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Decision letter and referee reports: first round

10th May 20

Dear Prof Perez-Jimenez,

Thank you for submitting your manuscript, "Obtention of high performance crystalline nanocellulose using an ancestral endoglucanase", to Communications Materials. It has now been seen by 3 referees. You will see from their comments below that while they find your work of interest, some important points are raised. We are interested in the possibility of publishing your study in Communications Materials, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

We therefore invite you to revise and resubmit your manuscript, taking into account the points raised.

We are committed to providing a fair and constructive peer-review process. Please don't hesitate to contact us if you wish to discuss the revision in more detail.

When submitting your revised manuscript, please include the following:

-A rebuttal letter with a point-by-point response to each of the referee comments and a description of changes made. Please include the complete referee report in the rebuttal letter. Please note that the rebuttal letter must be separate to the cover letter to the editors.

-A marked-up version of the manuscript with all changes to the text in red colored font. Please do not include tracked changes or comments. Please select the file type 'Revised Manuscript - Marked Up' when uploading the manuscript file to our online system.

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We hope to receive your revised paper within three months; please let us know if you aren't able to submit it within this time so that we can discuss how best to proceed. If we don't hear from you, and the revision process takes significantly longer, we will close your file. In this event, we will still be happy to reconsider your paper at a later date, as long as nothing similar has been accepted for publication at Communications Materials or published elsewhere in the meantime.

We understand that due to the current global situation, the time required for revision may be longer than usual. We would appreciate it if you could keep us informed about an estimated timescale for resubmission, to facilitate our planning. Of course, if you are unable to estimate, we are happy to accommodate necessary extensions nevertheless.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further. We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Best regards,

John Plummer, PhD
Chief Editor
orcid.org/0000-0003-4824-8497
Communications Materials

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this study, Alonso-Lerma et al. report the generation of cellulose nanocrystals (EnCNC) using a bacterial endoglucanase obtained previously from ancestral sequence reconstruction. The properties of EnCNC and its potential use in tissue engineering as well as fabrication of cellulose/graphene composites are investigated in detail. The work is thoroughly done and of general interest. I recommend publication, after the following questions and concerns have been addressed adequately:

1)The authors compared the performance of their ancestral enzyme (ANC EG) with modern enzymes from Bacillus subtilis, Thermotoga maritima, and Trichoderma reesei.

a)As pointed out, the carbohydrate binding module (CBM) is essential for high conversion. It is not specified whether the modern enzyme constructs used here also carry a CBM. This would be crucial for a fair comparison! As it was apparently not possible to reconstruct an ancestral CBM, the B. subtilis domain was used. Is it part of all constructs used here or do the other modern enzymes have their own CBM? If yes, how similar are they? A sequence alignment in the supplement may help.

b)The T. reesei enzyme seems to give better results than the other modern enzymes regarding conversion and size distribution. However, it is only shown in the supplement (Supp Fig 1 and 2). I don't see why and recommend including the data in the main text in Fig. 1.

c)Can you explain why the cellulose particle size increased again after 30 hours of enzymatic treatment with ANC EG (Fig. 1b/c)?

2)The authors use many different techniques to characterize the physicochemical properties of EnCNC

and compare to conventional nanocellulose obtained from acid hydrolysis (AcCNC). However, if the authors want to demonstrate the superior properties of the ancestral enzyme in generating this material, they should do the key characterizations also with the CNCs obtained from treatment with the modern enzymes, or at least the T. reesei enzyme, which gave a similar size distribution.

3)Similarly, in order to plausibly demonstrate that "high performance CNC" can only be obtained from treatment with the ancestral enzyme, key experiments for nanocomposite formation with graphene should also be repeated with the material generated by a modern enzyme. Only then a true assessment is possible.

4)Minor aspect: Reference 14 and 22 are identical!

Reviewer #2 (Remarks to the Author):

The work "Obtention of high-performance crystalline nanocellulose using ancestral endoglucanase" presents a detailed study on the production of cellulose nanocrystals employing a particular form of bacterial endoglucanase(EnCNC). The features of the obtained EnCNC are studied consistently and in detail. The EnCNC are tested in applications ranging from cell culture to nanocomposites.
The research deserves publication after addressing the following comments:

- The authors measure the zeta potential for the EnCNC. They should provide the zeta potential also for the inks produced adding graphene oxide.

