Enzymatic degradation of sulfite-pulped softwoods and the role of LPMOs

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Abstract

Background: Recent advances in the development of enzyme cocktails for degradation of lignocellulosic biomass, especially the discovery of lytic polysaccharide monooxygenases (LPMOs), have opened new perspectives for process design and optimization. Softwood biomass is an abundant resource in many parts of the world, including Scandinavia, but efficient pretreatment and subsequent enzymatic hydrolysis of softwoods are challenging. Sulfite pulping-based pretreatments, such as in the BALI™ process, yield substrates that are relatively easy to degrade. We have assessed how process conditions affect the efficiency of modern cellulase preparations in processing of such substrates.

Results: We show that efficient degradation of sulfite-pulped softwoods with modern, LPMO-containing cellulase preparations requires the use of conditions that promote LPMO activity, notably the presence of molecular oxygen and sufficient reducing power. Under LPMO activity-promoting conditions, glucan conversion after 48-h incubation with Cellic® CTec3 reached 73.7 and 84.3% for Norway spruce and loblolly pine, respectively, at an enzyme loading of 8 mg/g of glucan. The presence of free sulfite ions had a negative effect on hydrolysis efficiency. Lignosulfonates, produced from lignin during sulfite pretreatment, showed a potential to activate LPMOs. Spiking of Celluclast®, a cellulase cocktail with low LPMO activity, with monocomponent cellulases or an LPMO showed that the addition of the LPMO was clearly more beneficial than the addition of any classical cellulase. Addition of the LPMO in reactions with spruce increased the saccharification yield from approximately 60% to the levels obtained with Cellic® CTec3.

Conclusions: In this study, we have demonstrated the importance of LPMOs for efficient enzymatic degradation of sulfite-pulped softwood. We have also shown that to exploit the full potential of LPMO-rich cellulase preparations, conditions promoting LPMO activity, in particular the presence of oxygen and reducing equivalents are necessary, as is removal of residual sulfite from the pretreatment step. The use of lignosulfonates as reductants may reduce the costs related to the addition of small molecule reductants in sulfite pretreatment-based biorefineries.

Keywords: Lignocellulose, Sulfite, Pretreatment, Pulping, Cellulase, LPMO, AA9, GH61, Biofuel
(SPORL). Generally, pretreatment and subsequent enzymatic digestion of hardwoods is less demanding compared to softwoods, which contain more lignin and have more rigid structure [1, 3]. Relatively efficient pretreatment and enzymatic hydrolysis of softwoods has been demonstrated for organosolv- and SPORL-pretreated biomasses [4, 5]. The BALI™ process is an alternative sulfite-based pretreatment that yields cellulose-rich substrates containing little lignin and hemicellulose relative to, e.g., the SPORL pretreatment, whose enzymatic conversion to glucose is relatively easy [6].

Complete biochemical conversion of cellulose to monomeric sugars requires the synergistic action of several classes of enzymes [7]. By far, the most studied enzymes are cellulose-degrading glycosidase hydrolases, known as cellulases. Cellulases include enzymes acting on the ends of the cellulose chain, known as cellulase 1,4-β-cellobiosidases or celllobiohydrolases, cleaving off cellbiose units from reducing and non-reducing chain ends. Endo-β-1,4-glucanases cleave internal glycosidic bonds in the cellulose chains, thus generating novel chain ends, and β-glucosidases convert released soluble celloextrins and cellobiose to glucose [8]. In natural biomass-converting enzyme systems, the cellulases are accompanied by a wide range of hemicellulose- and lignin-degrading enzymes working in concert to fully decompose lignocellulose.

The discovery of lytic polysaccharide monooxygenases (LPMOs) has challenged the classical model of enzymatic decomposition of polysaccharides. Contrary to the cellulases, which rely on hydrolysis to perform catalysis, LPMOs are metalloenzymes breaking down glycosidic bonds by an oxidative mechanism involving molecular oxygen (or, in a recently suggested alternative mechanism, hydrogen peroxide [9]) and an electron donor [10, 11]. LPMO-catalyzed cleavage of the glycosidic bond results in the formation of oxidized chain ends, either at the C1 and/or at the C4 carbon position. So far, LPMOs cleaving glycosidic bonds in chitin [10, 12], cellulose [11, 13–16], cellobioextrins, and starch [20, 21] have been identified. LPMOs are classified in the Carbohydrate Active Enzymes (CAZy) database as auxiliary activities families AA9, AA10, AA11, and AA13 [22].

