# Supporting Information

# Chemo-enzymatic One-Pot Depolymerization of β-Chitin

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# Materials and Methods

β-chitin and the enzyme *N*-acetylglucosamine oxidase (NAGox) were sourced from France Chitin (Marseille, France) and Gecco Biotech BV (Groningen, Netherlands), respectively. The pET-30Xa/LIC vector containing the gene encoding chitobiase was a gift from the Protein Engineering and Proteomics Group, Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences. Palladium (II) chloride (99.995% purity), and gold (III) chloride (99.9% purity) were purchased from Merck and Strem Chemicals, respectively. Titanium dioxide (P25) was supplied by Degussa.

## Expression and purification of recombinant *sm*AA10A (CBP21)

A *pET3a* plasmid harboring a codon-optimized gene encoding *smAA10A* was synthesized by GenScript Biotech (UK) (Oxford, England) and transformed into BL21$^{STAR}$ (DE3) cells (25 µL). A single colony was used to inoculate LB media (10 mL) containing ampicillin (500 µg mL). After incubation with shaking (200 rpm) at 37 $^{o}$C for 16 h, this cell culture was diluted 1:100 into LB-ampicillin media and incubated with shaking (200 rpm) at 37 $^{o}$C for an additional 16 h. Cells were then harvested by centrifugation (3300*g*, 15 min) and stored at -80 °C until required for enzyme purification. *sm*AA10A was extracted from the cell pellets following a cold osmotic shock protocol, as previously described.[1] Thus, a cell pellet (100 g) was defrosted and resuspended in 50 mM Tris-HCl buffer (300 mL), pH 8.5, containing 20% (w/v) sucrose and lysozyme (7.5 mg), before being incubated with shaking (250 rpm) at 2 °C for 1 h. Solid $MgSO_4$ (180 mg) was then added to a final concentration of 5 mM, and the solution incubated with shaking (250 rpm) at 2°C for a further 30 min. This solution was clarified by centrifugation (3000 *g*, 20 mins) and stored on ice. Additional protein was obtained by resuspending the pellet in a minimum volume of ice-cold water, shaking at 2 °C for 1 h, and clarification by centrifugation (34220 *g* for 40 min). These extracts were combined and concentrated to give a solution (10 mL) that was mixed with 5 mM $CuSO_4$ (400 µL) at 4$^{o}$C for 16 h. This procedure yielded Cu(II)-containing *sm*AA10A, which was then applied at a flow rate of 2.5 mL min$^{-1}$ to a chitin bead column (New England Biolabs, Beverly, MA) that had been pre-equilibrated in 20 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 8.0. Holo-*sm*AA10A was eluted by changing the mobile phase to 20 mM aq. AcOH (pH 3.6). Fractions containing pure *sm*AA10A were combined, and buffer-exchanged into 20 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 8.0, using a HiPrep 26/10 desalting column (Sigma Aldrich) (Fig. S1). Aliquots (0.5 mL) of the purified, recombinant WT *sm*AA10A were flash frozen with liquid $N_2$ and stored at -80 °C.

## Expression and purification of WT b-*N*-acetylhexosaminidase (chitobiase)

Recombinant chitobiase was expressed and purified based on literature procedures.[2] A *pET-30Xa/LIC* vector containing the gene encoding *Serratia marcescens* chitobiase with a cleavable N-terminal His-tag was transformed into BL21$^{STAR}$ (DE3) cells (25 µL). A single colony was used to inoculate LB media (10 mL) containing kanamycin (50 µg mL$^{-1}$). After incubation with shaking (200 rpm) at 37 $^{o}$C for 16 h, this cell culture was diluted 1:100 into fresh LB-kanamycin media (1 L) and incubated with shaking (200 rpm) at 37 $^{o}$C until $OD_{600}$ = 0.5. Protein expression was induced by IPTG (0.1 mM), and the incubation temperature was lowered to 30 $^{o}$C. After 3 h, the cells were harvested by centrifugation (3300*g*, 15 min) and re-suspended in 20 mM Tris.HCl, pH 8, containing 20 mM imidazole. The cells were lysed by sonication (5 s sonication, 5 s pause for 4 mins at 30% amplitude, 4 °C), and the lysate clarified by centrifugation (34220*g*, 40 min) and subsequent filtration (0.2 µm). After loading the lysate onto Ni-sepharose 6 FF resin (Cytiva), pre-equilibrated with Tris.HCl buffer (20 mM, pH 8.0), containing imidazole (20 mM), the recombinant chitobiase was eluted from the column using 20 mM Tris.HCl buffer, pH 8.0, containing 500 mM imidazole. Samples containing the pure enzyme were concentrated (30 µM), and buffer-exchanged into 20 mM Tris.HCl buffer, pH 8.0, containing 20 mM imidazole using a

HiPrep 26/10 desalting column. The purified, N-terminally tagged chitobiase was flash frozen with liquid $N_2$ and stored at -80 °C (0.5 mL aliquots).

## pH-Dependence of recombinant WT *sm*AA10A activity

The pH-dependent peroxygenase activity of recombinant WT *sm*AA10A could be conveniently assayed using a chromogenic substrate, 2,6-dimethoxyphenol (2,6-DMP) (Fig. S2).[3] In these experiments, a cuvette was charged with 10 mM 2,6-DMP (100 µL) and 5 mM $H_2O_2$ (20 µL) in 100 mM buffer (33% NaOAc, 33% Tris.HCl, 33% BisTris.HCl) (860 µL) adjusted to the appropriate pH value. After incubating the mixture at 30 °C for 15 min, the reaction was initiated by adding 120 µM recombinant WT *sm*AA10A dissolved in the same buffer (20 µL). The rate of coerulignone production was continuously monitored at 469 nm ($\varepsilon$ = 53200 $M^{-1}.cm^{-1}$) for 5 min using a Shimadzu UV-1900 spectrophotometer.

## Preparation of a chitobionic acid standard

Solutions of 10 mM diacetylchitobiose in 50 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 8 (0.2 mL), 6 µM NAGox in 50 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 8 (0.5 mL) and >100U catalase (2 µL), were mixed with 50 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 8 (0.5 mL). After stirring (150 rpm) at 21°C for 4 h, acetonitrile (1 mL) was added to precipitate the protein. Filtration then gave chitobionic acid as a solid.[3]

## General procedure for making the chemo-catalyst

The procedure to produce 0.5%Pd–0.5%Au/$TiO_2$ (2 g) is based on previously reported protocols.[4] Briefly, aqueous acidified $PdCl_2$ solution (1.667 mL, 0.58 M HCl, [Pd] = 6 mg $mL^{-1}$) and aqueous $HAuCl_4 \cdot 3H_2O$ solution (0.8263 mL, [Au] = 12.25 mg $mL^{-1}$, Strem Chemicals) were mixed in a 50 mL round-bottom flask and heated to 65 °C with stirring (1000 rpm) in a thermostatically controlled oil bath, with total volume fixed to 16 mL using $H_2O$ (HPLC grade, Fischer Scientific). Upon reaching 65 °C, $TiO_2$ (1.98 g, P25) was added over the course of 5 min with constant stirring. The resulting slurry was stirred at 65 °C for a further 15 min, following this the temperature was raised to 95 °C for 16 h to allow for complete evaporation of water. The resulting solid was ground prior to a reductive heat treatment (5% $H_2$/Ar, 400 °C, 4 h, 10 °C $min^{-1}$). Brunauer-Emmett-Teller (BET) measurements showed that the surface area of the 0.5%Au-0.5%Pd/$TiO_2$ catalyst and the as-received $TiO_2$ were 50 and 58 $m^2\ g^{-1}$, respectively.

## pH-Dependence of chemo-catalyst-dependent $H_2O_2$ synthesis

Radley glass RBFs (50mL; certified to 5 bar) in a multi-reaction system were charged with the 0.5%Au-0.5%Pd/$TiO_2$ chemo-catalyst (1 mg) and $K_2HPO_4/KH_2PO_4$ buffer (100 mM, 10 mL). Each RBF was then purged and pressurized with $H_2$ (1.6 bar) and compressed air (0.4 bar). Reaction mixtures were stirred (250 rpm) at 21 °C and depressurized at appropriate timepoints (Fig. S3). After removing the solid material by filtration, an aliquot (1 mL) of the solution was added to an equal volume of 0.02 M aq. potassium titanium oxalate dihydrate acidified with 30 % $H_2SO_4$ to pH 1. Immediately after mixing, the $H_2O_2$ concentration was determined from the absorbance at 400 nm using a standard curve. These measurements were performed on a Shimadzu UV-1900 spectrophotometer.

## *sm*AA10A-catalyzed β-chitin degradation with *in situ* $H_2O_2$ generation

Reactions were carried out in 50 mL Radleys glass round bottom flasks (RBFs) (certified to 5 bar) on a multi-reaction system (Fig. S4). Under our standard conditions, each RBF was charged with the 0.5%Au-0.5%Pd/$TiO_2$ heterogeneous catalyst (1 mg), β-chitin (100 mg), and 0.5 μM *sm*AA10A and 1 mM ascorbate dissolved in 50 mM $K_2HPO_4$/$KH_2PO_4$ buffer, pH 6, (10 mL total volume). The reaction was initiated by pressurization with $H_2$ (1.6 bar) and compressed air (0.4 bar); *these values are outside the flammability limits of $H_2$ and $O_2$.* Reaction mixtures were stirred (250 rpm) at 21 $^oC$ and depressurized at appropriate timepoints (Fig. S5). After removing solid material by filtration, and an aliquot (100 μL) of the reaction mixture was incubated with 3 μM recombinant WT chitobiase in 20 mM Tris-HCl buffer, pH 8, at 37 °C for 2 h (110 uL total volume) (Figs. S6 and S7). The resulting concentrations of chitobionic acid and *N*-acetylglucosamine were determined by HPLC (Fig. S8). The amount of residual $H_2O_2$ in the post-reaction solution was also measured. All measurements for each reaction at a given timepoint were performed in triplicate.

## Quantifying the extent of β-chitin degradation

MALDI-TOF mass spectrometry was used to confirm the chitinolytic activity of recombinant WT *sm*AA10A under a given set of reaction conditions. In these measurements, 2,5- dihydroxybenzoic acid (18 μg) dissolved in 30% aq. acetonitrile (2 μL) was mixed with an aliquot of the reaction solution (1 μL) on an MTP 384 ground steel target plate TF (Bruker Daltonics), and the resulting solution dried under a stream of air. Samples were analyzed using an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics GmbH) operated in positive acquisition mode (Bruker FlexControl 3.4 software). Spectra were gathered with an acceleration voltage of 25 kV, a reflector voltage of 16 kV, and pulsed ion extraction of 120 ns in the positive ion mode. The acquisition range was m/z 0-5000, and reflector voltages 1 and 2 were set to 21 and 9.55 kV, respectively. Data were collected by averaging 400 laser pulses with the lowest laser energy necessary, and peak lists generated from the MS spectra (Bruker FlexAnalysis 3.4 software). MALDI-TOF MS analysis of post-reaction solutions revealed the presence of signals associated with the $Na^+$ and $K^+$ adducts of the aldonic acid forms of soluble oligomers built from 4, 6, and 8 saccharide units (DP) (Fig. S5), consistent with prior literature findings. No evidence for oxidative cleavage at the 4'-position of *N*-acetylglucosamine was seen in these experiments.