- The authors chemically reduced GO. Do they study if a thermal reduction was possible? Was, in this case, the EnCNC degraded?

- The authors prepare conductive samples with 2, 5, and 10 wt% rGO. The authors should try also lower rGO concentration and determine the electrical percolation threshold.

- The authors show the SEM morphology of the nanocomposites prepared with rGO. How was the cross-sectional SEM view of the samples? Was the rGO homogeneously dispersed trough the sample thickness?

Reviewer #3 (Remarks to the Author):

The manuscript reports the use of "ancestral" cellulase enzymes to produce cellulose nanocrystals. Cellulase enzymes have been used previously as both pretreatments and hydrolytic agents for the production of nanocellulose; however, the enzyme used in this study has not been previously investigated for the production of nanocellulose which does contribute aspects of novelty to the work. The authors also demonstrate several applications of the resultant nanocellulose and observe enhanced performance with the enzymatically produced nanocellulose relative to that obtained by sulfuric acid hydrolysis.
The study does seem to show that the morphology of the resultant nanocellulose can be altered by the choice of enzyme preparation which is used for hydrolysis. This is an interesting result which generally motivates investigations of novel enzyme systems for the NC production applications.
I have several concerns about the claims in the manuscript.
1. The authors conclude that enzymatically produced nanocellulose "is a unique nanocellulose that

conserves cellulose I structure with elevated stability, crystallinity and controlled aspect ratio," and further that "enzymatic obtention of nanocellulose renders EnCNC as an empty canvas for further surface modification as opposed to AcCNC that contains sulfate groups on the surface." However, these features can be obtained with other methods. For example, the use of HCl (instead of sulfuric acid) also preserves the native hydroxyl functionality and can produce CNC with very high crystallinity and size uniformity. This draws the conclusion of "unique nanocellulose" into question.

2. Characterization of the cellulose fragments as "CNC" (cellulose nanocrystals) is also questionable. While there is admittedly some ambiguity within the community about precise definition with the field, many of the particles shown in the AFM images appear more as cellulose nanofibrils (CNF) due to the very high aspect ratio. This also draws the claim about uniformity into question.

3. The comparison of the suspension stability (visualized in Figure 3A) is counter-intuitive. The sulfate groups on the surface of the so-called AC-CNC are known to stabilize their aqueous suspensions, while the native hydroxyl groups on CNCs produced by HCl typically result in less stable suspensions. These results seem to indicate the opposite effect. This result must be rectified with previous reports in the literature.

Response to reviewer #1

We deeply thank the reviewer for his/her feedback that has clearly improved the manuscript.

1)The authors compared the performance of their ancestral enzyme (ANC EG) with modern enzymes from Bacillus subtilis, Thermotoga maritima, and Trichoderma reesei.

a) As pointed out, the carbohydrate binding module (CBM) is essential for high conversion. It is not specified whether the modern enzyme constructs used here also carry a CBM. This would be crucial for a fair comparison! As it was apparently not possible to reconstruct an ancestral CBM, the B. subtilis domain was used. Is it part of all constructs used here or do the other modern enzymes have their own CBM? If yes, how similar are they? A sequence alignment in the supplement may help.

As the reviewer points out, the CBM cloned into the ancestral endoglucanase belongs to *B. subtilis* EG. Therefore, we have a direct comparison of the CBM role in the activity as the *B. subtilis* EG that we produced has this CBM as well. Thus, the differences in activity are only attributed to the catalytic domain. In the case of EG from *T. maritima*, this EG does not have a CBM in its wild type form[1]. Moreover, *T. reesei* EG has its own CBM like most cellulases from fungi have. The comparison of both CBM from *B. subtilis* (CBM3)[2] and *T.reesei* (CBM1)[3] renders a very low identity between the sequences, which is a constant feature when comparing CBMs regardless of the species, as they show little conservation. This low identity does not even allow to construct a consistent alignment to show. We have included a clarification regarding CBM in the results section, first paragraph.

b) The T. reesei enzyme seems to give better results than the other modern enzymes regarding conversion and size distribution. However, it is only shown in the supplement (Supp Fig 1 and 2). I don't see why and recommend including the data in the main text in Fig. 1.