Despite the fact that the boosting effect of LPMOs on cellulase activity was demonstrated early on [13, 15], exploration of the role and performance of LPMOs in applied settings has been limited. Cannella et al. [23] showed that LPMOs present in the commercial cellulase preparation Celluclast® CTeC2 enhanced hydrolysis of hydrothermally pretreated wheat straw. Müller and colleagues showed the importance of LPMOs in this enzyme preparation for degradation of steam-exploded birch [24]. Using both model cellulosic substrates and industrially relevant lignocellulosic biomass, Hu et al. [25] demonstrated synergy between AA9 LPMOs and cellulases.

In this study, we have investigated the impact of process conditions on the enzymatic degradation of sulfite-pulped softwood biomasses with particular focus on the role of LPMOs, the presence of oxygen and small molecule electron donors, and the effect of residual sulfite. We have utilized commercially available cellulase preparations, Celluclast® 1.5L (supplemented with Novozym 188) and Celic® CTeC3, as well as an in-house produced fungal LPMO from Thermoascus aurantiacus (TaLPMO9A) and individual major Trichoderma reesei cellulases.

**Methods**

**Cellulosic substrates, pretreatment, and compositional analysis**

Norway spruce (Picea abies) and loblolly pine (Pinus taeda) were subjected to the proprietary pretreatment technology developed by Borregaard AS (Sarpsborg, Norway) [6, 26]. The pretreatment included a sulfite cooking step utilizing calcium or sodium as a counterion, which converts lignin into water-soluble lignosulfonates and removes most of the hemicellulose that is washed out of the remaining cellulose pulp. The composition of the pretreated materials was determined following the National Renewable Energy Laboratory standardized protocol (NREL/TP-510-42618) and is presented in Table 1. The commercially available model cellulosic substrate Avicel PH-101 (Sigma Aldrich, St. Louis, MO, USA) was also used in enzymatic hydrolysis experiments. Dried lignosulfonates were kindly provided by Borregaard AS (Norway, Sarpsborg) and were obtained by the evaporation of the sulfite spent liquor (SSL) that had been stripped of soluble sugars by fermentation [6].

**Enzymes**

Celluclast® 1.5L, Novozym 188, and Celic® CTeC3 were all kindly provided by Novozymes A/S (Bagsværd, Denmark).

**Table 1 Compositional analysis of pretreated Norway spruce and Loblolly pine**

| Substrate     | Component (%) of DM | Glucan b | Xylan b | Mannan b | Acid insoluble lignin c |
|---------------|---------------------|----------|---------|----------|-------------------------|
| Norway spruce | 88.3                | 4.5      | 4.8     | 3.8      |                         |
| Loblolly pine | 80.4                | 3.0      | 3.0     | 8.9      |                         |
| Avicel        | 92.2                | 2.1      | 0.1     | 0.9      |                         |

Values are presented as a percentage of dry matter

a The presence of the potentially inhibitory sugar derivatives furfural and hydroxymethylfurfural was also analyzed, both were not detected

b Sugar monomers measured by HPLC and corrected by the hydration factor (*0.9)

c Klason lignin (not corrected for ash)
Denmark). The AA9 family LPMO from Neurospora crassa (NcLPM09C), used for generation of C4 oxidized standards, was produced and purified as described previously [24]. Trichoderma reesei Cel7A was purified from the culture filtrate of T. reesei QM 9414 (VTT Culture Collection, D-74075, Finland) essentially as described in [27]. TrCel7B and TrCel6A from T. reesei were purified as described by Suurnäkki et al. [28].

The gene encoding T. aurantiacus LPMO9A (also known as TaGH61A; [11]) including its native signal sequence was cloned into the Gateway vector destination pDONR221 (Invitrogen) and the pDONR plasmid was transformed into E. coli DH5α (Thermo Fisher Scientific, Waltham, MA, USA) utilizing bovine serum albumin (BSA) as a standard [31].

