Oxidation-reduction potential (ORP) as a tool for process monitoring of H_2O_2/LPMO assisted enzymatic hydrolysis of cellulose

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Conflicts of interest

None declared.

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Abstract

Oxidation-reduction potential (ORP) is an environmental factor of importance in several biological conversion processes. Lytic polysaccharide monooxygenases (LPMOs) catalyze oxidative disruption of the cellulose chain in the presence of oxygen or hydrogen peroxide and increase enzymatic hydrolysis yields. However, the enzymes are also sensitive to oxidative damage and the level of oxidative agent needs to be controlled to avoid inactivation of the LPMOs. In the current study, enzymatic hydrolysis of sulfite-pretreated softwood (12% DM loading) was carried out in lab scale reactors with gradual addition of hydrogen peroxide using an LPMO-containing commercial enzyme cocktail. The ORP was measured during enzymatic hydrolysis together with released glucose and the level of C4-oxidized dimer as a marker for LPMO activity. Arrests in LPMO activity coincided with a markedly changed ORP signal and this was utilized in subsequent experiments in which the feed rate of hydrogen peroxide was controlled by keeping the ORP at predetermined set-points of -40 mV, -80 mV and -120 mV. Under anaerobic conditions, the highest hydrolysis yield (78% after 72 h) was found for the ORP set-point of -80 mV. The results show that ORP can serve as an indicator of LPMO activity and may help optimizing overall process efficiency.

Keywords: cellulose, enzymatic hydrolysis, lytic polysaccharide monooxygenase (LPMO), oxidation-reduction potential (ORP), hydrogen peroxide, redox

1. Introduction

Efficient production of biofuels and biochemicals from lignocellulosic biomass is needed in order to establish a future low-carbon economy. Although the cost of the enzymatic hydrolysis of cellulose has come down quite significantly in recent years, it is still substantial and further cost reductions are needed to reach cost levels for cellulosic sugars comparable to those of starch derived sugars. Overcoming the recalcitrance of the cellulosic substrate by better utilizing the activity of lytic polysaccharide monooxygenases (LPMOs) present in commercial enzyme cocktails is one highly promising approach towards this goal.

The oxidative activity of LPMOs was first discovered in chitin-acting enzymes [1], closely followed by reports on cellulose acting enzymes [2–5]. The main findings of these early studies were: (1) LPMOs incorporate a single oxygen atom into the polysaccharide chain during oxidative cleavage, (2) LPMO activity requires electron donors (such as ascorbate) and (3) the catalytic center of LPMOs contains a copper ion. Subsequently, it was shown that lignin and/or lignin degradation products can serve as electron donors, eliminating the need for the addition of external electron donors during enzymatic hydrolysis of pretreated biomass [6–9]. Additionally, the importance of dissolved oxygen was confirmed by the fact that anaerobic conditions did not support LPMO activity [9,10].

The exact mechanism of LPMO action is still not fully elucidated. Recent results indicate that H_2O_2 is a co-substrate for the oxidation of cellulose by LPMOs [11]. Under aerobic conditions LPMOs that are not bound to substrate generate H_2O_2 from stoichiometric amounts of oxygen and reducing agent [12], and this could fuel H_2O_2-driven cellulose cleavage. A recent study has shown that continuous feeding of H_2O_2 to

saccharification reactions with Cellic CTec2 increases the rate of cellulose oxidation, without the need for stoichiometric addition of reducing agent [13]. These recent developments open up for new process alternatives in commercial hydrolysis of cellulose, in which hydrogen peroxide is used as the oxidant, either together with or instead of oxygen, to increase the rate of hydrolysis and/or ultimate glucose yields.

One key problem related to the use of LPMOs in biomass processing is their sensitivity to inactivation by oxidative damage. It is well known that H_2O_2 may damage enzymes, likely through generation of hydroxyl radicals upon reactions with transition metals, and it has been shown that enzyme inactivation during biomass processing may be alleviated by addition of catalases [14]. In this respect, LPMOs are special because their inactivation does not primarily result from these general processes but from a highly specific auto-catalytic process that only damages the LPMO [11]. This process, which leads to oxidative damage in the catalytic center of the LPMO [11], emerges when a reduced LPMO reacts with H_2O_2 in the absence of substrate. Thus, the rate of LPMO inactivation depends on the availability of both the polysaccharide substrate and the concentration of H_2O_2 [11,15], pointing to a need to control the levels of dissolved O_2 and/or H_2O_2 during enzymatic hydrolysis.

The potential of an electrode immersed in a solution can be taken as a measure of oxidizing or reducing conditions and referred to as the oxidation-reduction potential (ORP). The equilibrium cell potential is determined by the activities of the substances that are oxidized or reduced and can be calculated using the Nernst equation [16]. However, in practice this is difficult for complex systems. Thus, the most common approach used in process conditions is to directly measure the potential of an inert metal electrode (typically platinum) immersed in a solution of interest. The resulting ORP

measurements have been used for monitoring and control of various redox processes, including biological [17] and chemical wastewater treatment [18], and microbial fermentation [19,20].

The objective of the present study was to assess the usefulness of continuous *in situ* ORP measurements with a platinum electrode as a means to follow LPMO activity during enzymatic hydrolysis of an industrial cellulosic substrate. Furthermore, the effects of controlling the ORP at specific set-point values by feed-back regulated addition of hydrogen peroxide were examined under both anaerobic and aerobic conditions. The substrate used in the study was sulfite-pretreated Norway spruce, and hydrolysis was obtained with a commercial cellulase preparation (Cellic CTec3).

2. Materials and methods

2.1. Biomass pretreatment and composition analysis

Norway spruce (*Picea abies*) was pretreated by Borregaard AS (Sarpsborg, Norway). The pretreatment included a sulfite cooking step, which converts lignin into water soluble lignosulfonates and most of the hemicellulose into monosaccharides (the composition of the resulting cellulose-enriched material is shown in Table 1). The monosaccharides released during the pretreatment and the enzymatic hydrolysis described below were mostly D-glucose, D-mannose, D-xylose, D-galactose and L-arabinose, which, in the remainder of the text are referred to without the enantiomer prefix.

The pretreated solids were washed and then dewatered using a Strainpress SP4 screw press (HUBER SE, Berching, Germany). The liquid fraction from the pretreatment, referred to as spent sulfite liquor (SSL), was collected. The SSL and the solid pretreated

spruce material were kept stored at 4 °C until further use. Additional information on the pretreatment process is proprietary information of Borregaard A/S.

