Assessing the key role of Lytic Polysaccharide Monooxygenases on the enzyme-mediated preparation of nanocellulose from organosolv pretreated beechwood

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Introduction

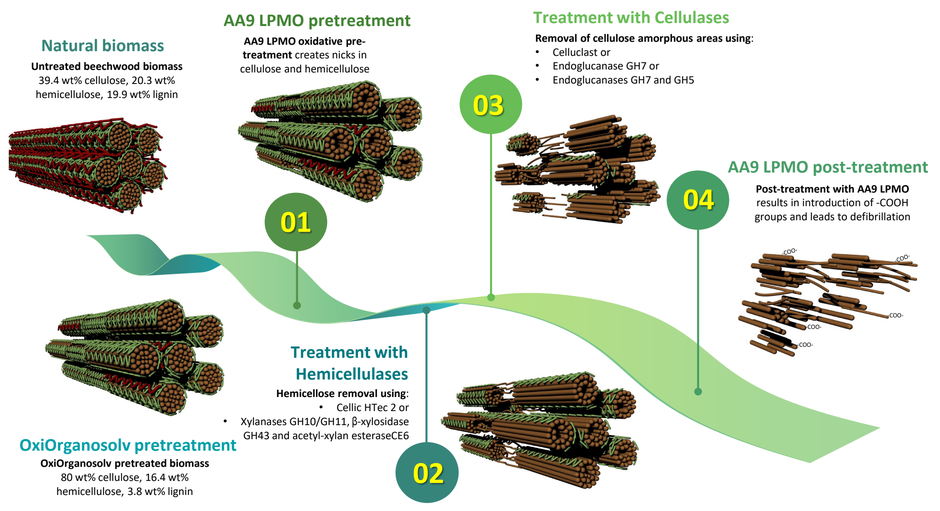
Lytic polysaccharide monooxygenases (LPMOs) are are copper-dependent enzymes that cleave polysaccharides through an oxidative mechanism.

- LPMO genes are found in the genome of a variety of lignocellulose-degrading microorganisms [1].
- Oxidative cleavage of glycosidic bonds after an hydroxylation of the C1, C4 or both C1/C4 carbon atoms (different regioselectivity).
- Their ability to act both as monooxygenases and peroxygenases has been highly debated, as they utilize both O_2 and H_2O_2 as a co-substrate.
- They target both crystalline substrates (cellulose, chitin), amorphous structures including xylan, mannan, pectin and oligosaccharides [2].

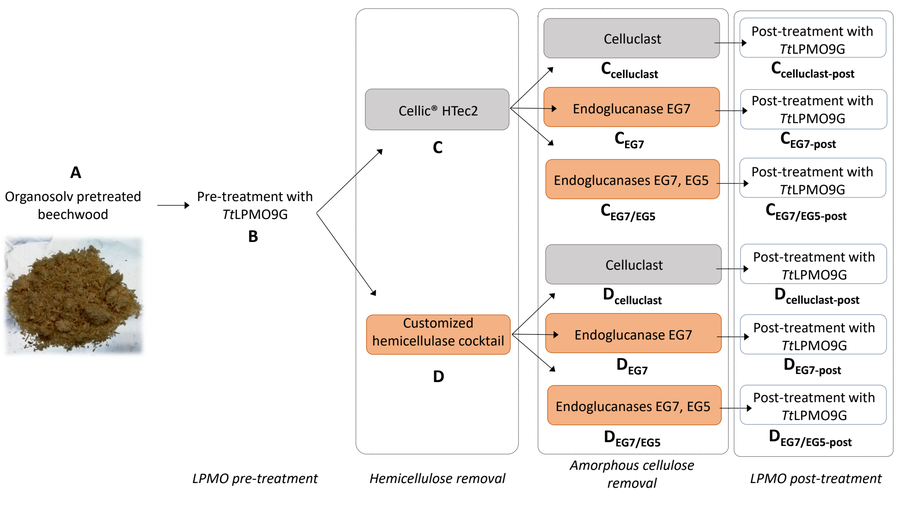
The key role of LPMOs towards the isolation of nanocellulose from natural substrates has been demonstrated [3].

- LPMOs promote amorphogenesis of the substrate and facilitate the defibrillation process by reducing fiber cohesiveness.
- They are used in combination with cellulases and/or hemicellulases towards the isolation of nanocellulose.
- The produced nanostructures are functionalized with –COOH groups which provides good colloidal stability.
- Enzyme-mediated nanocellulose preparation enables elimination of sugar degradation products, inhibitors and toxic compounds that could limit its application in food, medical and cosmetic industries.

In the present study, an AA9 LPMO from the thermophilic fungus *Thermothelomyces thermophilus* was employed both as a pre- and a post-treatment step alongside with commercially available and in-house produced tailored cocktails of hemicellulases and cellulases in four-step multi-enzymatic processes for the isolation of nanoscale cellulose from OxiOrganosolv pretreated beechwood.