We think this is a good point, and we added the *Trichoderma* data to the principal figure. In the first version of the manuscript, we left *T. reesei* EG in the supplementary because we wanted to compare the ancestral enzyme with two moderns bacterial EGs, produced in the laboratory in similar conditions. Besides, Trichoderma EG is a commercial enzyme that although its main activity is endoglucanase, glucose can be measured after hydrolysis, which means that traces of glucosidases might be present. The other EGs, ancestral and moderns, did not produce glucose as they have 100% endoglucanase activity. We thought that the fairest comparison was the use of modern bacterial EGs. Nevertheless, data from *T. reesei* EG is now provided in Figure 2. In consequence, figures of supplementary data were renumbered.

c)Can you explain why the cellulose particle size increased again after 30 hours of enzymatic treatment with ANC EG (Fig. 1b/c)?

We suspect that the increasing size is produced by CNC aggregation due to hydrogen bonding between crystals as they have no charge[4]. Also, the activity of ancestral endoglucanase deceased after 24 hours, leading to the agglomeration of the crystals at longer times. We have included this detail in the result section, second paragraph, page 3.

2)The authors use many different techniques to characterize the physicochemical properties of EnCNC and compare to conventional nanocellulose obtained from acid hydrolysis (AcCNC). However, if the authors want to demonstrate the superior properties of the ancestral enzyme in generating this material, they should do the key characterizations also with the CNCs

obtained from treatment with the modern enzymes, or at least the T. reesei enzyme, which gave a similar size distribution.

Thanks to the reviewer's suggestion, we performed the FTIR, XRD and TGA analysis of the EnCNC *T. reesei* sample. We observed how the FTIR spectra on *T. reesei* EnCNC was similar to the EnCNC produced with the ancestral EG, as expected. In the case of XRD analysis, we found out how the crystallinity was drastically reduced in the *T. reesei* CNC sample, as compared to the ancestral EnCNC, 77.2 vs. 87.9, for *T.reesei* and ancestral EG+CBM, respectively (Supplementary Figure 9). This shows that although *T. reesei EG* could produce nanocrystals with a size similar to ANC EG+CBM. The ancestral EG not only shows better yields but also generates CNC with higher crystallinity. We also measured thermal stability, and we observed a marked difference in To, 223 vs. 262 °C for *T. reesei* EnCNC and ANC EnCNC, respectively. Also, *T. reesei* EnCNC started to depredate faster than ANC EnCNC. These results remark the differences between both CNCs and are now discussed in the manuscript (page 4), and the supplementary information.

3)Similarly, in order to plausibly demonstrate that "high performance CNC" can only be obtained from treatment with the ancestral enzyme, key experiments for nanocomposite formation with graphene should also be repeated with the material generated by a modern enzyme. Only then a true assessment is possible.

We have followed the reviewer suggestion and have prepared GO composites using nanocellulose obtained with *T. reesei* EG. The nanopapers fabricated with EnCNC from *T. reesei* EG + 10 wt% rGO maintained similar conductivity, 20 S/m than that of nanopapers prepared with ANC EnCNC EG+CBM (25.2 S/m), with the same rGO content, which was expected given that the conductivity is mostly dependent on rGO content. However, we found a marked difference in the thermal stability of both EnCNC. While nanopapers prepared with *T. reesei* EG enCNC started degradation at 193°C, those fabricated with ANC EG EnCNC start degrading at 270 °C. We have added this new data to the manuscript.

4) Minor aspect: Reference 14 and 22 are identical!

Thanks for point this error; we removed it from the manuscript and renumbered refereces.

Response to Reviewer #2:

We are glad that the reviewer #2 gave excellent feedback to our work and also supports publication in the journal. He/she raised some concerns that we are happy to answer as follows:

- The authors measure the zeta potential for the EnCNC. They should provide the zeta potential also for the inks produced adding graphene oxide.