Enzymatic hydrolysis
Saccharification of lignocellulosic biomass and AviCel was conducted in 50-mL rubber sealed glass bottles (Wheaton, Millville, NJ, USA) with 10 mL working volume. The biomass obtained after the sulfite pulping process described above was not washed, milled, or dried prior to the saccharification experiments. Enzymatic hydrolysis was performed with 5% total solids loading in 50 mM sodium acetate buffer pH 5.0 at 50 °C, with 8 mg/g glucan total protein loading of either a 5:1 (w/w) Celluclast®:Novozym 188 mixture or CelloC® CTec3, in the presence or absence of an external electron donor. To facilitate efficient mixing, bottles were rotated at 38 rpm in a Multi RS-60 programmable rotator (Biosan, Riga, Latvia). Anaerobic conditions were reached by vigorously flushing the substrate-buffer suspension with nitrogen (Yara, Trondheim, Norway) for 3 min and addition
of 0.025% (w/v, final concentration) of L-cysteine hydrochloride monohydrate (Sigma Aldrich, St. Louis, MO, USA) to ensure complete removal of oxygen. In aerobic conditions, oxygen came from ambient air present in the headspace of the reaction bottles. Reactions were initiated by injection of 800 µL of enzyme preparations, appropriately diluted in 50 mM sodium acetate buffer pH 5.0, through the rubber septum. Reactions were terminated at different time points. To ensure reproducible sampling from the flasks, the entire reaction mixture was diluted threefold with ultrapure water (Merck Millipore, Billerica, MA, USA) and then transferred to new 50-mL Falcon tubes. Reactions were stopped by incubating the Falcon tubes at 100 °C for 15 min in a water bath. Supernatants were collected by centrifugation of the tubes for 15 min at 3803g and 4 °C, and these were then transferred to 1.5-mL tubes and stored at −20 °C prior to further analysis.

**Product analysis**

Glucose and cellobiose released during enzymatic hydrolysis were quantified with High-Performance Liquid Chromatography (HPLC) using a Dionex Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) coupled to a refractive index (RI) detector 101 (Shodex, Tokyo, Japan). Separation of hydrolysis products was achieved utilizing a Rezex ROA-Organic Acid H⁺ (8%), 300 × 7.8 mm analytical column equipped with SecureGuard Carbo-H⁺ 4 × 3.0 mm guard column (Phenomenex, Torrance, CA, USA), operated at 65 °C, with 5 mm H₂SO₄ as the mobile phase, and a flow rate of 0.6 mL/min. For quantification, the areas of peaks corresponding to glucose and cellobiose were compared to standard curves generated with known concentrations of glucose and cellobiose (in the range of 0.1–10 g/L). Hydrolysis yields were calculated based on detected glucose and cellobiose (typically less than 1% of the total) and expressed as a percentage of the theoretical maximum that would be obtained upon complete conversion of glucan to glucose.

Statistical significance of differences in glucan saccharification yields was determined using two-way ANOVA with Tukey’s post hoc test (95% confidence interval) and was carried out using R (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance is shown as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Native cello-oligosaccharides (DP 2 to DP 5) and oxidized sugars were analyzed with High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using a Dionex ICS 3000 system (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA1 2 × 250 mm analytical column with a CarboPac PA1 2 × 50 mm guard column, as described in [15]. Briefly, initial conditions were set to 0.1 M NaOH (100% eluent A), followed by a linear gradient of eluent B (1 M sodium acetate in 0.1 M NaOH), reaching 10% B 10 min after sample injection and 30% B at 35 min after injection. This was followed by a 5-min exponential gradient to 100% eluent B, after which the column was reconditioned by running the initial conditions for 9 min.

C4-oxidized standards were generated with NcLP-MO9C by cellopentaose degradation as described in [24]. The data were collected and analyzed using Chromeleon 7.0 software.

**Results**

Below, several series of hydrolysis experiments are described. Table 2 provides an overview of the hydrolysis yields obtained in these experiments.

**Saccharification of a model cellulosic substrate**

The model cellulosic substrate Avicel PH-101 was incubated for 48 h with an older generation, LPMO-poor, cellulase mixture (Celluclast® 1.5L supplemented with Novozym 188; 5:1, w/w), as well as a modern, LPMO-containing, cellulase preparation, Celliç® CTec3, under either aerobic or anaerobic conditions, in the presence or absence of an external electron donor (ascorbic acid). These experiments showed that Celliç® CTec3 gives higher hydrolysis yields, but only under aerobic conditions and when adding an external electron donor (Fig. 1a). Indeed, under these conditions only, considerable LPMO activity was detectable (Fig. 1b; the amount of C4-oxidized product amounts to approximately 1.5% of the amount of solubilized glucan). Saccharification of Avicel with the Celluclast® 1.5L:Novozym 188 mixture was hardly affected by the presence of oxygen or the addition of ascorbic acid (Fig. 1c), and under LPMO-promoting conditions, only low product levels were observed (Fig. 1d). Celluclast® 1.5L is a product based on the T. reesei secretome, in which three AA9 family LPMOs have been identified, which however, are expressed at low levels [13, 32].