The pretreated spruce solids were water-insoluble solids (WIS), while SSL contained water-soluble solids (WSS). The dry matter (DM) content was determined by drying at 105 °C for 24 hours. The DM contents of the pretreated spruce solids and SSL were 35.1±0.4% and 13.30±0.03%, respectively. The composition of the pretreated spruce solids and the SSL was analyzed according to NREL (National Renewable Energy Laboratories) standard procedures [21,22], and is shown in Table 1.

2.2. LPMO reactions with SSL as reductant

Thermoascus aurantiacus LPMO (*Ta*AA9A) was expressed in *Pichia pastoris* and purified essentially as described before [23]. Purified LPMO was saturated with Cu (II) by incubation with $CuSO_4$ (3:1 molar ratio of copper:enzyme) for 30 min at room temperature [24]. Subsequently, the protein solution was loaded onto a PD midiTrap G-25 desalting column (GE Healthcare, UK), equilibrated with 50 mM Bis-Tris/HCl buffer pH 6.5. Protein was eluted with 1 mL of equilibration buffer and enzyme was stored at 4 °C until further use. The protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) based on the Bradford method [25], using bovine serum albumin as a protein standard.

LPMO reaction mixtures contained 5 g/L of phosphoric acid-swollen cellulose (PASC), 1 µM *Ta*AA9A, 1 mM ascorbic acid (AscA) or 10 g/L of SSL as a reductant, and 50 mM sodium acetate pH 5.0. PASC was prepared from Avicel according to a previously described procedure [26]. Reactions were carried out in 2 mL reaction tubes with 200 µL total reaction volume incubated at 50 °C for 24 h, in a ThermoMixer (Eppendorf,

Hamburg, Germany) with orbital shaking at 750 rpm. Reactions were terminated by filtration in 96-well plates with 0.45 µm PES filter (Merck Millipore, Billerica, MA, USA). Supernatants were stored at -20 °C until further analysis. Control experiments were performed in the absence of either reductant or enzyme.

2.3. Enzymatic hydrolysis in bioreactors

The mass of the hydrolysis reaction was 1.0 kg, containing 120 g pretreated spruce solids (12% DM) and the rest (880 g) was defined as hydrolysis liquid. In all hydrolysis reactions, SSL was used as the reductant. In standard reactions, SSL was mixed with water and pretreated spruce solids to reach a final concentration of 10 g SSL DM per liter hydrolysis liquid.

The hydrolysis experiments were carried out in Biostat A Plus bioreactors (Sartorius, Melsungen, Germany). The reactor vessel with a diameter of 130 mm was equipped with a 70 mm diameter pitched-blade impeller. The impeller speed was maintained at 300 rpm. The solids were fed gradually to the reactor during the first 30 min of the hydrolysis reaction to improve initial mixing. All hydrolysis reactions were run for 72 h, and in some cases, the reaction time was prolonged to 144 h.

The enzyme preparation used (Cellic CTec3) was kindly provided by Novozymes A/S (Bagsvaerd, Denmark). The enzyme loading used was 0.04 g enzyme solution/g pretreated spruce solids (based on DM). The temperature was maintained at 50 °C in all experiments. The pH was maintained at 5.0 by controlled addition of 3 M NaOH. To create consistent aerobic or anaerobic conditions the hydrolysis liquid was sparged before the start of hydrolysis with either 200 mL/min air or nitrogen gas. To maintain aerobic or anaerobic conditions during hydrolysis the headspace was flushed with either

200 mL/min air or nitrogen gas. In this study concentrations of dissolved O_2 (DO) lower than 0.1% were considered as anaerobic conditions. However, even DO lower than 0.1% may affect oxidation-reduction potential (ORP) measurements.

In constant feed experiments H_2O_2 (0.5 wt%) was fed to the reactor at a feed rate of 1.2 mL/h using a Watson-Marlow 120 peristaltic pump (Watson-Marlow, Falmouth, Cornwall, England). This corresponds to a feed rate of 200 µmol/h of H_2O_2 per liter of hydrolysis liquid. The feed of H_2O_2 was started 16 hours after the start of hydrolysis in order to allow time for viscosity reduction and improve mixing. The 95% mixing time, i.e. the time for a normalized probe signal to reach and remain within 100±5% of the final value after a perturbation, was determined to be less than 10 seconds at the onset of H_2O_2 feeding, indicating that local accumulation of H_2O_2 can be neglected. In control experiments with no H_2O_2 feed, H_2O was fed instead at a feed rate of 1.2 mL/h.

2.4. Oxidation-reduction potential (ORP) measurement

The ORP was measured with a combination redox electrode (Pt4805-DPAS-SC-K8S/200, Mettler-Toledo, Greifensee, Switzerland) consisting of a Pt electrode and a reference equivalent to Ag / AgCl / 3 M KCl. The redox electrode was connected to a transmitter (Multi-parameter Transmitter M300, Mettler-Toledo, Greifensee, Switzerland) that provided the voltage reading. ORP is shown relative to the reference electrode; ORP relative to the standard hydrogen electrode (SHE) can be calculated according to: $ORP_{SHE} = ORP + 187.6$ mV.

For ORP controlled enzymatic hydrolysis experiments a feed-back regulated on/off control of ORP was implemented. In short, a 4 mV ORP dead-band was applied around the ORP set-point (± 2 mV), and if the ORP decreased below this value feeding of H_2O_2

(0.5 wt%) at a feed rate of 6 mL/h was initiated until the ORP increased above this value.

2.5. Analysis of monosaccharides and C4-oxidized dimer

Samples of the hydrolysis liquid in 2 mL Eppendorf tubes were separated by centrifugation at 14850 × g for 5 min. The supernatant was filtered through 0.2 μm filters. The samples were then heated in a water bath for 10 min at 100 °C to inactivate the enzymes. Following inactivation, the samples were cooled on ice and stored at -20 °C until analysis. Concentrations of monomeric sugars were measured by HPLC (high-performance liquid chromatography). In short, the sugars were separated on a polymer column (Aminex HPX-87P, Bio-Rad Laboratories, München, Germany) at 85 °C. Deionized water (Purelab Flex, Elga, Marlow, UK) was used as eluent at a flow rate of 0.6 mL/min. The sugars were detected with a refractive index detector (Waters 2410, Waters, Milford, MA, USA).

