE relative abundance is approximately 0.1%, compared to Cel7A, the most abundantly secreted CAZyme at ~15%. Despite its low relative abundance, CelOCE led to a 10-fold increase in cellobionic acid release from pretreated lignocellulose and resulted in a saccharification performance superior to the same strain expressing a well-characterized LPMO from *Lentinus similis* (*Ls*AA9A), which has an expression level of ~1.2%. This highlights the remarkable synergistic potential of CelOCE with *Trichoderma* CAZymes for efficient biomass breakdown.

- What enzyme loading is used in Figure 5 in mg enzyme/g enzyme? What solids loading is used? Put this in the caption.

R: We used an enzyme loading of 10 mg enzyme cocktail per gram of dried pretreated biomass and a total solids loading of 15%, reflecting industrially relevant conditions. This information has been added into the panel 5c.

- I’m surprised that the authors only show a single timepoint. Why not show a time course reaction for these reactions instead of a single timepoint? Usually these types of experiments are measured by “time to a conversion target” and a time course profile would be nice to see.

R: We appreciate the suggestion to include time-course data. We collected data over time, and we have now modified the new panel 5c to show the reaction time course in 24, 48 and 72 hrs, typical times shown for biomass saccharification assays under industrially relevant conditions. This will allow for a better understanding of the reaction kinetics and provide a more comprehensive picture of the enzyme cocktail performance. Furthermore, the cellobionic acid was quantified from all these reaction times as shown in the New Supplementary Fig. 40, highlighting the 10-fold increase in cellobionic acid production from pretreated biomasses.

- Is there a difference in selectivity of products with and without CellOx? In other words, can the authors please show the full product profile, including cellobionic acid, in Figure 5?

R: As Fig. 5 has been updated with time-course reaction data, we have then provided corresponding quantifications of cellobionic acid in the Supplementary Fig. 40 for the three time points as shown for panel 5c. Note that the remarkable increase of cellobionic acid generated by the CelOCE-containing cocktail. The HPAEC-PAD chromatograms were included for showing the product profile in the 24, 48 and 72 hrs in both biomasses, sugarcane bagasse and eucalyptus.

- Does beta-glucosidase cleave cellobionic acid to monomers? This would be nice to study.

R: As reported by Canella et al. (2012), aldonic acids are poor substrates for β-glucosidases. The accumulation of cellobionic acid in our saccharification assays over time supports this observation. Additionally, we conducted an experiment with cellobionic acid incubated with a highly active GH3 β-glucosidase from *Aspergillus fumigatus* (AfuBgl2, NCBI accession code: EAL86858), further demonstrating that cellobionic acid is cleaved very slowly (Fig. R1). As shown in Fig. R1, no statistically significant difference was observed compared to the control experiment.

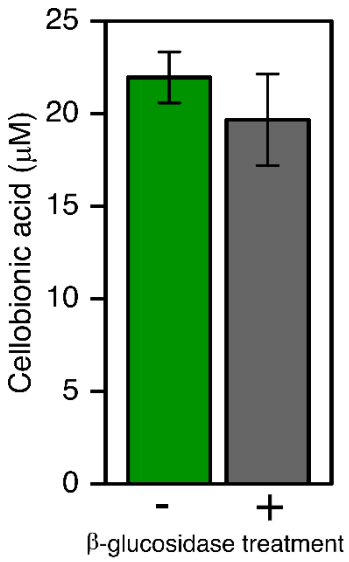


Fig. R1. Cellobionic acid is a poor substrate for a GH3 A1 β-glucosidase from *Aspergillus fumigatus* (*Afu*Bgl2). The panel shows no significant difference between the control and enzyme incubation (22 µM cellobionic acid, pH 5.0, 45 ºC, 1 h and 3.3 µg/mL *Afu*Bgl2).

The authors make several claims about the industrial relevance of this enzyme as well. In the absence of a process model, and in this case, in understanding the difference in product selectivity, I would suggest that the authors tone this language down. In no way does this diminish the exciting discovery of the work here, but I think it is important to not over-promise process relevance.

R: We appreciate your concern about overstating the industrial relevance of our findings at this stage. We have taken steps to address this by focusing on the fundamental scientific significance of the discovery while still highlighting its potential for future applications.