This is a good point. We have measured the zeta potential of all EnCNC + GO mixtures as the reviewer requested:

Sample	Zeta potential (mV)
EnCNC + 2% rGO	-35,3

EnCNC + 5% rGO	-34,8
EnCNC + 10% rGO	-33,1

The zeta potential of all the inks had similar values than EnCNC suspension (-32 mV), highlighting its good stability and supporting the use of these mixtures as inks. This data is added now to the supplementary information as Supp Table 4.

- The authors chemically reduced GO. Do they study if a thermal reduction was possible? Was, in this case, the EnCNC degraded?

We chemically reduced GO with ascorbic acid given that thermal reduction is not possible because cellulose materials degrade at temperatures below that needed for a proper thermal reduction[5]. In this direction, we are working on the reduction of GO with other physical treatments such as ultraviolet light reduction.

- The authors prepare conductive samples with 2, 5, and 10 wt% rGO. The authors should try also lower rGO concentration and determine the electrical percolation threshold.

We appreciate the reviewer suggestion. We prepared nanocomposites with lower concentrations of rGO, 0.3 and 1 wt% and also with higher concentration, 15%, and measured conductivity. The conductivity values were plotted against the volumetric content (%) of rGO and fitted to a power law following the percolation theory to determine the electrical percolation threshold, determined at 0.16 %, which is 0.25wt% of rGO in our system, a value similar to that reported for other rGO nanocomposites[6,7]. This has been added now to a new Fig 5c in the manuscript and discussed in the text.

- The authors show the SEM morphology of the nanocomposites prepared with rGO. How was the cross-sectional SEM view of the samples? Was the rGO homogeneously dispersed trough the sample thickness?

This is an excellent remark. We have performed more measurements on the cross-section of the nanocomposite. In the new supplementary information, we show the cross-section SEM images of the EnCNC + rGO nanocomposites. The samples seem more compact and homogeneous at 10% rGO concentration; this could also explain the higher conductivity in EnCNC + 10 rGO% nanopapers. This data is added to the new supplementary information in Supp Fig. 14

Response to Reviewer #3:

The reviewer #3 also remarks on the novelty of our work and the enhanced performance of our nanocellulose in the applications proposed. He/she has some concerns that we are happy to address:

1. The authors conclude that enzymatically produced nanocellulose “is a unique nanocellulose that conserves cellulose I structure with elevated stability, crystallinity and controlled aspect ratio,” and further that “enzymatic obtention of nanocellulose renders EnCNC as an empty canvas for further surface modification as opposed to AcCNC that contains sulfate groups on the surface.” However, these features can be obtained with other methods. For example, the use of HCl (instead of sulfuric acid) also preserves the native hydroxyl functionality and can produce CNC with very high crystallinity and size uniformity. This draws the conclusion of “unique nanocellulose” into question.

Although it is true that HCl can render nanocellulose with no charged groups, it is a still a very aggressive treatment that generates CNC of smaller size (~200 nm); this leads to a smaller aspect ratio as compared to EnCNC which is important for CNC mechanical reinforcement properties[8] and also affects its viscosity[9]. In fact, to obtain larger length to diameter ratios it is necessary to carry out HCl hydrolysis using inorganic chlorides[10]. Still, we agree with the reviewer that HCl would be the closest method to enzymatic treatment in terms of material features. An aspect that need to be also considered is the obtention method. In practical terms, HCl treatment is a very pollutant and dangerous method and not used for commercial or industrial purposes, also due to low yields[8]. Our method renders CNC with high stability and crystallinity and most importantly controlled size. In addition, it is biocompatible, and it is greener and sustainable process, making it different overall.

In order to translate this idea, the sentence "EnCNC is chemically pure, displays higher thermal stability, crystallinity and mechanical strength than standard CNC isolated by acid hydrolysis (AcCNC)." has been replaced by "EnCNC is chemically pure, displays higher thermal stability, crystallinity and mechanical strength than standard CNC isolated by acid hydrolysis (AcCNC), being a greener and sustainable process" in the revised version.