Glucan conversion, i.e. the percent of glucan converted to glucose, was calculated as follows. The density of the hydrolysis liquid (ρ) was calculated based on the measured concentrations of glucose (c_{Glc}), mannose (c_{Man}) and xylose (c_{Xyl}) only, as the concentrations of other sugars were negligible [27]:

$$\rho = 1000 + 0.35\left(c_{Glc} + c_{Man} + c_{Xyl}\right)$$

The volume of the hydrolysis liquid (V) was solved from a mass balance based on the initial mass of hydrolysis liquid ($m_0 = 880$ g), the mass added with the H_2O_2 feed (m_{feed}), the mass of monosaccharides released by hydrolysis ($m_{hyd\ sug}$) and the mass of water taken up during hydrolysis ($m_{hyd\ H_2O}$):

$$\rho V = m_0 + m_{feed} + m_{hyd\ sug} - m_{hyd\ H_2O}$$

$$m_{hyd\ sug} = (c_{Glc} + c_{Man} + c_{Xyl})V - (c_{Glc0} + c_{Man0} + c_{Xyl0})V_0$$

$$m_{hyd\ H_2O} = 0.1[(c_{Glc} + c_{Man})V - (c_{Glc0} + c_{Man0})V_0] + 0.12[c_{Xyl}V - c_{Xyl0}V_0]$$

Finally, the glucan conversion (C) was calculated based on the mass of glucose released by hydrolysis, initial mass of pretreated spruce solids ($m_{s0} = 120$ g) and the initial glucan fraction in the solids ($\chi_0 = 0.874$), according to:

$$C = 100\,\frac{c_{Glc}V - c_{Glc0}V_0}{1.111\chi_0 m_{s0}}$$

LPMOs acting on cellulose may oxidize the C1 or the C4 position in the scissile glycosidic bond, whereas some LPMOs generate mixtures of C1- and C4- oxidized products [12,28]. In the presence of cellulase and β-glucosidase activity, as is the case with commercial cellulase cocktails, two soluble oxidized products are formed: the C1-oxidized monomer, i.e. gluconic acid, and the C4-oxidized dimer, i.e. Glc4gemGlc. The type and amount of LPMOs present in Cellic CTec3 is unknown. Under our reaction conditions Glc4gemGlc was the main product. Due to the high signal background, adversely affecting the analysis of gluconic acid, only Glc4gemGlc was analyzed in the study, as a proxy of LPMO activity. Importantly, there are difficulties with analysis of Glc4gemGlc, as it is unstable, particularly at high pH, meaning that it decomposes during the reaction, sample processing, and on the column during product analysis [13,29]. These issues were minimized by keeping the standard and the samples in the same conditions during sample processing and analysis. Nonetheless, the measured values should serve more as indications of trends rather than absolute values. Overall, the amounts of detected C4-oxidized products never exceeded 0.8% of the total amount of glucan β-1,4 linkages in the reaction mixture.

Concentrations of the C4-oxidized dimer (Glc4gemGlc) were analyzed with HPAEC-PAD (high-performance anion exchange chromatography with pulsed amperometric detection) using a Dionex ICS 5000 system (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA1 4×250 mm analytical column and a CarboPac PA1 4×50 mm guard column operating at an eluent flow rate of 1 mL/min [9,30]. In short, initial conditions were set to 0.1 M NaOH (100% eluent A), followed by a linear gradient towards increasing concentrations 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 by degrading cellopentaose with *Nc*LPMO9C [9].

3. Results and discussion

3.1. SSL as reductant for LPMOs

Comparing product formation by *Ta*AA9A in reactions with PASC using either 1 mM AscA or 10 g/L of SSL showed no difference in released oxidized sugars (Fig. 1), indicating that SSL is capable to reduce LPMOs and drive the oxidative cleavage of insoluble cellulosic substrate. It had been shown previously that lignosulfonates, which are derived from SSL by fermentation and subsequent evaporation, can activate the LPMO-containing Cellic CTec3 preparation releasing oxidized sugars during aerobic hydrolysis of sulfite-pulped Norway spruce [23]. It is interesting to note that the release of oxidized sugars from PASC, which is nearly devoid of lignin, suggests that SSL is able to drive the LPMO reaction without the need of solid lignin bound to the substrate. It is plausible that the interplay between sulfonated lignin fractions of different

molecular weight, analogous to the interplay between low and high molecular lignin fractions [7], enables LPMO reduction by the SSL.

3.2. Monitoring ORP during enzymatic hydrolysis

In this study, we used a platinum electrode for monitoring the ORP during enzymatic hydrolysis reactions in laboratory bioreactors under conditions similar to those expected in industrial processing. As previously mentioned, O_2 and H_2O_2 are both possible co-substrates for the LPMO catalyzed oxidation of cellulose. The ORP measured by a platinum electrode is affected by the concentration of dissolved O_2 (DO) and H_2O_2 during enzymatic hydrolysis, and the electrode signal may therefore provide useful information on the redox state of the solution, which likely affects the capacity for LPMO reactions to take place. It is, however, important to keep in mind that the measured ORP may not depend directly on the concentration of H_2O_2, but may reflect changes in the ratio of other redox couples present in the system [31].

In a series of initial experiments, the ORP was measured for enzymatic hydrolysis reactions under aerobic and anaerobic conditions, and with and without H_2O_2 feeding (200 µmol/Lh starting at 16 h). The enzymatic hydrolysis reaction under anaerobic conditions without H_2O_2 feeding represented a control experiment without LPMO catalyzed oxidation. Glucan conversion during the initial phase of hydrolysis was similar in all experiments (Fig. 2A). In the reactions without addition of H_2O_2, the curves started to diverge after 16 hours, showing more rapid hydrolysis under aerobic conditions. The higher efficiency of the aerobic reaction was expected since Cellic CTec3 contains LPMOs whose activity is affected by oxygen and similar observations have been made before [23]. H_2O_2 feeding starting at 16 hours significantly increased the hydrolysis rate in both aerobic and anaerobic conditions. Importantly, this higher

hydrolysis rate was not sustained during the last 24 hours of the reaction carried out under aerobic conditions.