**Saccharification of sulfate-pulped softwoods**

Sulfite-pulped Norway spruce was incubated with both cellulase preparations for 48 h, under conditions identical to those used for Avicel, with strikingly similar results (Fig. 2a–d). Again, Celliç® CTec3 was the most efficient and, again, only under aerobic conditions and when adding an external source of electrons (Fig. 2a). Saccharification of pretreated Norway spruce with the LPMO-poor cocktail was not improved by aerobic conditions or the presence of electron donor (Fig. 2c). Oxidized products were only observed for reactions with oxygen and externally added electron donor present, and the levels of oxidized product were much higher for Celliç® CTec3 (Fig. 2b), compared
to the Celluclast® reaction (Fig. 2d). This result shows that the heavily delignified sulfite-pretreated softwood biomass lacks the potential to activate LPMOs.

Saccharification of sulfite-pulped loblolly pine with Cellic® CTec3 using the same conditions and varying the same parameters showed the same trends as observed for Norway spruce and Avicel. Interestingly, saccharification of pretreated loblolly pine was clearly more efficient than identically pretreated Norway spruce (compare Fig. 2e with a). The higher conversion of loblolly pine was achieved despite a seemingly lower LPMO activity (compare Fig. 2f with b).

The importance of oxygen, which affects LPMO activity, was explored further using extended reaction times (up to 144 h). The results showed that enzymatic hydrolysis of sulfite-pulped Norway spruce under anaerobic conditions never reached the yields obtained under conditions promoting LPMO activity, the final yields being about 65 and 85%, respectively (Fig. 3).

Reduced glutathione and gallic acid can also drive LPMO activity by acting as electron donors [10, 15, 25]. Similarly to the addition of ascorbic acid, addition of each of these reducing agents improved enzymatic degradation of pretreated Norway spruce under aerobic conditions (Additional file 1: Figure S1, a) albeit less efficiently than ascorbic acid (Fig. 2a).

Repeating the experiments with sulfite-pulped Norway spruce, in the absence of L-cysteine hydrochloride monohydrate (0.025%, w/v), showed that the addition of this compound, used to ensure anaerobic conditions, had no effect on hydrolysis yields (Additional file 2: Figure S2).

**Effect of residual sulfite on softwood saccharification**

The sulfite-pretreated biomasses used in the experiments described above had been stored for some time and were likely devoid of residual sulfite, which is readily oxidized to sulfate in a reaction that involves oxygen and leads to the generation of various reactive intermediate species [33]. Sulfite pretreatment of lignocelluloses in a factory may result in residual sulfite still being present in the material that is subjected to subsequent enzymatic hydrolysis. To test how residual sulfite would affect enzymatic hydrolysis, we studied the effect of sulfite on hydrolysis of Avicel (Additional file 3: Figure S3) and pretreated Norway spruce (Fig. 4) by Cellic® CTec3 and the Celluclast®-based cocktail. We tested the effect of adding 1000 ppm (12.5 mM) of sulfite ions (in the form of freshly added sodium sulfite), which was considered to be at the higher end of the concentrations that could be expected to be present in freshly pretreated and washed biomass, based on process data from the BALI™ pilot plant at Borregaard AS. Generally, sulfite had a negative effect on enzymatic hydrolysis of all substrates and no LPMO products could be detected (Fig. 4; see also Additional file 1: Figure S1, a; Additional file 2: Figure S2, a; Additional file 3: Figure S3). Maximal conversion with Cellic® CTec3 (i.e., aerobic, with AscA, 48 h) was reduced from 69.0 and 73.7% to 31.6 and 34.1% for Avicel and Norway spruce,