For those samples containing soluble oligomers built from 4, 6, and 8 saccharide units, the extent of β-chitin degradation was determined by incubating two aliquots of the reaction solution (100 μL) for 24 h at 37 °C with 3 μM recombinant chitobiase in 20 mM Tris-HCl buffer, pH 8 (10 μL), (total volume 110 μL). One of these solutions as used to quantify chitobionic acid by hydrophilic interaction chromatography (HILIC) on an Agilent 1260 infinity HPLC system. Analytes were eluted isocratically (0.4 mL $min^{-1}$) at 60 °C on an Acquity BEH Amide column (2.1 mm x 50 mm) with a 78:22 mixture of acetonitrile and 15 mM Tris.HCl buffer, pH 8 (Fig. S8). Similarly, the second solution was used to determine the amount of N-acetyl glucosamine by ion exchange chromatography. Analytes were eluted isocratically (0.6 mL $min^{-1}$) at 60 °C on a Hi Plex $Na^{2+}$ column (7.7 mm x 50 mm) with water. Residual ascorbate was measured from the absorbance of the post-reaction solution at 266 nm and comparison with a standard curve of ascorbate in the reaction solution.

## *sm*AA10A-catalyzed β-chitin degradation with pre-prepared $H_2O_2$ solutions

Reactions were carried out in 50 mL RBFs sealed by a rubber septum (Fig. S9). Each RBF was charged with β-chitin (100 mg) and *sm*AA10A (0.5 μM) dissolved in 50 mM $K_2HPO_4$/$KH_2PO_4$ buffer, pH 6 (10 mL total volume), unless otherwise stated. After injecting 1 mM ascorbate (100 μL) dissolved in 50 mM $K_2HPO_4$/$KH_2PO_4$ buffer, pH 6, the reaction was initiated by either (i) the immediate introduction of flow-

fed 10 mM $H_2O_2$ (0.252 μmol $min^{-1}$, 25 μL $min^{-1}$ in 50 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 6), over a time of 4 hours (determined from examining the effect of reactants on *in situ* $H_2O_2$ production (Fig. S11)), or a single injection of 1 mM $H_2O_2$ (605 μL) in 50 mM $K_2HPO_4/KH_2PO_4$ buffe, pH 6. Each reaction was stirred (250 rpm) at 21 °C with β-chitin degradation being monitored by incubating filtered aliquots (100 μL) with chitobiase (3 μM) in 20 mM Tris-HCl buffer, pH 8, at 37 °C for 2 h (110 μL total volume). The analysis of reaction products by HPLC and residual $H_2O_2$ in the post-reaction solution was then carried out. All measurements for each reaction at a given timepoint were performed in triplicate. All of these measurements were made in individual experiments. The reaction mixtures were not sampled online.

## *sm*AA10A-catalyzed β-chitin degradation under aerobic conditions

Reactions were carried out in 50 mL Radleys glass round bottom flasks (RBFs) (certified to 5 bar) on a multi-reaction system. Under our standard conditions, each RBF was charged with β-chitin (100 mg), 0.5 μM *sm*AA10A and 1 mM ascorbate dissolved in 50 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 6, (10 mL total volume) that was not de-gassed. After pressurization with $N_2$ (2.0 bar), reaction mixtures were stirred (250 rpm) at 21 °C and depressurized at appropriate timepoints. Aliquots (100 μL) were then taken and incubated with recombinant WT chitobiase (3 μM) in Tris-HCl buffer (20 mM, pH 8), at 37 °C for 2 h (110 uL total volume). The analysis of reaction products by HPLC and residual $H_2O_2$ in the post-reaction solution was then carried out. All measurements for each reaction at a given timepoint were performed in triplicate.

## Top-up experiments with β-chitin and ascorbate

Reactions were carried out in 50 mL Radleys glass round bottom flasks (RBFs) (certified to 5 bar) on a multi-reaction system (Fig. S9). An identical procedure to that outlined above for the chemo-enzymatic degradation of β-chitin was followed for a reaction time of 2 h. Stirring was then stopped and the RBF depressurized prior to the addition of ascorbate (1.0 mM) or *sm*AA10A (0.5 μM). After being re-pressurized with $H_2$ (1.6 bar) and compressed air (0.4 bar), the reaction mixture was stirred (250 rpm) for a further 2 h and the products analyzed following the procedures outlined above.

## Characterization of the 0.5%Au-0.5%Pd/$TiO_2$ chemo-catalyst

Aberration-corrected scanning transmission electron microscopy was performed using a probe-corrected Thermo Fisher Scientific Spectra 200 Cold-FEG operating at 200 kV. The instrument was equipped with a HAADF detector, and the imaging was done at a probe current of 120 pA and convergence angle of 30 mrad. Samples were dry dispersed onto 300 mesh copper grids coated with a holey carbon film. Energy-dispersive X-ray (EDX) mapping was performed using a Super-X G2 detector at a dwell time of 25 μs. All images and EDX data were processed using Velox software (Fig. 4, Figs. S13-S15, S17 and S18)

XPS measurements (Fig. S16) were performed on a Thermo Scientific K-Alpha$^+$ photoelectron spectrometer was used to collect XP spectra utilizing a micro-focused monochromatic Al Kα X-ray source operating at 72 W. Data were collected over an elliptical area of ~400 $μm^2$ at pass energies of 40 and 150 eV for high-resolution and survey spectra, respectively. Sample charging effects were minimized through a combination of low-energy electrons and $Ar^+$ ions; consequently, this resulted in a C (1s) line at 284.8 eV for all samples. All data were processed using CasaXPS v2.3.24 using a Shirley background,[5] Scofield sensitivity factors,[6] and an electron energy dependence of −0.6 as recommended by the manufacturer.

## Metal leaching studies

To provide an indication of the extent of metal leaching during the *in situ* chemo-enzymatic degradation of β-chitin reaction, indicative studies were conducted in the absence of β-chitin (to remove the potential for homogeneous metal adsorption). The heterogeneous catalyst (10 mg, *i.e*. ten times that utilized for the chemo-enzymatic degradation of β-chitin reaction) was added to an RBF containing ascorbic acid (1 mM) and *sm*AA10A (0.5 μM) dissolved in 50 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 6 (10 mL total volume). The RBF was pressurized with $H_2$ (1.6 bar) and compressed air (0.4 bar) and stirred (250 rpm) for the desired reaction time (up to 96 h) at 21 $^oC$ (table S5). Post-reaction, the chemo-catalyst was removed by filtration and the reaction solution analyzed using an Agilent 7900 ICP-MS equipped with an I-AS auto-sampler (5-point calibration using certified reference materials (Perkin Elmer) and a certified internal standard (Agilent). All calibrants were matrix matched.

The role of leached metal species on the efficiency of *sm*AA10A-catalyzed β-chitin degradation was assessed using the following procedure. Ascorbate (1 mM) was added to a heterogeneous mixture of β-chitin (100 mg) in 50 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 6, (10 mL total volume) containing *sm*AA10A (0.5 μM) and $PdCl_2$ (0.148 μg $L^{-1}$) or $HAuCl_4$ (0.068 μg $L^{-1}$) or NaCl (2.32 μM). The reaction was then stirred (250 rpm) for 2 h at 21 °C after pressurization with $H_2$ (1.6 bar) and compressed air (0.4 bar). The RBF was then depressurized, and substrate was removed by filtration. Aliquots of the supernatant were then analyzed for chitobionic acid and *N*-acetylglucosamine concentration using standard procedures as outlined above (Table S6).

## Catalyst reusability measurements

Using our standard method, 0.5%Au-0.5%Pd/$TiO_2$ chemo-catalyst (1 mg), β-chitin (100 mg), and 0.5 μM *sm*AA10A and 1 mM ascorbate dissolved in 50 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 6, (10 mL total volume) was stirred (250 rpm) under an atmosphere of $H_2$ (1.6 bar) and compressed air (0.4 bar) at 21 $^oC$ for 2 h. After depressurization, solid material (consisting of the chemo-catalyst and β-chitin) was separated from the post-reaction solution by filtration, washed (2 x 5 mL HPLC grade water) and dried under vacuum (30 °C, 16 h). The concentrations of CBA and residual $H_2O_2$ in the filtrate were then determined using the procedures described above. The dried solid material was then added to a solution of 0.5 μM *sm*AA10A and 1 mM ascorbate dissolved in 50 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 6, (10 mL total volume). The resulting mixture was then stirred (250 rpm) under an atmosphere of $H_2$ (1.6 bar) and compressed air (0.4 bar) at 21 $^oC$ for 2 h, and the solid material isolated and dried again. This procedure was repeated again to evaluate the stability of the 0.5%Au-0.5%Pd/$TiO_2$ chemo-catalyst (table S7).

## Supplementary Text

## Technical reasons for not performing product inhibition studies

LPMO's such as CBP-21 are processive, surface active enzymes, which act on polymeric substrates, such as β-chitin, in their crystalline form that result in the stepwise shortening by oligomeric unit composed of 4, 6, or 8 residues, as confirmed by mass spectrometry Fig S5). Given the scarcity of chain ends (*i.e.* the low effective substrate concentration) there is a preference for the enzyme to remain attached to the polymeric substrate, while cleaving these dimeric units. Despite our best efforts we were unable to source the oligomeric units, which would have allowed for product inhibition studies.