Measurements of the levels of C4-oxidized dimers (Glc4gemGlc) showed interesting correlations between LPMO activity and glucan conversion (Fig. 2B). In agreement with previous reports on sulfite pretreated spruce [13], there was no Glc4gemGlc production under anaerobic conditions in the absence of H_2O_2 (i.e. condition that gave the lowest glucose yields; Fig. 2A). During aerobic hydrolysis without added H_2O_2 the production of Glc4gemGlc was slow and steady, likely driven by H_2O_2 generated from dissolved oxygen and reducing agents in the spent sulfite liquor (SSL). Feeding of H_2O_2 led to a drastic increase in the rate of Glc4gemGlc production (and an increase in glucose yields; Fig. 2A). In the aerobic reaction with H_2O_2 feed, the rate of Glc4gemGlc production started decreasing in the period around 48 hours, soon after which the levels of Glc4gemGlc started decreasing due to the instability of this compound [13]. The stop in Glc4gemGlc production coincided with a decrease in the enzymatic hydrolysis rate (Fig. 2A). Interestingly, LPMO activity lasted longer under anaerobic conditions (Fig. 2B), and this was reflected in a higher glucose release rate in the final phase of the reaction (Fig. 2A). The levels of Glc4gemGlc in the anaerobic reaction started decreasing after the 72 hour time-point due to the instability of C4-oxidized dimer (Suppl. fig. 1).

It seems that, with this H_2O_2 feeding rate, oxidative inactivation of LPMO is promoted by the presence of oxygen, possibly because overall H_2O_2 levels are higher due to reactions between O_2 and reducing compounds (likely including reduced LPMOs) in the hydrolysis mixture [32]. Overall, the results depicted in Figs. 2A & 2B support the

importance of LPMO activity for the overall hydrolysis rate, particularly in the late phases of the hydrolysis reaction.

Fig. 2C shows that, as expected, the ORP was higher under aerobic compared to anaerobic conditions. In the absence of H_2O_2 feeding, the ORP increased very slowly during the aerobic hydrolysis and was essentially constant. With H_2O_2 feeding, the ORP initially had a similar profile, but it started increasing rapidly in the period around 48 hours, coinciding with the loss of LPMO activity visible in Fig. 2B. Fig. 2C further shows that in anaerobic hydrolysis without H_2O_2 feed, the ORP decreased during the initial phase of the reaction. At the start of H_2O_2 feeding in anaerobic conditions the ORP increased rapidly, after which it stabilized at a relatively constant level. In the period around 48 hours the ORP started increasing again, and this coincided with the reduced LPMO activity (Fig. 2B).

The initial decrease in ORP during anaerobic hydrolysis without H_2O_2 feed was most likely caused by gradual removal of remaining dissolved oxygen (DO). Schuldiner et al. observed a decrease in ORP of 59.1 mV for a 10-fold decrease in DO, by measuring the potential of a platinum electrode immersed in a solution of 1 M H_2SO_4 at 25 °C, while varying the partial pressure of oxygen in the range of 10^{-6} to 10^{-2} atm [33]. The low ORP in the anaerobic hydrolysis reactions may promote the reduction of LPMOs, as the formal potentials of LPMOs vs Ag/AgCl (45 mV for *Sc*AA10C and 54 mV for *Sc*AA10B [34]; -42 mV for *Pa*AA9E and 129 mV for *Pa*AA9H [35]; -2 mV for *Ta*AA9A [36]; 80 mV for *Fo*AA9 and 125 mV for *Mt*AA9 [37]) are in most cases much higher.

Fig. 2C shows that an increase in ORP occurs *during H_2O_2 feeding* in three different cases: (1) aerobic hydrolysis when the LPMO activity starts to decrease, (2) anaerobic

hydrolysis at the start of H_2O_2 feeding, and (3) anaerobic hydrolysis when the LPMO activity starts to decrease. Control experiments without added enzyme showed that the ORP increases directly at the start of H_2O_2 feeding in both aerobic and anaerobic conditions, and continues to increase during feeding (Suppl. fig. 2). One could envisage that the three cases of ORP increase shown in Fig. 2C are due to: (1) and (3) a slowdown and eventual termination of LPMO activity, meaning that added H_2O_2 is no longer consumed and is accumulating, and (2) a short period of H_2O_2 "overshoot" directly after initiation of H_2O_2 feeding, before a steady-state is reached.

3.3. ORP controlled feeding of H_2O_2

Since LPMO activity apparently is connected to the ORP value, it was of interest to investigate the possibility of controlling the feed rate of H_2O_2 based on the ORP. The feed rate was therefore manipulated to keep the ORP value at different set-points using a simple dead-band feed-back control. In this way, the effect of the different set-points on the LPMO catalyzed oxidation could be assessed.

In anaerobic reactions, the ORP was kept at -120 mV, -80 mV or -40 mV, i.e. above the low ORP plateau of -180 mV that was reached during anaerobic hydrolysis with no feeding (Fig. 2C). In aerobic conditions the range of feasible ORP set-points was more limited due to the large effect of oxygen on the ORP signal. ORP control was started after 2 hours (ORP at 110 mV) or after 42 hours (ORP at 120 mV). These ORP set-points were slightly higher than the ORP measured for aerobic hydrolysis without H_2O_2 feed (Fig. 2C). As the ORP increases gradually during enzymatic hydrolysis in aerobic conditions making it difficult to maintain an ORP set-point during the whole reaction, an additional experiment was performed during which the ORP was controlled according to a linear profile between 110 mV at 2 hours and 150 mV at 72 hours.

In the anaerobic reactions, the initial rate of enzymatic hydrolysis (0 to 24 h) was higher, the higher the ORP set-point (Fig. 3A). However, control at the highest ORP (-40 mV), which implies the largest amount of added H_2O_2, led to early enzyme inactivation, as observed by a reduced hydrolysis rate and cessation of LPMO activity (Figs. 3A,B). Control at -80 mV led to a maintained hydrolysis rate throughout the complete incubation period and a higher final glucose yield. Maintaining the ORP at even lower level (-120 mV) led to slower hydrolysis when compared to control at -80 mV.