Experimental part



1. *Tt*LPMO9G was heterologously produced in *Pichia pastoris* and biochemically characterized.

- Evaluation of temperature and pH stability and optimal activity conditions.
- Evaluation of susbstrate specificity of different polysaccharides (cellulose, glucuronoxylan, arabinoxylan, xyloglucan).

2. *Tt*LPMO9G was employed both as a pre- and a post-treatment step alongside with hemicellulases and cellulases in four-step multi-enzymatic processes for nanocellulose isolation

- Hemicellulose removal by the commercial enzyme mixture Cellic HTec2 (Novozymes) or a specified and well-determined in-house produced cocktail comprised of four glycosyl-hydrolases of families GH10, GH11, GH30, GH43 and an acetyl xylan esterase.
- Cellulose removal either by the commercial enzyme mixture Celluclast (Sigma Aldrich) or endoglucanases of families GH7, GH5 [4,5].
- Delignified OxiOrganosolv pretreated beechwood was used as a substrate [6].

3. Nanostructures obtained from each process were examined for their morphological features and dimensions, crystallinity, colloidal stability and the presence of carboxylate groups with atomic force microscopy (AFM), X-ray diffraction analysis (XRD), dynamic light scattering (DLS) and conductometric titration, respectively.

Results and Discussion

Substrate specificity, thermal stability and pH dependency of *Tt*LPMO9G

- The enzyme has a C1-regioselectivity and a dual cellulolytic/xylanolytic activity.
- The enzyme showed the highest activity at pH 7.0, with >80% of the optimum activity shown at pH 6.0, while it remained stable in the pH range of 3–10 after 72 h, retaining 100% of its initial activity.
- The optimal temperature was 40 ◦C, while the enzyme remained fairly stable up to 40 °C after pre-incubation for 48 h, and at 40, 50, 60 and 70 °C, the half-life was 170 h, 50 h, 20 h and 10 min, respectively.

Production of nanoscale cellulose through a four-step enzymatic process

- Cellulose nano-structures were isolated after different enzymatic processes from pretreated beechwood (Figure 1).
- Samples after treatment with tailored hemicellulose cocktail (processes 4-6) showed lower zeta-potential values than their counterparts after treatment with Cellic HTec2 (Figure 2), which is attributed to limited hemicellulose removal. High absolute zeta-potential values indicate stable nanofibers with good quality colloidal features.
- AFM analysis (Figure 3) showed a broad range of dimensions and features in the resulting nanostructured material, which is a characteristic property of nanocellulose produced after enzymatic treatment.
- LPMO post-treatment reduced the fibers diameter, eliminated agglomeration, and yielded stable colloidal structures with sufficient mutual repulsion due to the introduction of carboxyl groups on the surface of cellulose fibers.

Taking into consideration that *high solid fraction recovery*, *low soluble reducing sugar loss*, *high product crystallinity* and *stability of the colloidal suspension* (zeta-potential <-20 mV) are required for designing an efficient process for nanocellulose isolation from OxiOrganosolv pretreated beechwood:

i. hemicellulose removal with the commercially available Cellic HTec2 mixture and

ii. targeted cellulose degradation with EG7 and EG7-EG5

seems to be the most appealing strategy for the isolation of nanocellulose from beechwood biomass.

The results demonstrate the formation of well-dispersed nanoscale cellulose in the complete absence of any chemical or mechanical treatment step and verify the importance of efficient hemicellulose removal

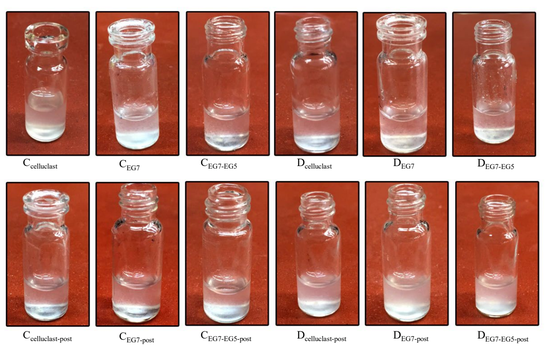


Figure 1. **Nanoscale cellulose isolated from different enzyme-mediated processes described in this study at a concentration of 0.2 wt%**