## Determination of β-Chitin saccharification in the 0.5%Au-0.5%Pd/$TiO_2$ /*sm*AA10A system.

The number of moles of *N*-acetylglucosamine (221.21 g $mol^{-1}$) present in the initial mass of β-chitin is calculated using the following expression (Eqn. S1):

$$\text{Initial } N\text{-acetylglucosamine (mol)} = \text{mass of β-chitin (g)}/221.21 \text{ (g mol}^{-1}\text{)}$$

The final number of moles of *N*-acetylglucosamine after oxidative cleavage of β-chitin by *sm*AA10A and subsequent treatment of the fragments with chitobiase is calculated, based on HPLC measurements, using the following expressions (Eqn. S2):

$$\text{Final } N\text{-acetylglucosamine (mol)} = \text{measured } \{N\text{-acetylglucosamine (mol)} + 2 \times \text{chitobionic acid (mol)}\}$$

$$\% \text{ saccharification} = 100 \times \text{Initial } N\text{-acetylglucosamine (mol)}/\text{Final } N\text{-acetylglucosamine (mol)}$$

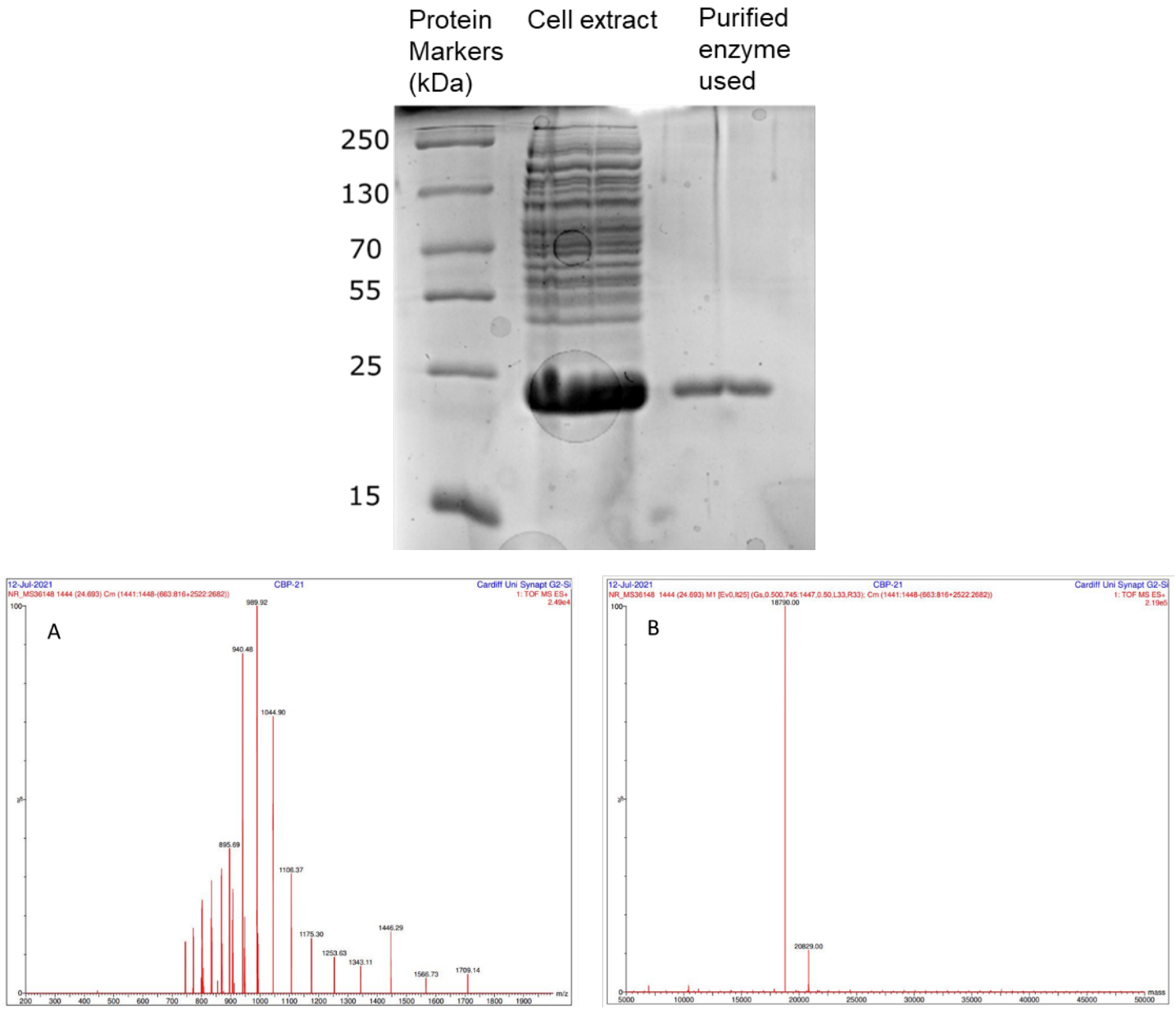



**Figure S1. Characterization of recombinant WT *sm*AA10A. (top)** SDS-PAGE gel showing the purity of the enzyme used in the chemo-catalyst/*sm*AA10A system; **(bottom)** MALDI-TOF mass spectra of purified, recombinant WT *sm*AA10A **(left)** and the deconvoluted spectrum **(right)**. Observed mass = 18790 Da; Expected mass = 18793 Da.

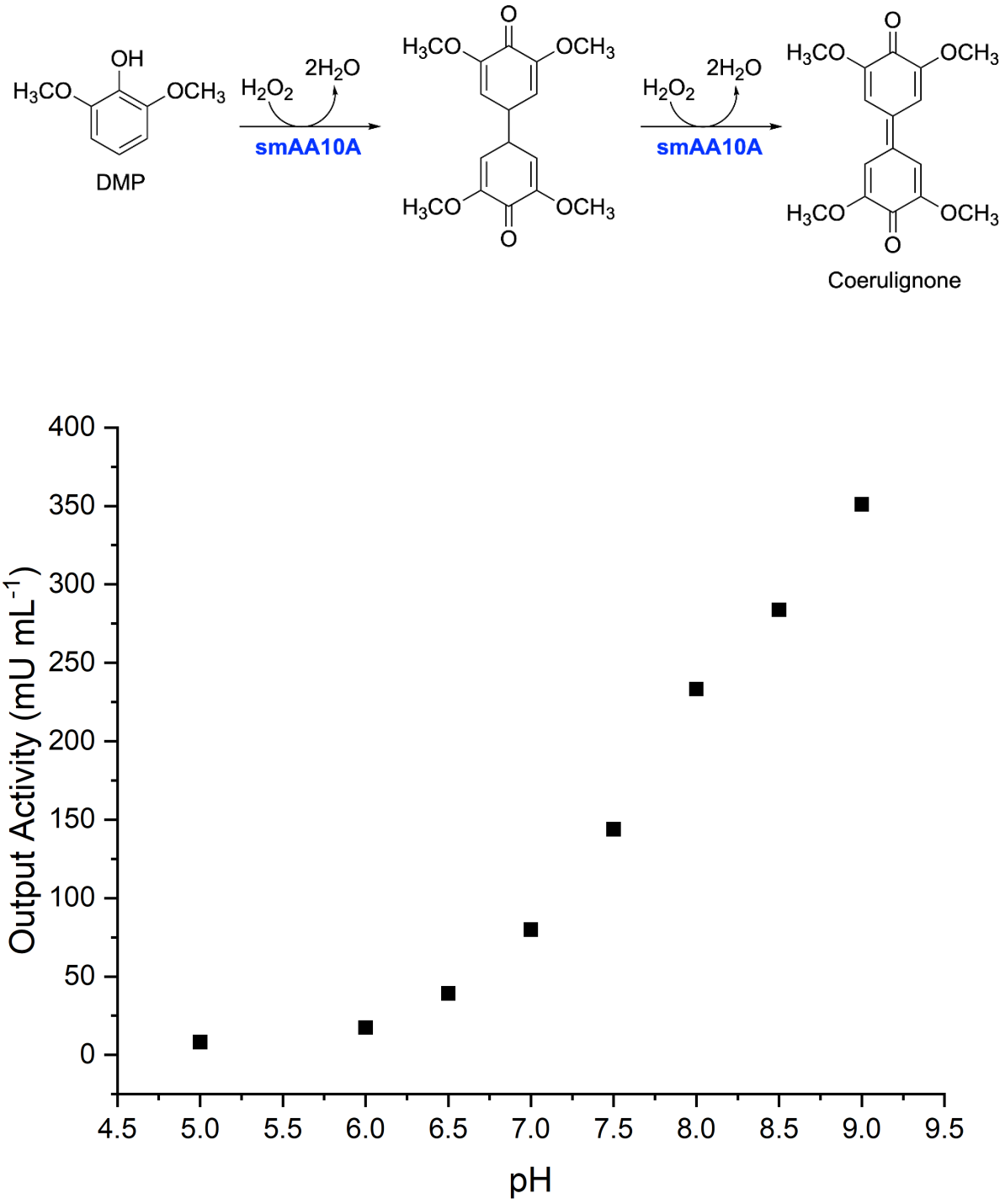



**Figure S2. Effect of pH on *sm*AA10A activity. (top)** Enzyme activity in these experiments was measured by monitoring the conversion of 2,6-dimethoxyphenol (DMP) into coerulignone at 469 nm. Reaction mixtures contained DMP (1 mM), and $H_2O_2$ (100 µM), dissolved in universal buffer (100 mM, consisting of 33.3mM NaAc, 33.3 mM Tris-HCl, 3.33 mM Bris Tris) adjusted to the desired pH (980 µL total volume), and were initiated by the addition of smAA10A (20 µL) and incubated at 30 °C for 5 min; **(bottom) Coerulignone production rates (1 U is equivalent to coerulignone formation of 1 µmol/min) as a function of pH.** Concentrations were obtained from absorbance values using an extinction coefficient of 53,200 $M^{-1}cm^{-1}$) at 469 nm.