The rate of Glc4gemGlc production was higher, the higher the ORP set-points (Fig. 3B), which was likely due to higher resulting H_2O_2 feed rates (Fig. 3D). Compared to the -40 mV reaction, controlling the ORP at a lower level (-80 mV) led to a prolonged period of LPMO-catalyzed oxidation. The prolonged LPMO activity was reflected in higher final glucan conversion (Fig. 3A). Higher LPMO reaction rates were accompanied by an earlier stop in Glc4gemGlc production. The -40 mV LPMO reaction stopped within the first 48 hours (Fig. 3B), while the -80 mV reaction stopped after the 72 hour time-point and the -120 mV reaction was maintained for 144 hours (Suppl. fig. 1). Fig. 3C shows the measured ORP during the ORP controlled anaerobic reactions; the feeding started later at lower ORP set-points as it took more time to reach these lower ORP values during hydrolysis (Fig. 3D).

The two ORP set-point control experiments performed in aerobic conditions showed clear differences (Fig. 4). When starting the control at 2 hours (110 mV), the initial rate of enzymatic hydrolysis (0 to 24 h) was slightly higher (Fig. 4A). However, this set-up caused an early stop in Glc4gemGlc production (Fig. 4B), with a concomitant decrease in enzymatic hydrolysis rate in the later stages of the reaction and decreased final

glucose yields. In contrast, starting the ORP control and H_2O_2 feeding at 42 hours (120 mV) allowed the reaction to reach a higher glucan conversion after 72 hours (72%). In this case the stop in LPMO activity occurred after the 72 hour time-point (Suppl. Fig. 1). The ORP control ramp experiment (110 to 150 mV) gave overall very similar results to the ORP set-point control experiment starting at 2 hours (Fig. 4).

3.4. Loss of LPMO activity or lack of electron donor?

The seemingly abrupt stop in LPMO activity in certain hydrolysis reactions with H_2O_2 feeding (e.g. Fig. 2B) is an interesting phenomenon. This was first reported by Müller et al. who suggested that inactivation is caused by high feed rates of H_2O_2, which would lead to LPMO self-inactivation in situations where the LPMO substrate becomes depleted [13]. Although a more fundamental inquiry into this phenomenon was outside the scope of the present study, a set of hydrolysis experiments were performed to explore the phenomenon: (1) hydrolysis with a double dose of added SSL, thus containing a double initial dose of the electron donor, (2) hydrolysis with an ORP-controlled feed of SSL, thus preventing the electron donor to be exhausted from the medium during the hydrolysis, (3) hydrolysis with a second dose of enzyme added close to the time-point (42 h) when the LPMO activity starts decreasing in aerobic reactions with H_2O_2 feeding. The experiments were performed under aerobic conditions with a constant H_2O_2 feed of 200 µmol/Lh.

Use of a double dose of SSL or ORP-controlled feeding of SSL had little effect on glucan conversion (Fig. 5A), which was similar to the results from the baseline aerobic H_2O_2 feeding case (Fig. 2A). Addition of a 2nd dose of CTec3 at 42 hours significantly increased the glucan conversion and the reaction reached almost full conversion after 72 hours. Neither a double initial dose of SSL nor controlled SSL feeding extended the

period of Glc4gemGlc production, indicating that the stop in LPMO activity was not caused by a lack of electron donor (Fig. 5B). Addition of a second dose of CTec3 at 42 hours prolonged Glc4gemGlc production, indicating that fresh LPMOs - but not more electron donors - were needed to continue the reaction.

Enzymatic hydrolysis with a double dose of SSL underwent a rapid increase in ORP associated with a stop in Glc4gemGlc production (Fig. 5C), very similar to the baseline aerobic H_2O_2 feeding case (Fig. 2C). ORP-controlled addition of SSL prevented this increase in ORP and kept it constant at 110 mV, likely because H_2O_2 that would otherwise accumulate due to the cessation of LPMO activity reacts with the added reductant in SSL. The second addition of CTec3 at 42 hours delayed the increase in ORP to around 60 hours, indicating that the addition of fresh LPMO extended the enzymatic consumption of H_2O_2. In this latter case, the ORP started increasing as glucan conversion approached 100%, meaning that there would be hardly any LPMO substrate left.

4. Conclusions

The present results show that ORP is a useful on-line measurement tool, which can also be used for feed-back control of hydrogen peroxide addition. The precision is better and the span of control set-points is greater for anaerobic conditions, since the redox signal is not affected (“polluted”) by oxygen from the air. The oxidative damage of LPMOs can therefore likely be better controlled under anaerobic conditions with hydrogen peroxide addition. However, there is no reason to believe that a fixed set-point represents a global optimum. We believe that further optimization should be possible, perhaps fueled by growing insight into the determinants of LPMO stability, and of the interplay between LPMOs and other enzymes in the cellulase cocktail.

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Figure legends

Figure 1. The activity of *Ta*AA9A on phosphoric acid-swollen cellulose (PASC) in the presence of ascorbic acid (AscA) or spent sulfite liquor (SSL). High-performance anion exchange chromatography (HPAEC) traces show reaction products released from PASC (5 g/L) upon incubation with *Ta*AA9A (1 µM) in the presence of 1 mM AscA (orange lines) or 10 g/L SSL (blue lines). The black solid line represents PASC incubated with *Ta*AA9A in the absence of reductant. Dashed lines represent PASC incubated in the presence of corresponding reductant and in the absence of LPMO. Reactions were carried out in 50 mM sodium acetate buffer pH 5.0, at 50 °C, with orbital shaking at 750 rpm, for 24 h.

Figure 2. (**A**) Glucan to glucose conversion, (**B**) Glc4gemGlc concentration and (**C**) oxidation-reduction potential (ORP) during bioreactor enzymatic hydrolysis of sulfite-pretreated spruce at 12% DM. Circle indicates 200 µmol/Lh H_2O_2 feed in aerobic conditions, triangle up indicates no H_2O_2 feed in aerobic conditions, triangle down indicates 200 µmol/Lh H_2O_2 feed in anaerobic conditions and square indicates no H_2O_2 feed in anaerobic conditions. H_2O_2 feed was started at 16 h. Dashed lines indicate the 16 and 42 h time-points.

Figure 3. (**A**) Glucan to glucose conversion, (**B**) Glc4gemGlc concentration, (**C**) oxidation-reduction potential (ORP) and (**D**) amount of H_2O_2 added during bioreactor enzymatic hydrolysis of sulfite-pretreated spruce at 12% DM with ORP-controlled H_2O_2 feeding in anaerobic conditions. Circle indicates ORP set-point at -40 mV, triangle down indicates ORP set-point at -80 mV and triangle up indicates ORP set-point at -120 mV. The ORP control was started at 2 h.