In addition, we have conducted further experiments to strengthen the evidence for potential biotechnological relevance:

- **Pilot-scale production:** We successfully scaled up the cultivation of the CelOCE-expressing strain in both 65-L and 300-L pilot-plant bioreactors using an industrial molasses-based medium (six independent cultivations). This demonstrates the feasibility of producing the enzyme along with multiple other CAZymes at a larger scale under industrially relevant conditions.
- **Comparative performance:** We engineered a strain expressing a well-known LPMO (*Ls*AA9A) and compared its performance to the CelOCE-expressing strain. The CelOCE cocktail resulted in overall higher glucose yields, evidencing its potential in biomass saccharification.
- **Binding isotherms:** We have conducted binding isotherm experiments to quantify CelOCE affinity for cellulose, demonstrating its effective binding and providing data relevant for its use.

While we acknowledge that a comprehensive process model is necessary to fully assess the industrial implications, we believe these additional experiments provide additional support for the potential of CelOCE in biotechnological processes. We have carefully revised the manuscript to ensure a balanced presentation of both the fundamental scientific discovery and its potential future applications.

On a similar note, what would cellobionic acid do in the presence of an ethanologen? Are these substrates able to be utilized and converted to EtOH?

R: We acknowledge the importance of considering the impact of cellobionic acid on ethanol production. Currently, most industrial yeast strains used for ethanol fermentation are unable to metabolize cellobionic acid directly. However, in our experiments, the levels of cellobionic acid produced were low ($mg.L^{-1}$) and did not negatively impact the overall glucose yield (typically in the range 45-70 $g.L^{-1}$). This suggests that the CelOCE activity is positive in terms of boosting the production of fermentable sugars for typical ethanologen strains.

I would suggest that the authors consider changing the name of the enzyme to be more descriptive of what it is doing. CellOx is not very descriptive. Perhaps exocellulo-peroxygenase? I am not sure what the right name should be, but it might be nice, given that the authors have so much mechanistic information already, to name the enzyme more appropriately.

R: We appreciate your suggestion for a more descriptive enzyme name. In line with all Reviewers comments, we propose renaming the enzyme to Cellulose Oxidative Cleaving Enzyme (CelOCE), which properly reflects its activity.

Overall, this is excellent and extremely comprehensive work. I hope that these comments are useful for the authors to further improve their paper.

R: We are sincerely grateful for the positive assessment of our work. We appreciate the time and effort taken to provide such detailed and constructive feedback. We have carefully considered all the comments and believe that the revisions made in response will significantly enhance the clarity, accuracy, and impact of our manuscript. Thank you again for your valuable insights and support.

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Response to referees' comments

Referee #1 (Remarks to the Author):

First of all, I commend you for the many experiments you conducted in response to the points I raised in my previous comments. After reading the revised manuscript, I think many of my raised points have been answered. On the other hand, there are some points in the newly added data that I do not understand, so I would like you to answer them properly.

R: We thank Reviewer #1 for acknowledging the revisions made to the manuscript and for carefully reviewing the newly added data. We are pleased to clarify the questions raised regarding the new experiments and analysis.

Why is the adsorption behavior sigmoidal in Fig. 2c? I think you should give the Hill coefficient and other values for this.

R: We appreciate the Reviewer's comment regarding the fitting of the binding isotherms. Despite several works in literature using the Langmuir model to fit binding isotherms for CBMs and cellulases, we opted for the Langmuir-Freundlich model. This model can better account for the heterogeneity of cellulose and for the complex adsorption process of CelOCE on cellulose that might involve multiple interactions, including hydrogen bonding, hydrophobic interactions, and electrostatic forces. As shown in Fig. R1, the attempt to use the simple Langmuir or Freundlich models resulted in poor coefficient of determination (R^2) contrasting to the hybrid Langmuir-Freundlich model.

The heterogeneous nature of cellulose results in a distribution of binding sites with varying affinities for the enzyme. Furthermore, the specific binding of CelOCE to cellulose chain ends limits the available binding sites. This leads to a faster saturation of binding sites compared to endo-acting enzymes like glucanases and LPMOs, heading to adsorption isotherms to reach a plateau quickly. The Langmuir-Freundlich model better captures this adsorption behavior by accounting for both surface heterogeneity and limited binding site availability.

Additionally, we cannot exclude the possibility of an allosteric behavior, as the enzyme biological unit is a homodimer, further complicating the binding process. In this scenario, it is conceivable that one subunit binds productively to cellulose and promotes oxidative cleavage, while the other binds non-productively, potentially serving as an *in situ* hydrogen peroxide supplier. This would be consistent with our proposed mechanistic model.