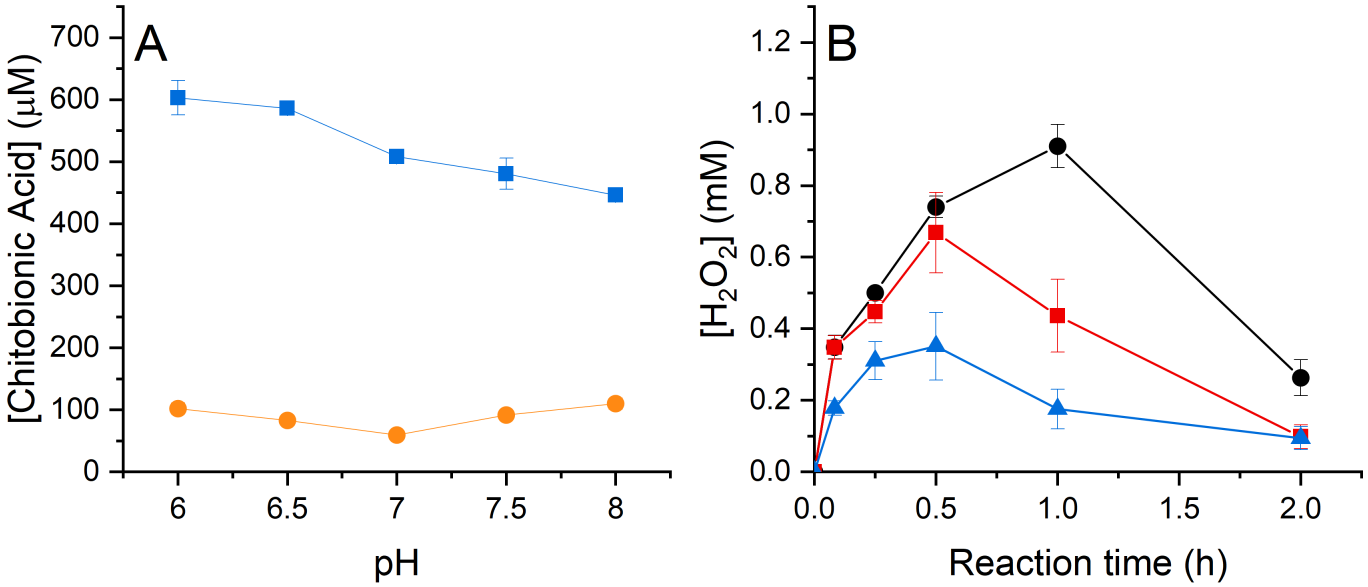



**Figure S3. Effect of pH on the chemo-catalyst/*sm*AA10A cascade.** (**A**) pH-Dependence of the ability of *sm*AA10A to degrade β-chitin with (■) and without (●) *in situ* $H_2O_2$ generation by the 0.5%Au-0.5%Pd/$TiO_2$ chemo-catalyst. Reaction mixtures included *sm*AA10A (0.5 μM), ascorbate (1 mM), β-chitin (100 mg), in $K_2HPO_4$/$KH_2PO_4$ buffer (50 mM, 10 mL final volume) adjusted to the desired pH under an $H_2$ (1.6 bar)/air (0.4 bar) atmosphere. Reactions were stirred (250 rpm) at 21 °C for 2 h; (**B**) pH-Dependence of *in situ* $H_2O_2$ generation by the 0.5%Au-0.5%Pd/$TiO_2$ chemo-catalyst (1 mg) under an $H_2$ (1.6 bar)/air (0.4 bar) atmosphere in 50 mM $K_2HPO_4$/$KH_2PO_4$ buffer adjusted to pH 6 (●), pH 7 (■) or pH 8 (▲) (10 mL total volume). Reaction mixtures were stirred (250 rpm) at 21 °C.

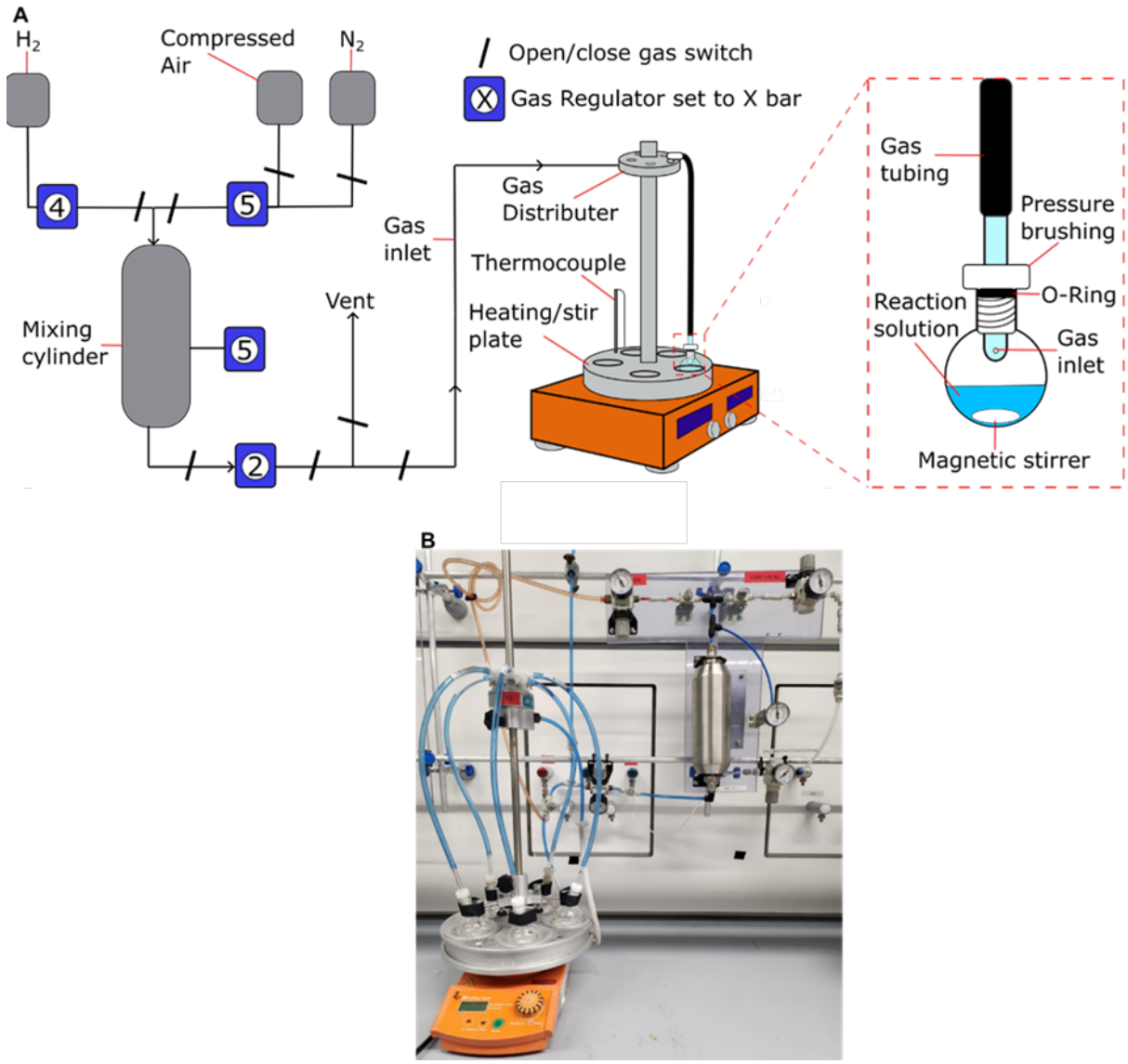
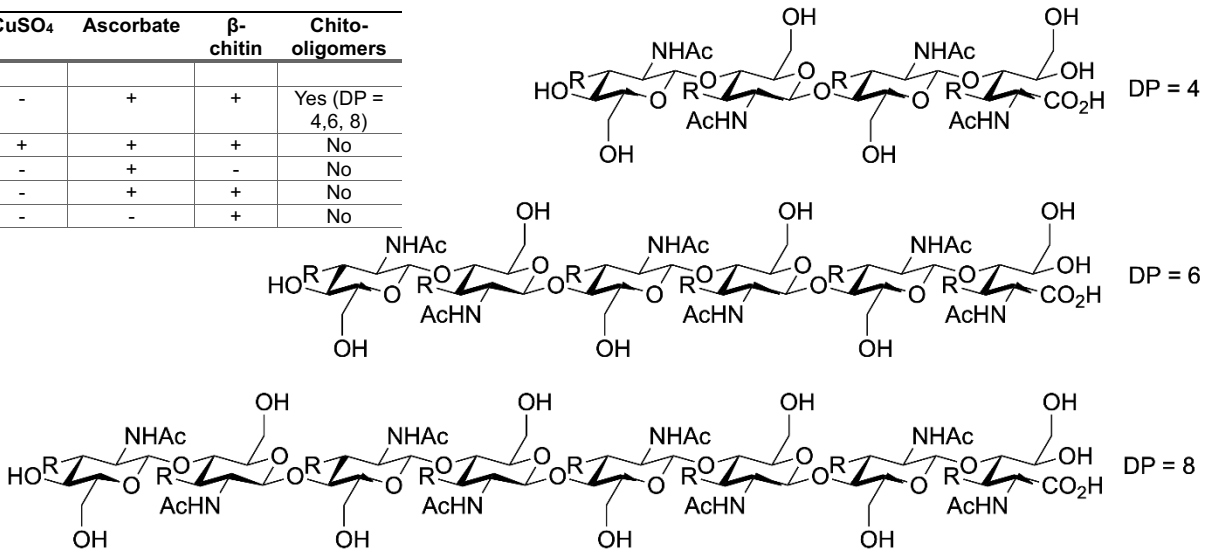



**Figure S4. Experimental setup used to evaluate the chemo-enzymatic cascade.**

| *sm*AA10A | $CuSO_4$ | Ascorbate | β-chitin | Chito-oligomers |
|---|---|---|---|---|
| | | | | |
| + | - | + | + | Yes (DP = 4,6, 8) |
| - | + | + | + | No |
| + | - | + | - | No |
| - | - | + | + | No |
| + | - | - | + | No |



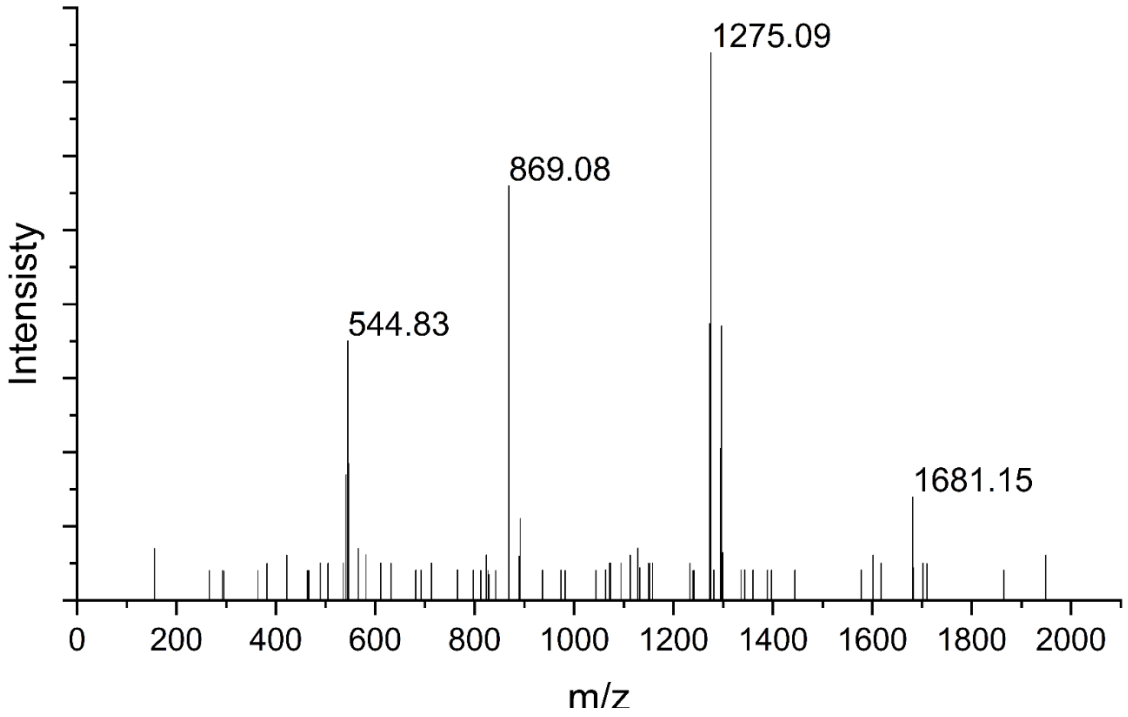




**Figure S5. MALDI-TOF mass spectrometric characterization of *sm*AA10A-catalyzed β-chitin breakdown under aerobic conditions. (top)** Oligomeric products identified in the reaction mixture only when the enzyme is present (table inset). Replacing *sm*AA10A with $CuSO_4$ (10 μM ), did not result in β-chitin oxidation. R = OH and DP is the degree of polymerization. Reaction mixtures containing *sm*AA10A (0.5 μM), ascorbate (1 mM) and β-chitin (10 mg) in Tris.HCl, (50 mM , pH 8, total volume 1 mL) were shaken in a thermomixer at 40 $^{o}$C for 16 h; **(bottom)** MALDI-TOF spectrum showing the Na and K adducts of the aldonic acid forms of chito-oligomers with DP = 4, 6 or 8. Ions of m/z less than 400 are suppressed.