Figure 4. (**A**) Glucan to glucose conversion, (**B**) Glc4gemGlc concentration, (**C**) oxidation-reduction potential (ORP) and (**D**) amount of H_2O_2 added during bioreactor enzymatic hydrolysis of sulfite-pretreated spruce at 12% DM with ORP-controlled H_2O_2 feeding in aerobic conditions. Circle indicates ORP control at 110 mV starting at 2 h, triangle down indicates ORP

control at 120 mV starting at 42 h and triangle up indicates ORP control ramp from 110 to 150 mV. Dashed line indicates the 42 h time-point.

Figure 5. (**A**) Glucan to glucose conversion, (**B**) Glc4gemGlc concentration and (**C**) oxidation-reduction potential (ORP) during bioreactor enzymatic hydrolysis of sulfite-pretreated spruce at 12% DM. Circle indicates a double dose of spent sulfite liquor (20 g/L SSL), triangle down indicates ORP controlled at 110 mV by SSL feeding and triangle up indicates a 2nd dose of Cellic CTec3 added at 42 h. All experiments were done in aerobic conditions with a 200 µmol/Lh H_2O_2 feed starting at 16 h. Dashed lines indicate the 16 and 42 h time-points. Quantitative comparison of panels A and B allows an approximation of the fraction of oxidized products to be made; such a comparison shows that in the experiment with two dosages of Cellic CTec3, after 72 hours, there is approximately 1 oxidized dimer per 150 released glucose molecules.

Table 1. Composition of the sulfite pretreated spruce solids and spent sulfite liquor (SSL). The percentages of sugars refer to anhydro sugars. The full composition of the SSL is proprietary information of Borregaard A/S.

	Pretreated spruce solids (% of DM†)		**Dissolved sugars in SSL (g/L)**
Glucan	87.4±0.6	Glucose	6.02±0.05
Mannan	5.2±0.2	Mannose	20.66±0.61
Galactan	nd‡	Galactose	3.95±0.10
Xylan	2.7±0.1	Xylose	8.57±0.31
Arabinan	nd	Arabinose	1.44±0.05
Acid insoluble lignin	3.3±0.3		

†dry-matter, ‡not detected

1

Figure 1

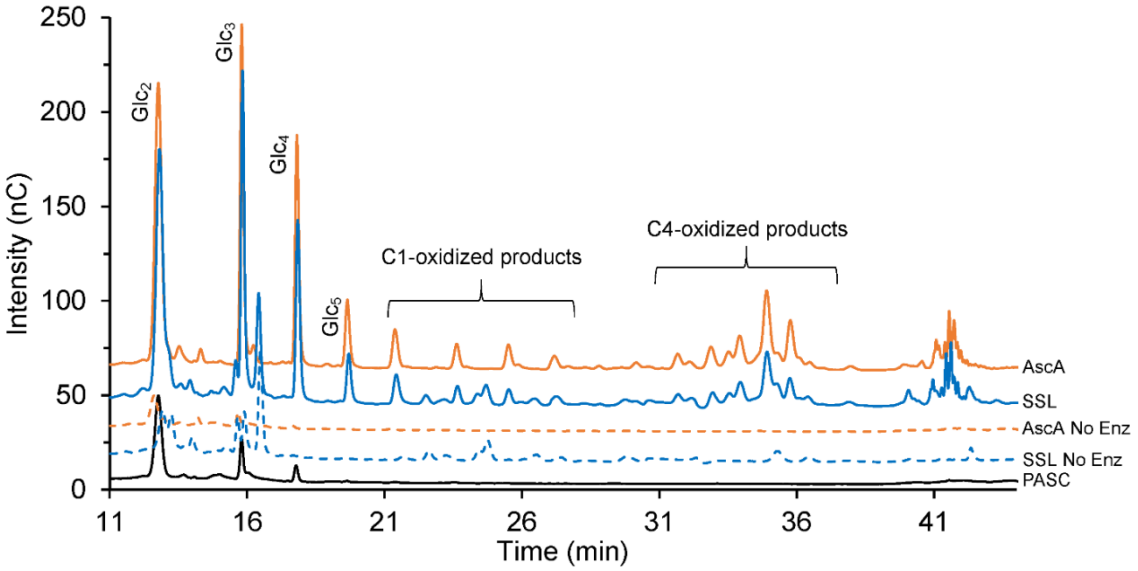

Intensity (nC)
250
200
150
100
50
0
11
16
21
26
31
36
41
Time (min)
Glc2
Glc3
Glc4
Glc5
C1-oxidized products
C4-oxidized products
AscA
SSL
AscA No Enz
SSL No Enz
PASC