Based on these facts, we believe that the Langmuir-Freundlich equation provides a more suitable empirical description of the observed binding isotherms. We have included this information in the experimental procedures and the Langmuir-Freundlich parameters in the legend of Fig. 2.

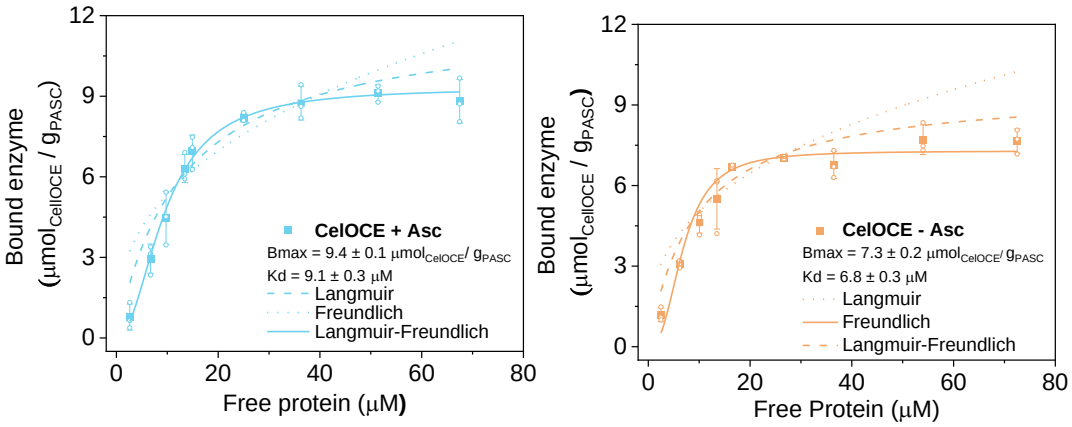


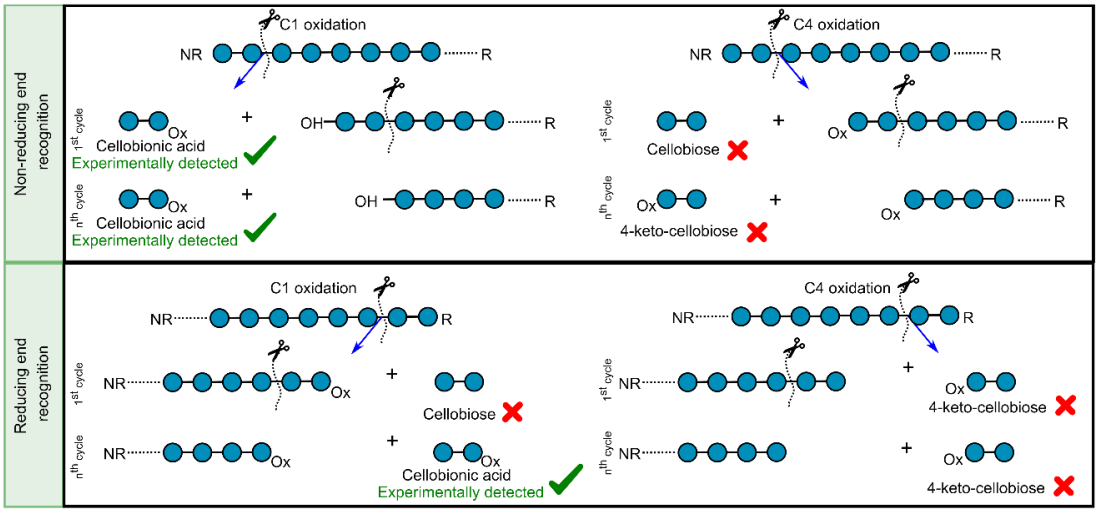
Figure R1: Binding isotherms on PASC of CelOCE with (left) or without (right) ascorbate. Note that the simple Langmuir or Freundlich models do not properly describe the experimental data.

Table R1: Parameters associated with the Langmuir, Freundlich and Langmuir-Freundlich models using the experimental data obtained from CelOCE with and without ascorbic acid.