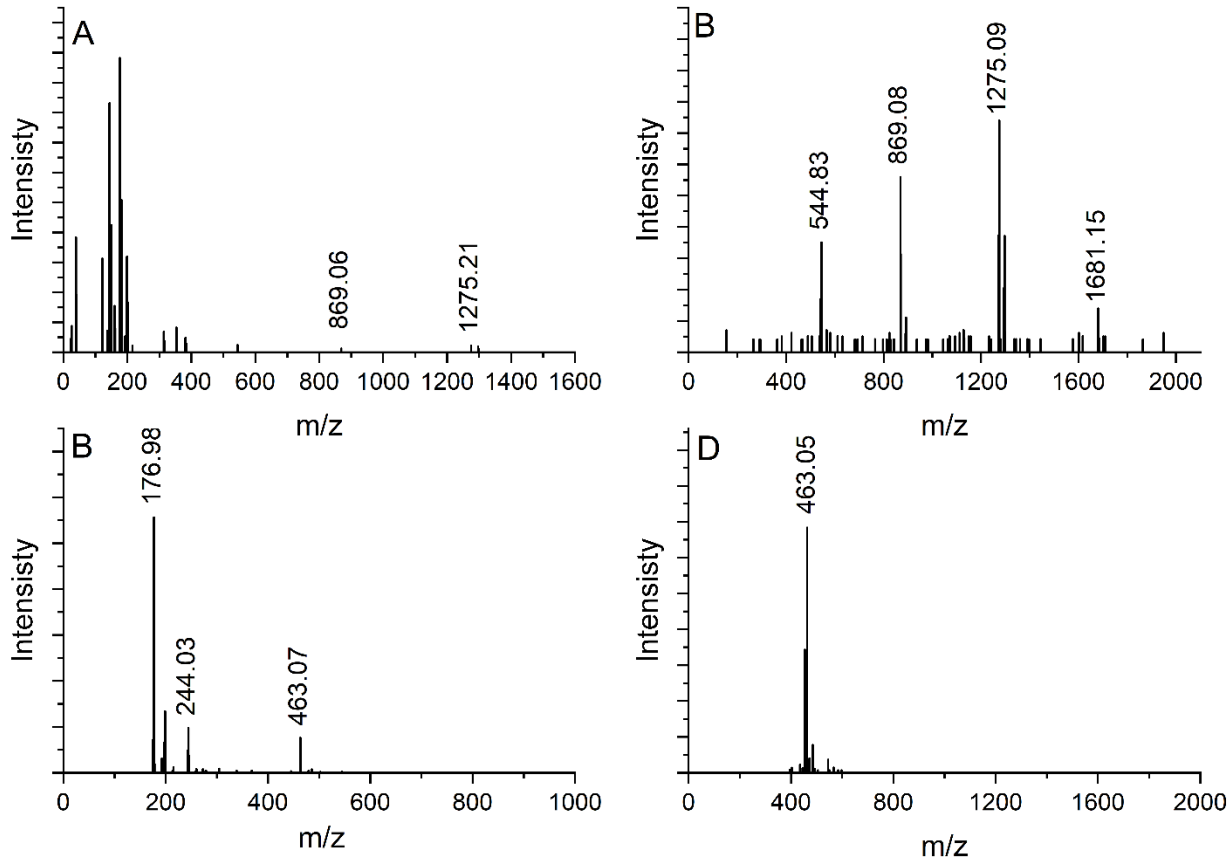



**Figure S6. MALDI-TOF mass spectra showing the effect of adding recombinant WT chitobiase to products formed by *sm*AA10A-catalyzed β-chitin degradation under aerobic conditions.** Reaction mixtures containing *sm*AA10A (0.5 μM), ascorbate (1 mM) and β-chitin (10 mg) in Tris.HCl, (50 mM , pH 8, total volume 1 mL), were stirred (1000 rpm) at 40 °C for 16 h. (**A**) Mass spectrum of initial product mixture without suppression of Ions of m/z less than 400; (**B**) Mass spectrum of initial product mixture with suppression of Ions of m/z less than 400; (**C**) Mass spectrum (without suppression of Ions of m/z less than 400) of products formed by incubating the post-reaction solution with 3 μM chitobiase at 37 °C for 2 h. Peaks with m/z = 244 and 463 correspond to the $Na^+$ adducts of N-acetylglucosamine and chitobionic acid, respectively. (**D**) Mass spectrum (with suppression of Ions of m/z less than 400) of products formed by incubating the post-reaction solution with 3 μM chitobiase at 37 °C for 2 h. Peak with m/z = 463 corresponds to the $Na^+$ adduct of chitobionic acid.

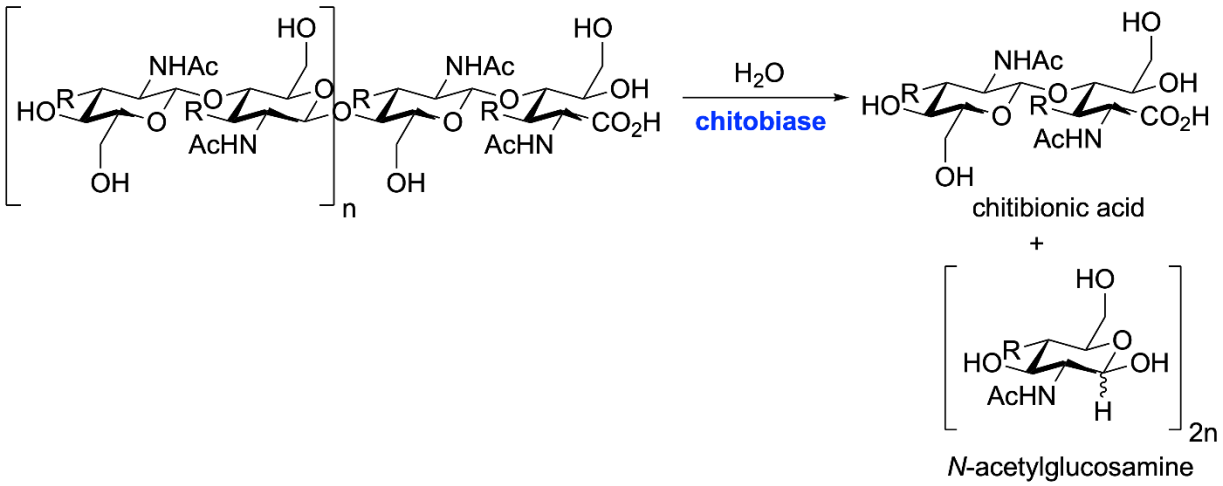



**Figure S7. Chitobiase-catalyzed hydrolysis of the chito-oligomers produced by *sm*AA10A-catalyzed β-chitin oxidation.** The concentration of chitobionic acid is equivalent to the number of oxidative cleavage sites and can be used to calculate the % saccharification in combination with the final concentration of *N*-acetylglucosamine.

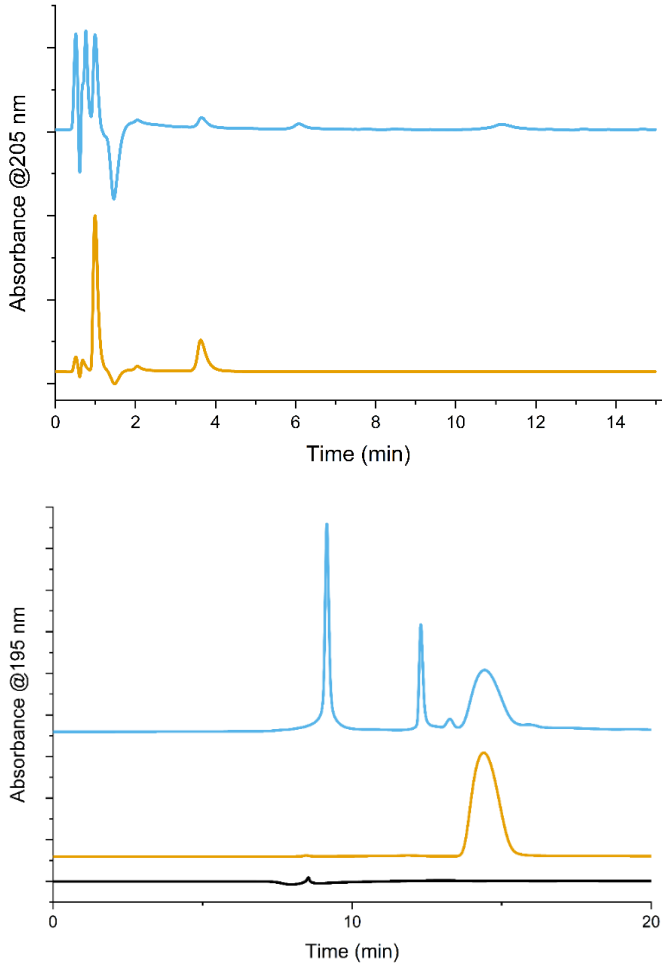



**Figure S8. HPLC-based separations of reaction products after treatment with chitobiase**. (**top**) Chromatograms of the reaction mixture with (---) and without (---) chitobiase incubation. The identity of the new peak (retention time of approx. 4 min) as chitobionic acid was established by comparison to an authentic standard. Analytes were eluted isocratically (0.4 mL $min^{-1}$) at 60 °C on an Acquity BEH Amide column (2.1 mm x 50 mm) with a 78:22 mixture of acetonitrile and 15 mM Tris.HCl buffer, pH 8; (**bottom**) Chromatograms of the solvent matrix (---), an authentic standard of *N*-acetylglucosamine (---), and the reaction mixture after chitobiase incubation (---). Analytes were eluted isocratically (0.6 mL $min^{-1}$) at 60 °C on a Hi Plex $Na^{2+}$ column (7.7 mm x 50 mm) with water.