Figure 2

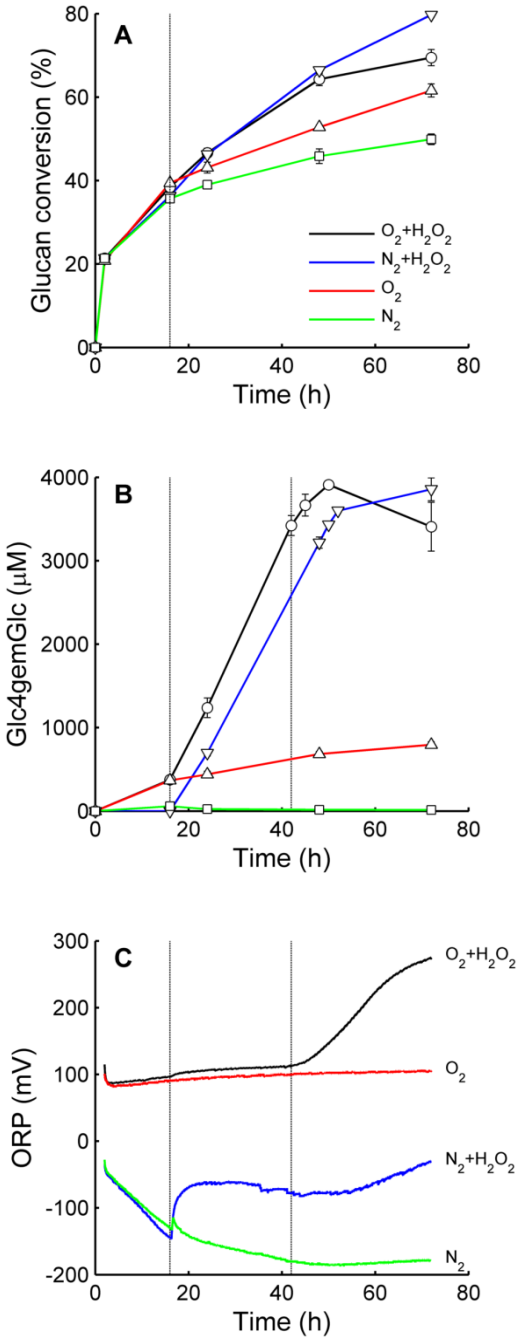

A
Glucan conversion (%)
Time (h)
O_2+H_2O_2
N_2+H_2O_2
O_2
N_2
B
Glc4gemGlc (μM)
Time (h)
C
ORP (mV)
Time (h)
O_2+H_2O_2
O_2
N_2+H_2O_2
N_2

Figure 3

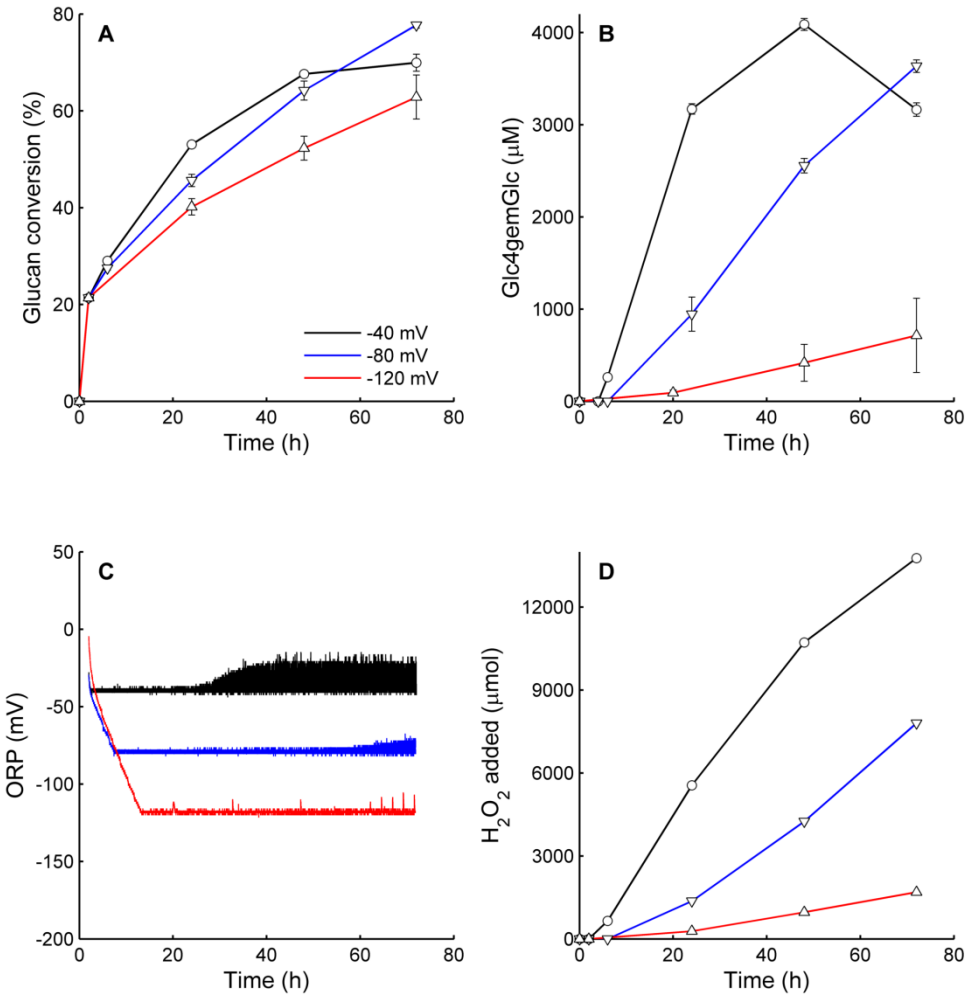

A
Glucan conversion (%)
Time (h)
-40 mV
-80 mV
-120 mV
B
Glc4gemGlc (µM)
Time (h)
C
ORP (mV)
Time (h)
D
H_2O_2 added (µmol)
Time (h)