| Raw material  | Enzyme          | Conditions | AscA | Sulfite (12.5 mM) | Glucan solubilization after 48 h (% of theoretical) |
|--------------|-----------------|------------|------|------------------|-----------------------------------------------|
| Avicel PH-101| Cellic® CTec3    | Aerobic    | +    | −                | 69.0 ± 1.9                                     |
|              |                 | Aerobic    | +    | +                | 31.6 ± 0.4                                     |
|              |                 | Anaerobic  | +    | −                | 50.0 ± 1.4                                     |
|              |                 | Anaerobic  | +    | +                | 44.1 ± 1.2                                     |
|              | CellulasteNovozym 188 | Aerobic    | +    | −                | 55.6 ± 0.9                                     |
|              |                 | Aerobic    | +    | +                | 39.5 ± 0.5                                     |
|              |                 | Anaerobic  | +    | −                | 57.1 ± 0.7                                     |
|              |                 | Anaerobic  | +    | +                | 38.6 ± 4.3                                     |
| Norway spruce| Cellic® CTec3    | Aerobic    | +    | −                | 73.7 ± 0.7                                     |
|              |                 | Aerobic    | +    | +                | 34.1 ± 0.4                                     |
|              |                 | Anaerobic  | +    | −                | 50.6 ± 3.8                                     |
|              |                 | Anaerobic  | +    | +                | 55.6 ± 2.3                                     |
|              | CellulasteNovozym 188 | Aerobic    | +    | −                | 64.9 ± 2.1                                     |
|              |                 | Aerobic    | +    | +                | 38.7 ± 1.5                                     |
|              |                 | Anaerobic  | +    | −                | 60.2 ± 1.4                                     |
|              |                 | Anaerobic  | +    | +                | 50.3 ± 2.6                                     |
| Loblolly pine| Cellic® CTec3    | Aerobic    | +    | −                | 84.3 ± 1.2                                     |
|              |                 | Anaerobic  | +    | −                | 60.3 ± 0.7                                     |
respectively (Table 2). These decreases in yields are larger than the effects of oxygen and ascorbic acid discussed above and are thus not likely to be only “LPMO effects.” Indeed, the efficiency of the LPMO-poor Celluclast® mixture was also affected by sulfite, albeit to a lesser extent (Table 2; Fig. 4; Additional file 3: Figure S3). The effect of sulfite was clearly less pronounced under anaerobic conditions, which could be due to the absence of reactions between oxygen and sulfite that may lead to the formation of various compounds, including the sulfite radical and other radicals [33] that inhibit the enzymes. In line with this, the addition of reducing agent (or “antioxidant”) to the aerobic reactions had a clear positive effect on yields, despite the absence of LPMO activity (e.g., Figure 4). In the absence of reducing agent and in the presence of sulfite, hydrolysis yields were generally very low.

**Effect of lignosulfonates on degradation of sulfite-pretreated softwoods**

Besides a cellulose-rich pulp, sulfite pretreatment also yields lignosulfonates (LS), representing a valuable commodity from the lignin fraction of softwood biomass. It has been shown that LS can improve enzymatic saccharification of pretreated lignocellulosic materials [34, 35]. Using enzymatic hydrolysis of sulfite-pulped Norway spruce as a test case, we assessed whether LS could replace ascorbic acid. It can be observed from Fig. 5 that 0.25% (w/w) LS led to increased hydrolysis yields under aerobic conditions, but to a smaller extent than the addition of ascorbic acid (Fig. 2). The increase in yield obtained by adding LS was accompanied by the production of oxidized products (Fig. 5b), albeit, again, at lower levels compared to reactions with ascorbic acid (Fig. 2b).
Fig. 2  Saccharification of pretreated Norway spruce (a–d) and loblolly pine (e–f) under aerobic (blue bars) or anaerobic (red bars) conditions in the presence or absence of 1 mM ascorbic acid (±AscA). The left panels show glucan conversion (as a percentage of theoretical glucan conversion) and the right panels show the concentration of Glc4gemGlc, at two time points. Enzymatic hydrolysis was carried out with Cellic® CTe3 (a, b, e, f) and Celluclast®:Novozym 188 (mixed at 5:1 ratio, w/w) (c, d) at 8 mg/g glucan total protein loading using reaction mixtures containing 5% DM in 50 mM sodium acetate pH 5.0 that were incubated at 50 °C. The data points represent the average value of three independent experiments with one technical replicate per experiment. The error bars represent standard deviations of the three independent experiments. The statistical significance of differences in the 48-h saccharification yields was analyzed using two-way ANOVA with Tukey’s post hoc test (95% confidence interval) and is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.
Addition of LS helped also to mitigate the negative effect of sulfite ions on hydrolysis efficiency (Fig. 5c), but to a lower extent than ascorbic acid (Fig. 4a) and other tested reducing agents (Additional file 1: Figure S1c, d).