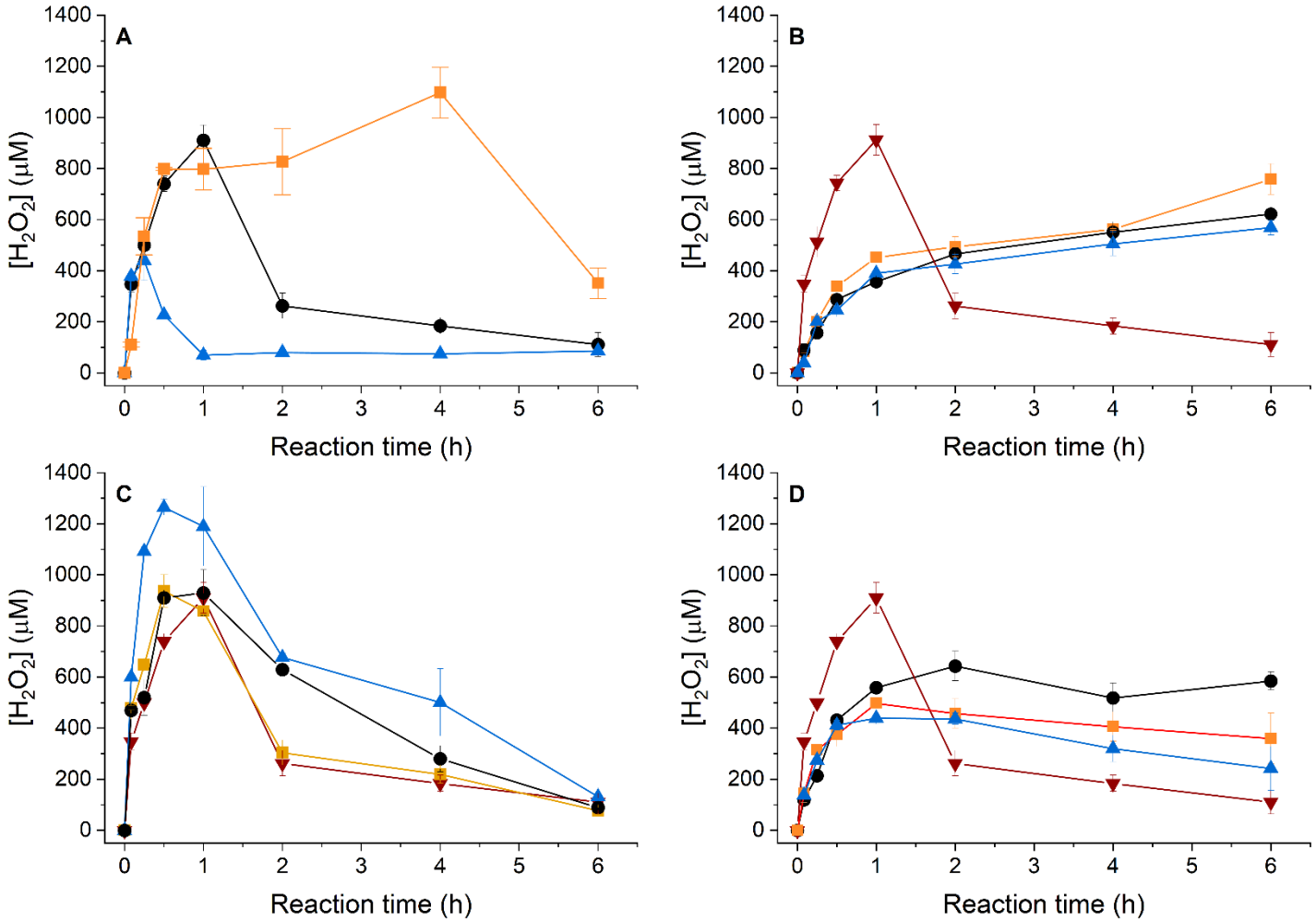



**Figure S9. Effects of reactants on *in situ* $H_2O_2$ production.** Unless stated otherwise, all reactions were performed using 0.5%Au-0.5%Pd/$TiO_2$ catalyst (1 mg) in 50 mM $K_2HPO_4$/$KH_2PO_4$ buffer, pH 6 (10 mL) at 21 °C with stirring (250 rpm) under a $H_2$ (1.6 bar) and air (0.4 bar) gas mixture. Variable amounts of (**A**) the chemo-catalyst at 0.5 mg (■), 1 mg (●), and 2 mg (▲); (**B**) β-chitin at 0 mg (▼), 50 mg (■), 100 mg (●), and 200 mg (▲); (**C**) ascorbate at 0 mM (▼), 0.5 mM (■), 1 mM (●), and 2 mM (▲); (**D**) *sm*AA10A at 0 mM (▼), 0.25 μM (■), 0.5 μM (●), and 1 μM (▲).

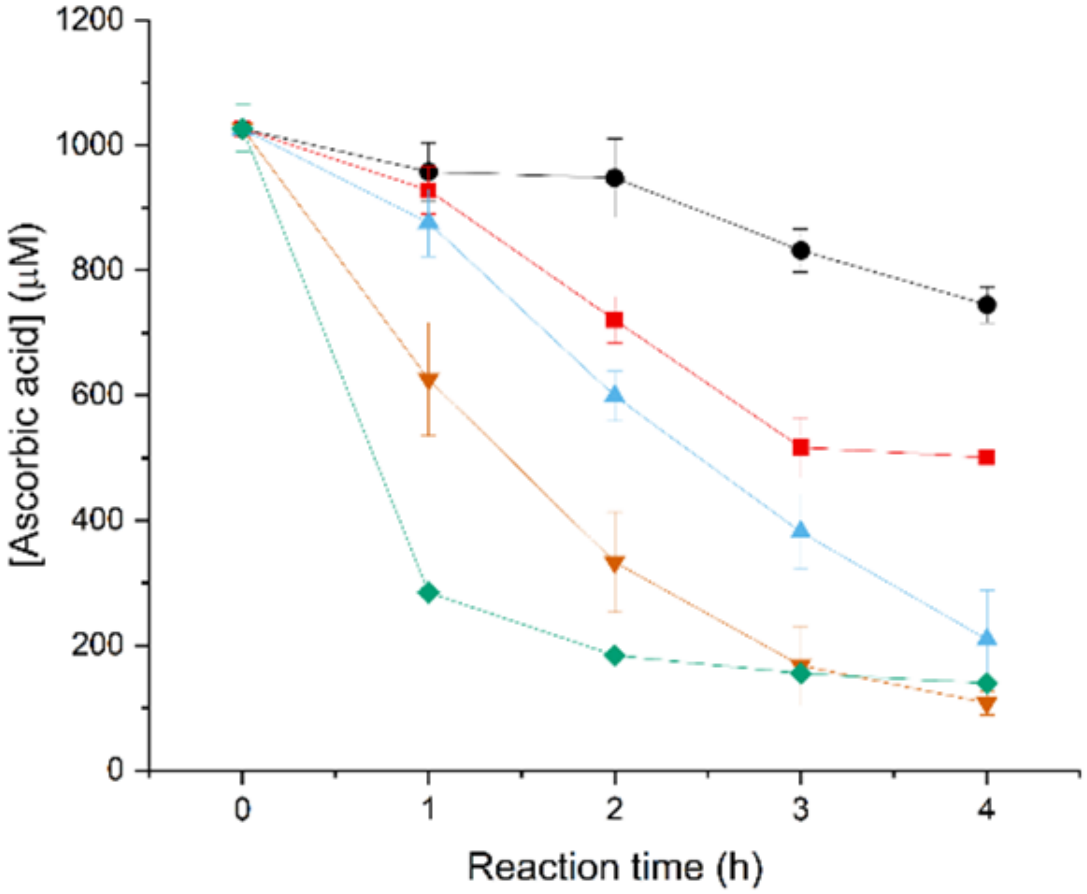



**Figure S10.** Ascorbic acid breakdown in the presence of 0.5%Au-0.5%Pd/$TiO_2$. Ascorbate (1 mM) was incubated with (●) 0 mg, (■) 0.5 mg, (▲) 1.0 mg, (▼), 2.0 mg, or (♦) 4.0 mg of the 0.5%Au-0.5%Pd/$TiO_2$ catalyst in $K_2HPO_4$/$KH_2PO_4$ buffer (50 mM, pH 6, 10mL) with stirring (250 rpm) at 21 °C.