Figure 4

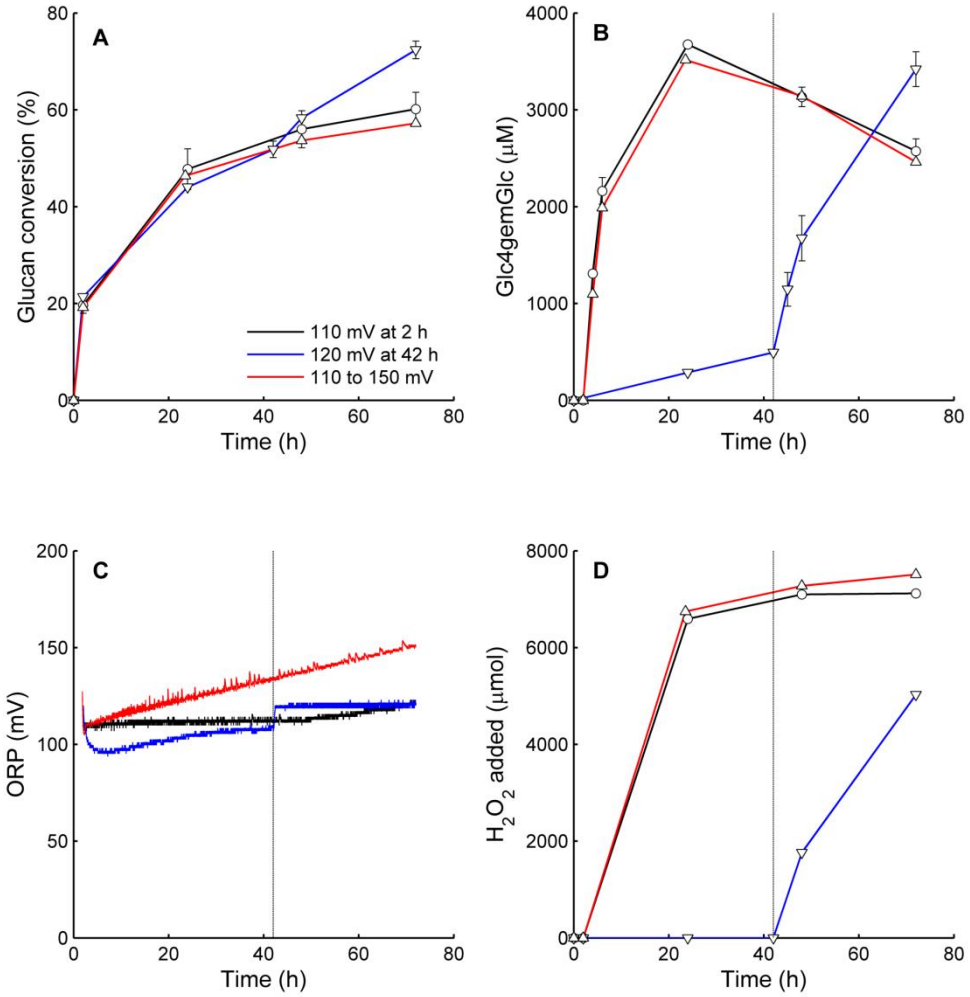
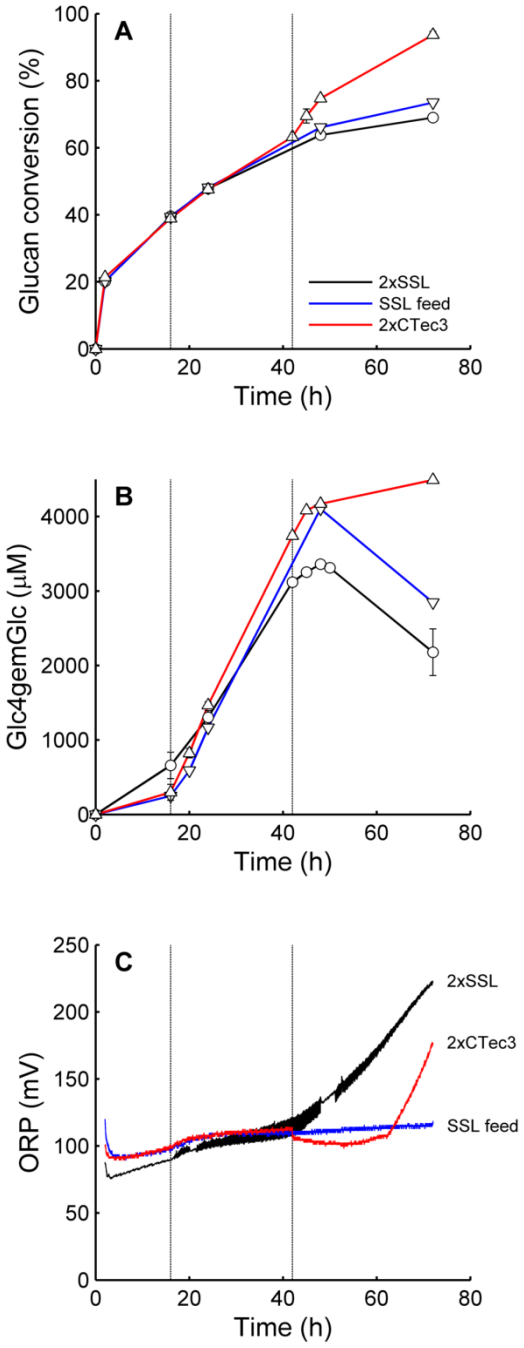
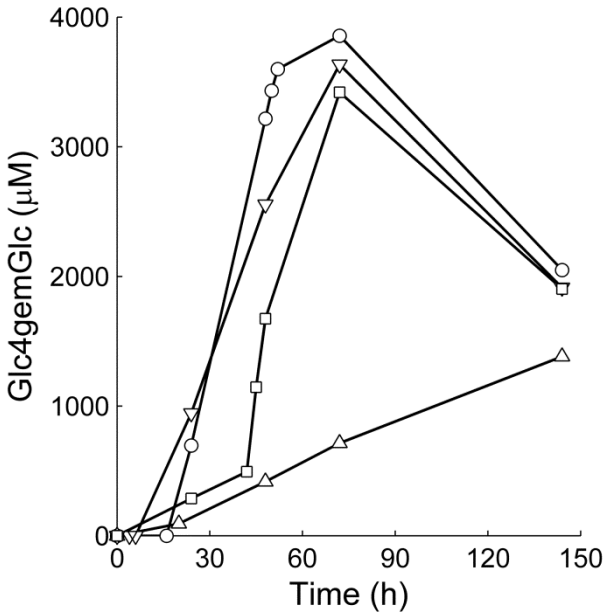

A
Glucan conversion (%)
Time (h)
110 mV at 2 h
120 mV at 42 h
110 to 150 mV
B
Glc4gemGlc (µM)
Time (h)
C
ORP (mV)
Time (h)
D
H_2O_2 added (µmol)
Time (h)

Figure 5


A
Glucan conversion (%)
Time (h)
2xSSL
SSL feed
2xCTec3
B
Glc4gemGlc (µM)
Time (h)
C
ORP (mV)
Time (h)
2xSSL
2xCTec3
SSL feed

Supplementary figure 1. Glc4gemGlc concentration during bioreactor enzymatic hydrolysis of sulfite-pretreated spruce at 12% DM. Circle represents 200 µmol/Lh H_2O_2 feed in anaerobic conditions. Triangle down represents ORP-controlled H_2O_2 feeding in anaerobic conditions with ORP set at -80 mV. Triangle up represents ORP-controlled H_2O_2 feeding in anaerobic conditions with ORP set at -120 mV. Square represents ORP-controlled H_2O_2 feeding in aerobic conditions with ORP set at 120 mV starting at 42 h.



Supplementary figure 2. Oxidation-reduction potential (ORP) during control experiments with sulfite pretreated spruce (1% DM), 10 g/L spent sulfite liquor (SSL) and no added enzyme. Black line indicates 200 µmol/Lh H_2O_2 feed in aerobic conditions and blue line indicates 200 µmol/Lh H_2O_2 feed in anaerobic conditions. Dashed line indicates the start of H_2O_2 feed at 16 h.