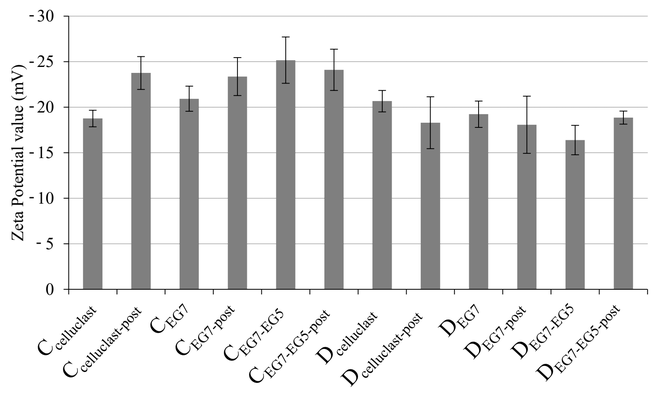


Figure 2. **Zeta-potential values for the isolated nanocellulose from different enzymatic processes.**

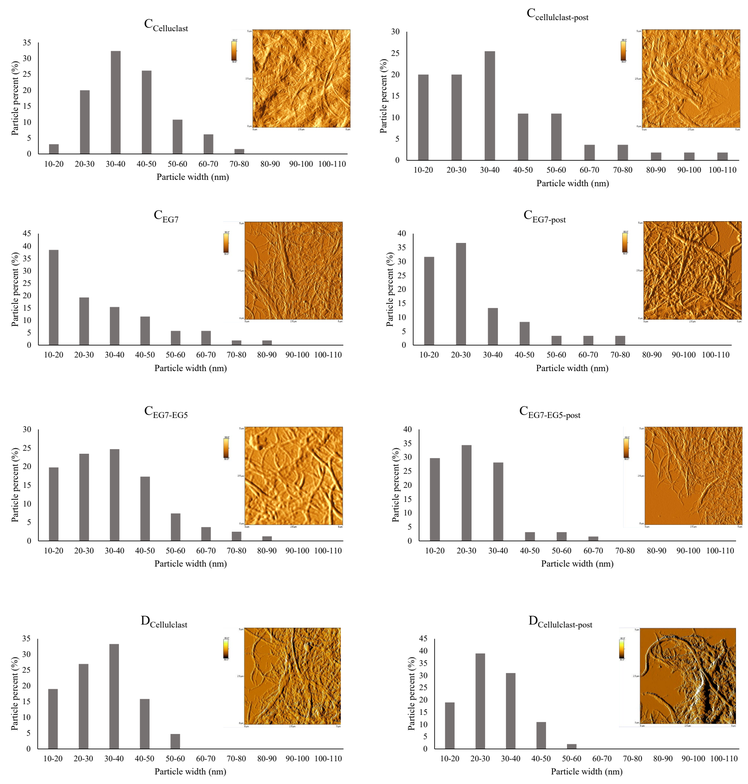


Figure 3. **AFM images and width values of isolated nanoscale cellulose from different enzymatic processes, after hemicellulose removal with Cellic Htec2.**

Table 1. % Solid recovery and characteristics of the isolated nanostructures after different steps of enzymatic treatment.

Sample	% Yield of solids	AFM analysis			CrI	Surface charge density- C_{COOH} (mmol/g)
		Height range (min-max values)	Average height	N of samples		
$C_{Celluclast}$	43.0	21.7 / 76.7	40.6	63	87.1	0.47
C_{EG7}	58.7	9.2 / 83.4	30.4	52	82.7	0.58
$C_{EG7/EG5}$	54.8	9.0 / 92.3	34.5	52	73.2	0.52
$D_{Celluclast}$	72.7	10.0 / 56.9	30.9	63	73.0	0.50
D_{EG7}	79.6	N.D	N.D.	N.D.	75.7	0.68
$D_{EG7/EG5}$	79.7	N.D.	N.D.	N.D.	67.0	0.52
$C_{celluclast-post}$	77.8	6.9 /110.3	37.7	55	-	1.43
$C_{EG7-post}$	77.2	6.6 / 79.0	28.4	57	-	3.48
$C_{EG7/EG5-post}$	81.6	8.5 / 81.8	30.2	54	-	2.81
$D_{celluclast-post}$	82.5	7.6 / 59.2	29.17	36	-	1.97
$D_{EG7-post}$	91.6	N.D	N.D.	N.D.	-	3.81
$D_{EG7/EG5-post}$	83.8	N.D.	N.D.	N.D.	-	3.05

Conclusions

- The C1-acting *Tt*LPMO9G from the thermophilic fungus *T. thermophilus* showed a high ability to oxidize cellulose and xylan, as well as organosolv pretreated beechwood biomass, as verified by the release of oxidized soluble products, indicating that *Tt*LPMO9G is a promising candidate to facilitate nanocellulose isolation in concert with hemicellulases and cellulases.
- Employment of LPMO both as a pre- and a post-treatment step in a multi-enzymatic process resulted in well-dispersed nanoscale cellulosic fibers, as indicated by the zeta-potential values and the presence of carboxylate groups.
- The results verify also the importance of efficient hemicellulose removal for the isolation of nanocellulose.

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