**Discussion**

Since the discovery of the true nature of LPMOs [10], their role in the degradation of cellulose and pretreated lignocellulosic biomasses has been the subject of several studies [13, 23–25, 37]. For example, it has been shown that harnessing LPMO activity improves the saccharification of hydrothermally pretreated wheat straw [23] and steam-exploled birch [24] with an LPMO-rich cellulase preparation, Cellic® CTec2. Both these studies also showed that LPMO activity does not require the presence of externally added small molecule reducing agents, as long as the substrate is rich in lignin. A similar conclusion was drawn by Hu et al. [25] in their study focused on spiking cellulase preparations with an AA9 LPMO in the degradation of several pretreated lignocelluloses. Importantly, the study by Müller et al. (2015) established the importance of the presence of oxygen in harnessing the full potential of LPMOs.

From previous work, it is known that sulfite-pretreated softwoods can be degraded quite efficiently with commercially available cellulase preparations [5, 38]. Sulfite pulping using the BALI™ pretreatment yields cellulose-rich and almost lignin-free materials that are also relatively easy to degrade [6]. So far, the potential role of LPMOs in the degradation of sulfite-pretreated biomasses has not been addressed, nor have the implications of a possible role of LPMOs on process design. The present experiments, using both a model cellulolytic substrate and industrially relevant, sulfite-pulped softwoods, shed light on these issues. The data show a clear correlation between the efficiency of saccharification and the detection of LPMO-generated products, underpinning the major contribution of LPMOs to the saccharification process, which amounts to increases in saccharification yield by up to 24% (in the case of sulfite-pulped loblolly pine). The presence of an externally added electron donor and aerobic conditions during enzymatic hydrolysis are both crucial for LPMO activity and for fully exploiting the cellulosic potential of modern cellulase preparations. The requirement for an external electron donor to harness LPMO activity separates sulfite-pulped lignocelluloses from biomasses subjected to other physicochemical pretreatments and is likely due to extensive lignin removal during sulfite pulping (Table 1). The small amount of remaining lignin bound to the cellulose is most likely sulfonated, which will alter
its properties, e.g., the degree of hydrophilicity, relative to, e.g., the lignin present in steam-exploded materials [3]. Dimarogona et al. [39] and Westereng et al. [40] have demonstrated that lignin is capable of transferring electrons to LPMOs and stimulate their activity in cellulose decomposition, whereas Hu et al. [25] have shown that complete lignin removal from steam-pretreated lodgepole pine nearly completely eliminated the boosting effect of adding an LPMO. Finally, by studying enzymatic saccharification of sugarcane bagasse samples obtained from different pretreatments and with varying degrees of delignification, Rodríguez-Zúñiga et al. [41] showed a positive correlation between lignin content and LPMO activity. All these results confirm early observations by Harris et al. [13], who showed that the enigmatic boosting effect of a GH61 protein (which later turned out to be an LPMO) on cellulase activity depended on the presence of lignin.

In the presence of ascorbate and under aerobic conditions, improvement of the enzymatic degradation of sulfite-pretreated softwoods was correlated with the accumulation of oxidized products. The positive effect of LPMO-promoting conditions was only observed when using LPMO-rich cellulase preparation Cellic® CTec3 and when spiking an older cellulase preparation, LPMO-poor Celluclast® 1.5L, with TaLPMO9A. The dependence on oxygen is evident from both the present study and earlier work on steam-exploded birch by Müller et al. [24]. Interestingly, there are recent indications that LPMO reactions may be driven by H2O2 instead of O2.