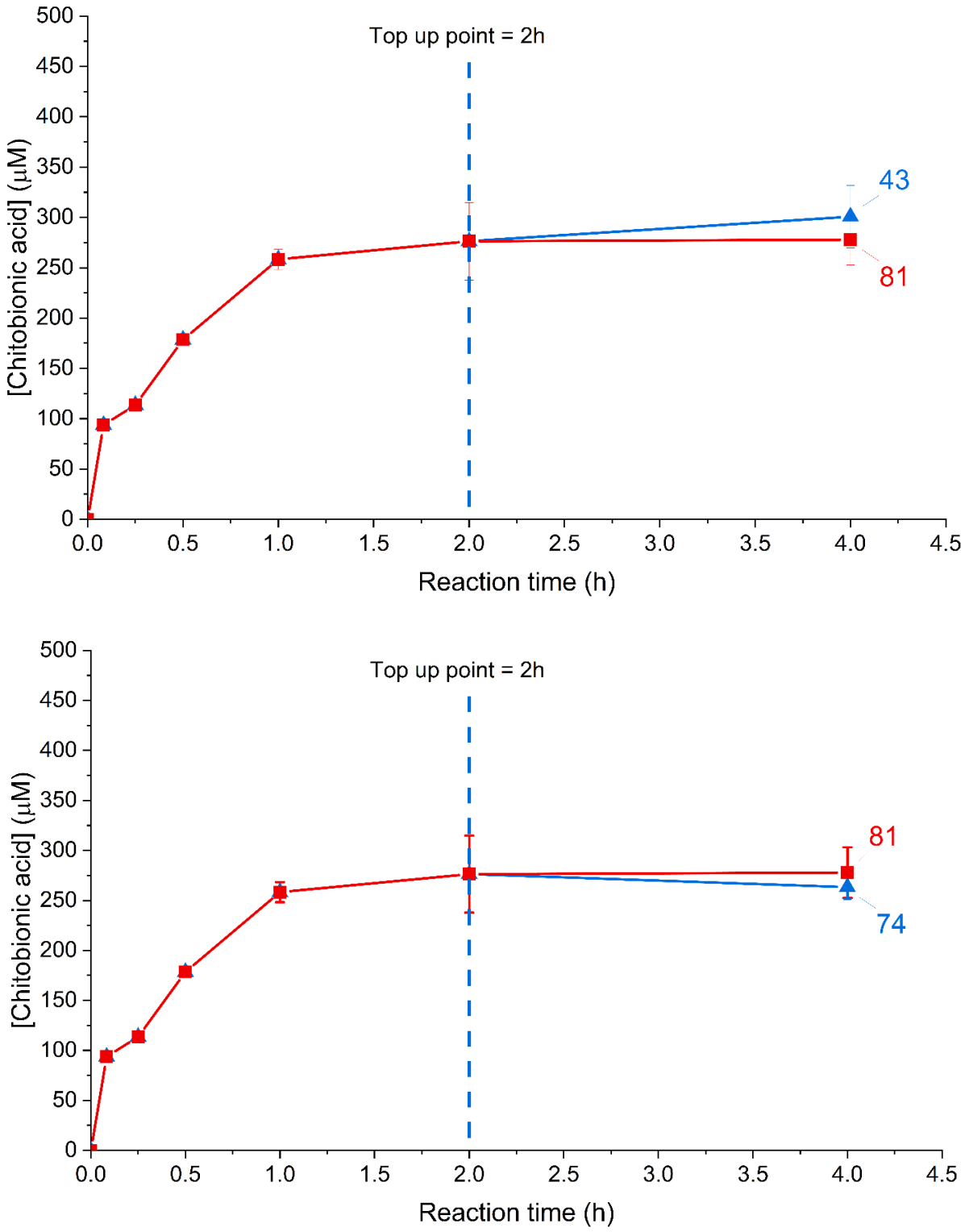



**Figure S11. Adding ascorbate or *sm*AA10A does not restore β-chitin degradation after cessation of chitobionic acid production in our chemo-enzymatic system.** (**top**) Addition of 1.0 mM (▲) and 0.0 mM ascorbate (■) at t = 2 h; (**bottom**) Addition of 0.5 µM (▲) and 0.0 µM (■) *sm*AA10A at t = 2 h. Numerical values indicate the amount of residual $H_2O_2$ (µM) in the reaction mixtures after 4 h. Initial conditions (t = 0 h): 0.5%Au-0.5%Pd/$TiO_2$ (1 mg), β-chitin (50 mg), *sm*AA10A (0.5 µM), and ascorbate (1 mM) in 50 mM $K_2HPO_4$/$KH_2PO_4$ buffer, pH 6 (10 mL). Reaction mixtures were stirred (250 rpm) under a $H_2$ (1.6 bar) and air (0.4 bar) gas mixture at 21°C.

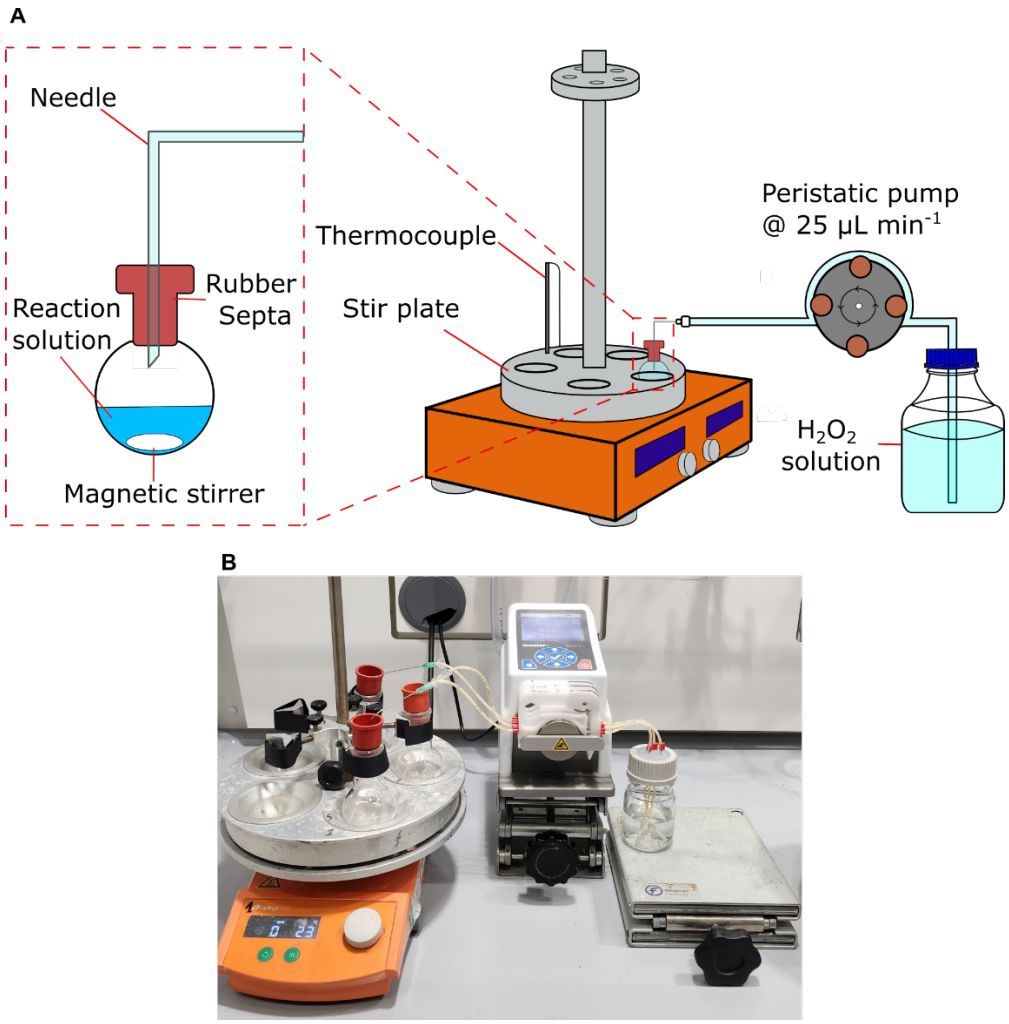



**Figure S12. Experimental setup used to flow-feed $H_2O_2$ (252 nmol $min^{-1}$) into *sm*AA10A-containing reaction mixtures.**

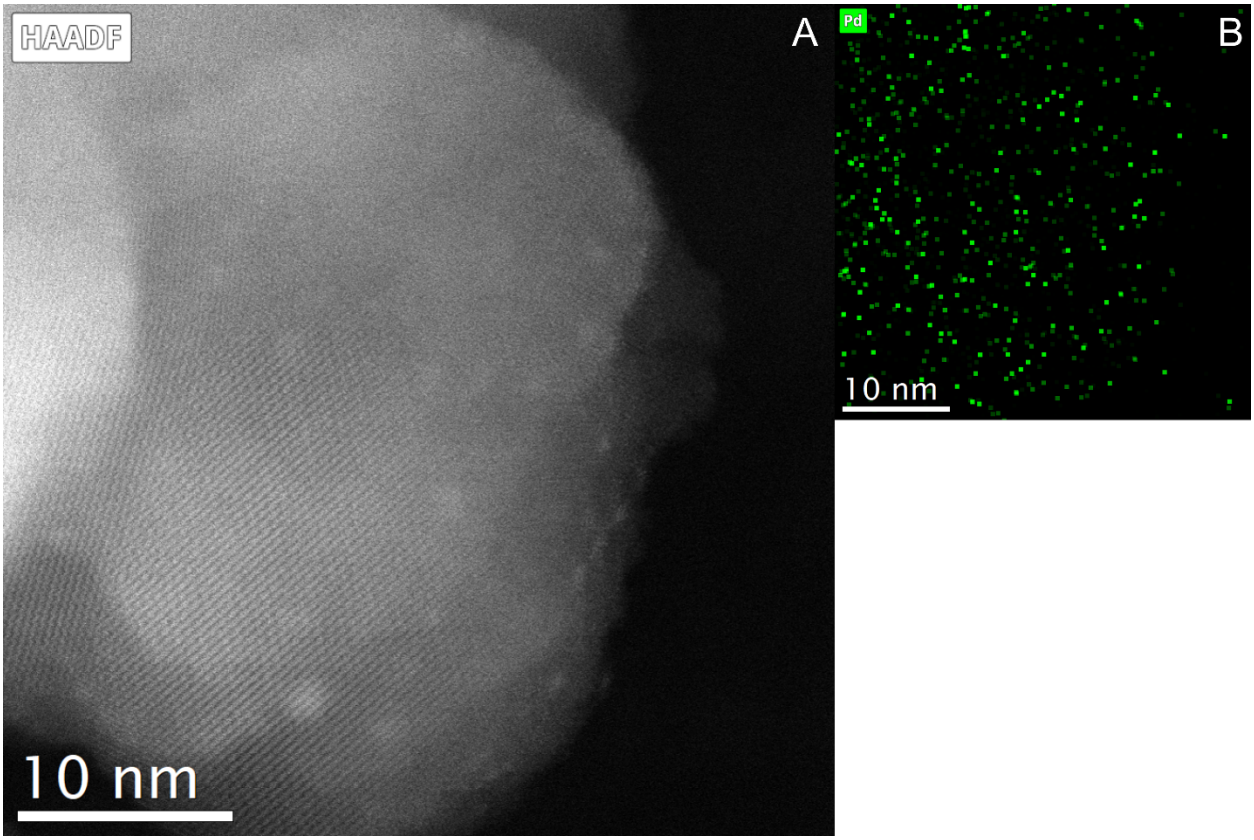



**Figure S13.** Catalyst surface structure and morphology of the fresh 0.5%Au-0.5%Pd/$TiO_2$ catalyst using (**A**) (High-angle annular dark-field scanning transmission electron microscopy, paired with (**B**) X-EDS imaging showing the presence of highly dispersed sub-nano (< 1 nm, Pd-only) particles.