	Langmuir Fit	**Freundlich Fit**	**Langmuir-Freundlich Fit**
CelOCE + Asc	R^2 = 0.94	R2 = 0.79	R^2 = 0.99 n = 2.1
CelOCE - Asc	R^2 = 0.90	R2 = 0.77	R^2 = 0.99 n = 2.5

Reducing or non-reducing is explained in Supplementary Fig. 34, although only cellobiose is produced in the first cleavage, cellobionic acid is produced after the second cleavage. In other words, unless the enzyme is an endo-type, the yield of cellobiose from cellulose with a degree of polymerization of 200 is only 1%, so it is unlikely to be detected as a product. Therefore, it is impossible to determine the activity direction from these experiments. In particular, the substrate-bound structure has not been obtained, and I think it is hasty to determine the direction of activity based on the docking of sugars alone.

R: We appreciate the insightful comment regarding the determination of CelOCE activity direction. Although docking studies indicate that the enzyme likely recognizes the non-reducing end, we agree that the determination of the activity direction can only be confirmed by solving the crystal structure of the substrate-enzyme complex. As the Reviewer correctly points out, if the enzyme were to act from the reducing end, subsequent catalytic cycles on the same cellulose chain could also generate cellobionic acid, albeit with the requirement of recognizing an oxidized sugar. Indeed, the low yield of cellobiose from a long cellulose chain, which would be expected if the enzyme acted from the reducing end, might make it very difficult to detect. Accordingly, the manuscript has been revised and an updated version (now Extended Figure 8) was included to illustrate the potential for cellobionic acid production regardless of the initial directionality, acknowledging the ambiguity (see the new figure below).



Extended Fig. 8: Schematic representation of the putative modes of substrate recognition and sugar regioselectivity for CelOCE. Cellobionic acid was the only product detected, supporting a C1 sugar regioselectivity. Although molecular docking studies indicate that the enzyme likely recognizes the non-reducing end of cellulose, we cannot rule out the possibility of reducing end recognition since subsequent catalytic cycles on the same cellulose chain could also generate cellobionic acid.

Furthermore, in the experiment (Fig. 5a) that showed that there was a synergistic effect with endo-type enzymes but not with CBH, I think that the synergy plot is usually carried out by varying the mixing ratio of the two (e.g. 1/9, 2/8, etc.). Just adding them together is an “additive effect”, so it is not clear whether it is a “synergistic effect” or not.

R: We acknowledge our data in Fig. 5a support an additive effect rather than a definitively synergistic one. To clarify this, we have revised the manuscript now describing the observed outcome as an "additive effect" rather than conclusively claiming synergy. The literature has been demonstrating that the cooperation mechanisms between redox enzymes and classical cellulases are complex with a certain degree of ambiguity. Therefore, further studies are required to fully elucidate the specific cooperation mechanisms between this novel class of metalloenzymes with classical cellulases. Furthermore, as mentioned by the Reviewer #3, this work will “spark a lot of follow-up mechanistic studies”.

Basically, I am quite positive to accept this manuscript, so please address the above questions.

R: We are pleased that the additional results included in the revised manuscript have clarified most of your questions.

Referee #2 (Remarks to the Author):

The revised version of "A metalloenzyme discivered from metagenomic dark matter promotes oxidative cellulose breakdown" has been improved according the the reviewer's suggestions. The questions have been answered and supported by additional experiments. The additional experiments verify the previous conclusions. This includes: affinity measurements for cellobiose with ITC and cellulose binding isotherms as well as a competitive binding assay; additional HPAEC-PAD experiments to rule out cellobiose as a substrate and better cellobionic acid quantitation; calculated turnover numbers and substrate docking. The accumulated evidence support the previous data and show a more complete picture of the enzyme. The changed enzyme name is much better than the initially used one. Considering all changes made, I support the publication of this manuscript.

R: We are grateful for the reviewer's thorough assessment and valuable feedback on the revised manuscript. We appreciate the reviewer taking the time to carefully consider our responses to the initial comments and acknowledge the new data provided. We also appreciate the positive comment on the revised enzyme name, which we believe now better reflects its function. We are delighted to have the reviewer's support for the publication of our manuscript.

Referee #3 (Remarks to the Author):

It is clear from the response letter and revised document that the authors took all of the feedback from the reviewers very seriously. This was already a tour de force in terms of the vast amount of work in the paper, and the new added experiments have made this work even better. This paper is excellent, and it will spark a lot of follow-up mechanistic studies. I have no additional comments.

R: We are pleased with the positive comments from Reviewer #3 and particularly appreciate their recognition of the effort invested in addressing the reviewers' feedback and conducting substantial additional experiments. We are very enthusiastic about the potential of this work to stimulate further mechanistic studies in this area.