**Fig. 4** Saccharification of pretreated Norway spruce in the presence of sulfite under aerobic (blue bars) or anaerobic (red bars) conditions, in the presence or absence of 1 mM ascorbic acid (+AscA). The left panels show glucan conversion (as a percentage of theoretical glucan conversion) and the right panels show concentrations of Glc4gemGlc, at two time points. Enzymatic hydrolysis was carried out with Cellic® CTec3 (a, b) and Celluclast® Novozym 188 (mixed at 5:1 ratio, w/w) (c, d) at 8 mg/g glucan total protein loading in reactions containing 5% DM in 50 mM sodium acetate pH 5.0 that were incubated at 50 °C. Data for similar reactions without sulfite are presented in Fig. 2 and data for the effect of sulfite on hydrolysis of Avicel are presented in Additional file 3: Figure S3. The data points represent the average value of three independent experiments with one technical replicate per experiment. The error bars represent standard deviations of the three independent experiments. The statistical significance of differences in the 48-h saccharification yields was analyzed using two-way ANOVA with Tukey’s post hoc test (95% confidence interval) and is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.
and that in such a set-up the LPMO requires only substoichiometric amounts of reductant [9]. While further work is needed to study the implications of this novel way to drive LPMO activity, it is worth noting that the use of H₂O₂ may change or even abolish the need for supplying molecular oxygen and reductant in industrial biorefining. Supplying and controlling hydrogen peroxide, which is soluble in water, in high dry matter hydrolysis reactors may be easier, compared to using oxygen.

Careful inspection of Figs. 1 (hydrolysis of Avicel) and 2 (hydrolysis of sulfite-pulped Norway spruce) shows that under conditions that do not lead to LPMO activity, the Celluclast®:Novozym 188 cocktail is more efficient than Cellic® CTec3. Under these conditions, the LPMO fraction of the Cellic® CTec3 enzyme cocktail, perhaps amounting to 15% of the protein, is not active, meaning a reduced effective enzyme load. This observation underpins the importance of harnessing the power of LPMOs in modern cellulase cocktails.

While sulfite pulping generally seems to be a good pretreatment for softwoods, the present data show that residual sulfite that might be carried over from the pretreatment step to the enzymatic hydrolysis step has a negative effect on saccharification efficiency. The oxidation of sulfite to sulfate happens via a multi-step pathway involving the formation of several reactive intermediates [33] that are known to interact with, e.g., proteins [33, 42, 43]. For example, radicals formed during oxidation of sulfite react with methionine and tryptophan in proteins, which could easily lead to enzyme inactivation [33, 43]. Reducing agents, such as ascorbic acid, are commonly used in the food industry to protect from...
oxidative damage caused by reactive oxygen species, and indeed our results show that reducing agents prevent the enzyme inactivation happening under aerobic conditions in the presence of sulfite. The lack of LPMO products under these conditions shows that the positive effect of reductants is rather due to the scavenging of free radicals formed during oxidation of sulfite than to the promotion of LPMO activity. Obviously, utilization of large amounts of costly chemicals such as reductants is not economically feasible. Thus, the development of enzymes resistant to high sulfite concentrations or introduction of process steps for removing residual sulfite should be considered.

Notably, further research on actual sulfite levels within the biorefinery is needed and so are studies on sulfite stability. The conditions used in this study, where we used high amounts of freshly added sodium sulfite, are not representative of the conditions in a real biorefinery.

It was known from before that lignosulfonates may have a positive effect on the saccharification of sulfite-pretreated lignocellulosic biomasses [34, 35]. These positive effects have been ascribed to the surfactant properties of LS [34] or to the formation of lignosulfonate–cellulase complexes [35], where both effects were thought to lead to reduced non-productive binding of cellulases to the substrate. In the present study, we observed that the positive effect of LS on hydrolysis efficiency was only present under aerobic conditions (Fig. 5a) and was linked to the promotion of LPMO activity (Fig. 5b). Interestingly, we also observed that LS reduced the negative effect of sulfite ions, though to a lesser extent than ascorbate. The exact mechanism of the stimulation of LPMO activity by LS addition is unknown and warrants further research. Notably, this effect could involve the interplay between (soluble) LS and residual lignin bound to the cellulose, which would be analogous to the interplay between solid and soluble lignin described by Westereng et al. [40].

Conclusions
In this study, we have demonstrated efficient enzymatic conversion of sulfite-pretreated softwoods by Cellic® CTec3 and we have demonstrated the importance of LPMOs in this process. We have also shown that, in order to obtain the high saccharification yields that are possible due to LPMO action, it is crucial that process conditions are adapted to the LPMOs, which need oxygen and reducing equivalents. The removal of residual sulfite from pretreatments is of major importance and both assessment of typical sulfite levels and methods to deal with these need further attention. Lignosulfonates have a potential to act as the electron donor for LPMOs, which could reduce the costs related to the addition of small molecule reductants in sulfite pretreatment-based biorefineries.