2. Characterization of the cellulose fragments as "CNC" (cellulose nanocrystals) is also questionable. While there is admittedly some ambiguity within the community about precise definition with the field, many of the particles shown in the AFM images appear more as cellulose nanofibrils (CNF) due to the very high aspect ratio. This also draws the claim about uniformity into question.

As the reviewer remarks, the definition of CNC is ambiguous within the community; CNC morphology usually depends on the production method and the cellulosic source. The AFM images could show a few CNFs between CNCs because we show that raw data with no particle separation after hydrolysis. However, we measured the lengths and diameters of each image to provide the average of each sample. By plotting the size population we measured an average length of 408 nm; this length is described as CNC[11]. Besides, the aspect ratio of our CNC corresponded to CNC with Cellulose I polymorph[12] and it is within the interval usually measured in CNC (<100)[13]. This is now clarifies in page 3 of the manuscript.

Regarding the homogeneity, we found that the enzymatic method produced particles with less uniformity than sulfuric acid. Acid hydrolysis produced an aggressive degradation of the amorphous part of cellulose. Nevertheless, Ancestral endoglucanase with CBM still produced a quite homogeneous CNC preparation as shown in the size distribution plots. We would like to point out that our method uses only one enzyme, that clearly show distinct capabilities to produce CNC, in aqueous solution and very mild conditions. We have some very preliminary results showing that combining our enzyme with other enzymes such as LPMO or xylanase can further reduce size and improve homogeneity, and even better with ancestral variants; however, we wanted to keep our focus on using one single ancestral EG enzyme, which is the only one that generate better CNCs *per se*.

3. The comparison of the suspension stability (visualized in Figure 3A) is counter-intuitive. The sulfate groups on the surface of the so-called AC-CNC are known to stabilize their aqueous suspensions, while the native hydroxyl groups on CNCs produced by HCl typically result in less stable suspensions. These results seem to indicate the opposite effect. This result must be rectified with previous reports in the literature.

As the reviewer points out, AcCNC generates a stable aqueous colloidal suspension, although EnCNC was still well dispersed. We measured the zeta potential of the suspensions to calculate the stability, for EnCNC was -32 mV and for AcCNC was -54 mV. As expected, AcCNC forms a more stable colloidal suspension in water due to sulfate groups. EnCNC (blue diagram in Figure 3a) clearly shows more aggregates and sedimentation. We have made this clear in the manuscript and supplementary information in page 3.

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Decision letter and referee reports: second round

1st Jul 20

Dear Prof Perez-Jimenez,

Your manuscript titled "Obtention of high performance crystalline nanocellulose using an ancestral endoglucanase" has now been seen again by the three referees, whose comments appear below. In light of their advice I am delighted to say that we are happy, in principle, to publish a suitably revised version in Communications Materials under the open access CC BY license (Creative Commons Attribution v4.0 International License).

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* I suggest simplifying the title to "High performance crystalline nanocellulose using an ancestral endoglucanase". Alternatively, "obtention" should be replaced with "obtaining".

* I suggest adding sub-headings in the Results section, the first of which should appear appear straight after the Results heading.

* Please title the Materials and Methods section as 'Methods'.

* Italic font should not be used for "versus".

* When referring to information in the Supplementary Materials please, for example, use "Supplementary Figure 1" rather than "Supp Fig. 1".

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"Enzymes are effective at upgrading natural materials to high-performance biomaterials. Here, an ancestral endoglucanase is used to obtain highly crystalline cellulose nanocrystals, which can act as a matrix for cell growth and be combined with graphene for conducting inks."

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John Plummer, PhD
Chief Editor
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REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The revised version of the manuscript addresses my questions and concerns adequately. Clarifications and restructuring of figures were done as suggested and additional experimental data have been included. I thus recommend to publish this version of the manuscript.

Reviewer #2 (Remarks to the Author):

The authors reviewed the manuscript taking into account all the comments. The manuscript can be published without any further modification.

Reviewer #3 (Remarks to the Author):

The Authors have made extensive revisions in response to my concerns stated in the original review. I feel that the revised manuscript and supplemental data are suitable for publication.