Additional files

Additional file 1: Figure S1. Saccharification of pretreated Norway spruce in the absence (a, b) or presence (c, d) of sulfite under aerobic (blue bars) or anaerobic (red bars) conditions in the presence or absence of 1 mM gallic acid (−/− GA; left panels) or reduced glutathione (+/− RG; right panels). Enzymatic hydrolysis was carried out with Cellic® CTec3 at 8 mg/g glucan total protein loading in reaction mixtures containing 5% DM that were incubated in 50 mM sodium acetate pH 5.0, at 50 °C. Reactions with sulfite (lower panels) contained 1000 ppm of sulfite ions added as a sodium sulfite (12.5 mM). The data points represent the average value of three independent experiments with one technical replicate per experiment. The error bars represent standard deviations of the three independent experiments. The statistical significance of differences in glucan conversion between the control reactions with BSA and reactions with added individual enzymes (in the presence or absence of 1 mM ascorbic acid) was analyzed using two-way ANOVA with Tukey’s post hoc test (95% confidence interval) and is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.
Additional file 2: Figure S2. Saccharification of sulfite-pretreated Norway spruce without addition of -cysteine hydrochloride monohydrate (0.025 % w/v) in the anaerobic reactions, in the absence (a, b) or presence (c, d) of sulfite under aerobic (blue bars) or anaerobic (red bars) conditions, in the presence or absence of 1 mM ascorbic acid (+/− AsCA). The left panels show glucan conversion (as a percentage of theoretical glucan conversion) and the right panels show the concentration of Glc4gemGlc, at two time points. Enzymatic hydrolysis was carried out with Cellulases Clic3 at 8 mg/g glucan total protein loading in reaction mixtures containing 5% DM in 50 mM sodium acetate pH 5.0 that were incubated at 50 °C. Reactions with sulfite (lower panels) contained 1000 ppm of sulfite ions added as a sodium sulfite (12.5 mM). The data points represent the average value of three independent experiments with one technical replicate per experiment. The error bars represent standard deviations of the three independent experiments. The statistical significance of differences in the 48 hour saccharification yields was analyzed using two-way ANOVA with Tukey’s post hoc test (95% confidence interval), and is indicated as follows: *, p<0.05; **, p<0.01; ***, p<0.001.

Additional file 3: Figure S3. Saccharification of Avicel PH-101 in the presence of sulfite under aerobic (blue bars) or anaerobic (red bars) conditions, in the presence or absence of 1 mM ascorbic acid (+/− AsCA). The left panels show glucan conversion (as a percentage of theoretical glucan conversion) and the right panels show concentration of Glc4gemGlc, at two time points. Enzymatic hydrolysis was carried out with Cellic® Clic3 (a, b) and Celluclast-Novozym 188 (mixed at 5:1 ratio, w/w) (c, d) at 8 mg/g glucan total protein loading in reaction mixtures containing 5% DM in 50 mM sodium acetate pH 5.0 that were incubated at 50 °C. Data for similar reactions without sulfite are presented in Fig. 1. The data points represent the average value of three independent experiments with one technical replicate per experiment. The error bars represent standard deviations of the three independent experiments. The statistical significance of differences in the 48 hour saccharification yields was analyzed using two-way ANOVA with Tukey’s post hoc test (95% confidence interval), and is indicated as follows: *, p<0.05; **, p<0.01; ***, p<0.001.

Abbreviations
AA, auxiliary activity; AsCA, ascorbic acid; Glc4gemGlc, 4-hydroxy-β-D-xyl‑hexopyranosyl-(1→4)-β-D-glucopyranosyl; HPAEC, high-performance anion exchange chromatography; HPLC, high-performance liquid chromatography; LPMO, lytic polysaccharide monooxygenase; LS, lignosulfonates.

Authors’ contributions
PC, MD, OB, ML, SJH, and VGHE designed the experiments and interpreted the results; SJH and VGHE supervised the study; PC did most of the experimental work; DP, GM, and MS-A contributed with experiments, materials, and ideas. PC drafted the manuscript, with help from GM, SJH, and VGHE; PC and VGHE finalized the manuscript. All authors read and approved the final manuscript.

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Competing interests
Lersch and Bengtsson are employees of Borregaard AS, Sarpsborg, Norway. Marie Dahlstrom was at the time of completing this work an employee of Borregaard AS, Sarpsborg, Norway.

Availability of supporting data
Supporting data are provided in Additional files 1, 2, and 3.

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