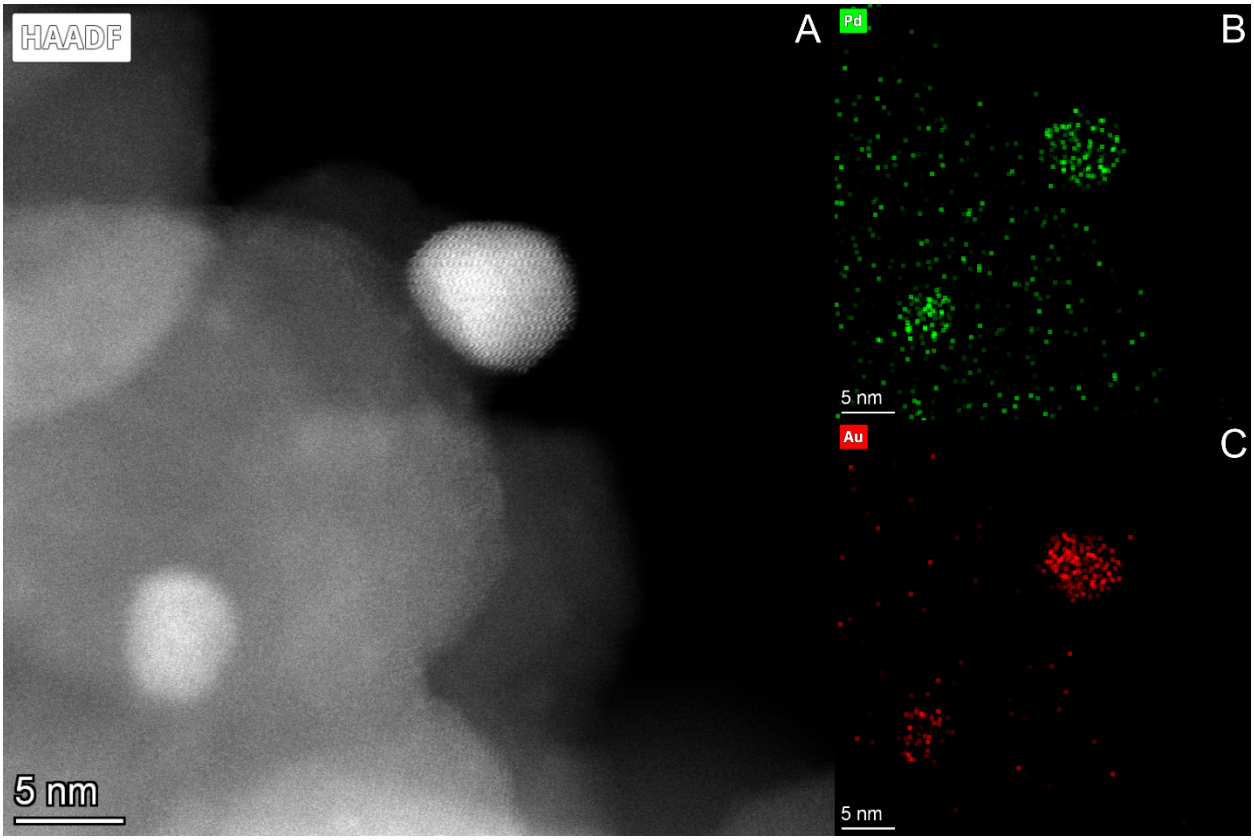



**Figure S14.** Catalyst surface structure and morphology of the fresh 0.5%Au-0.5%Pd/$TiO_2$ catalyst using (**A**) High-angle annular dark-field scanning transmission electron microscopy, paired with (**B, C**) X-EDS imaging showing the presence of Au-Pd nanoalloys of intermediate size (5-10 nm).

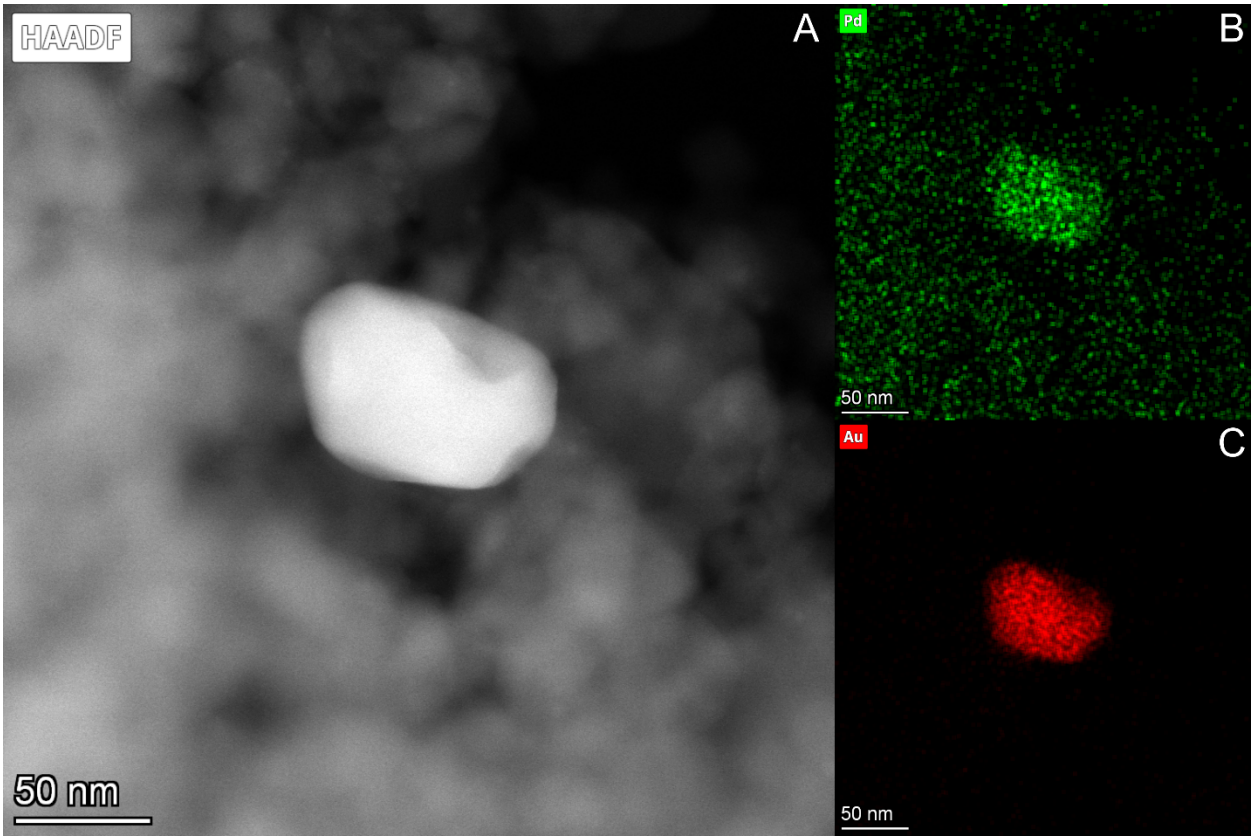



**Figure S15.** Catalyst surface structure and morphology of the fresh 0.5%Au-0.5%Pd/$TiO_2$ catalyst using (**A**) High-angle annular dark-field scanning transmission electron microscopy, paired with (**B, C**) X-EDS imaging showing the presence of larger (>50 nm) Au-Pd nanoalloys.

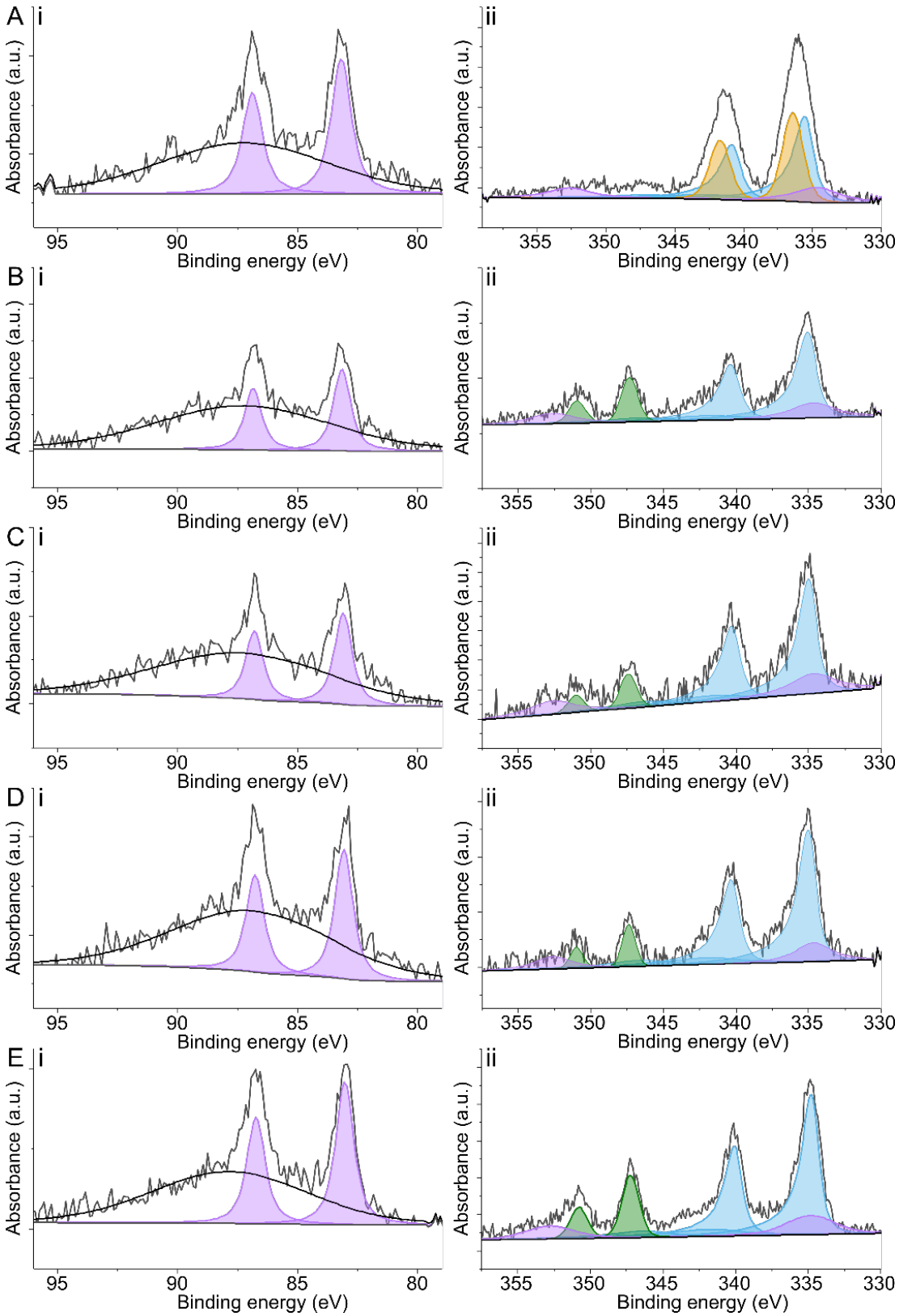



**Figure S16.** Surface atomic compositions of the 0.5%Au-0.5%Pd/$TiO_2$ catalyst both fresh (**A**) and used after 5 min (**B**), 30 min (**C**), 2 hours (**D**), 96 hours (**E**) as determined by X-ray photoelectron spectroscopy using the Au 4f (**i**) and Pd 3d/Au 4d (**ii**) regions. Key: $Au^0$, purple; $Pd^0$, blue; $Pd^{2+}$, orange; $Ca^{2+}$, green; loss of structure and Pd 4s (black line). a.u means arbitrary units.

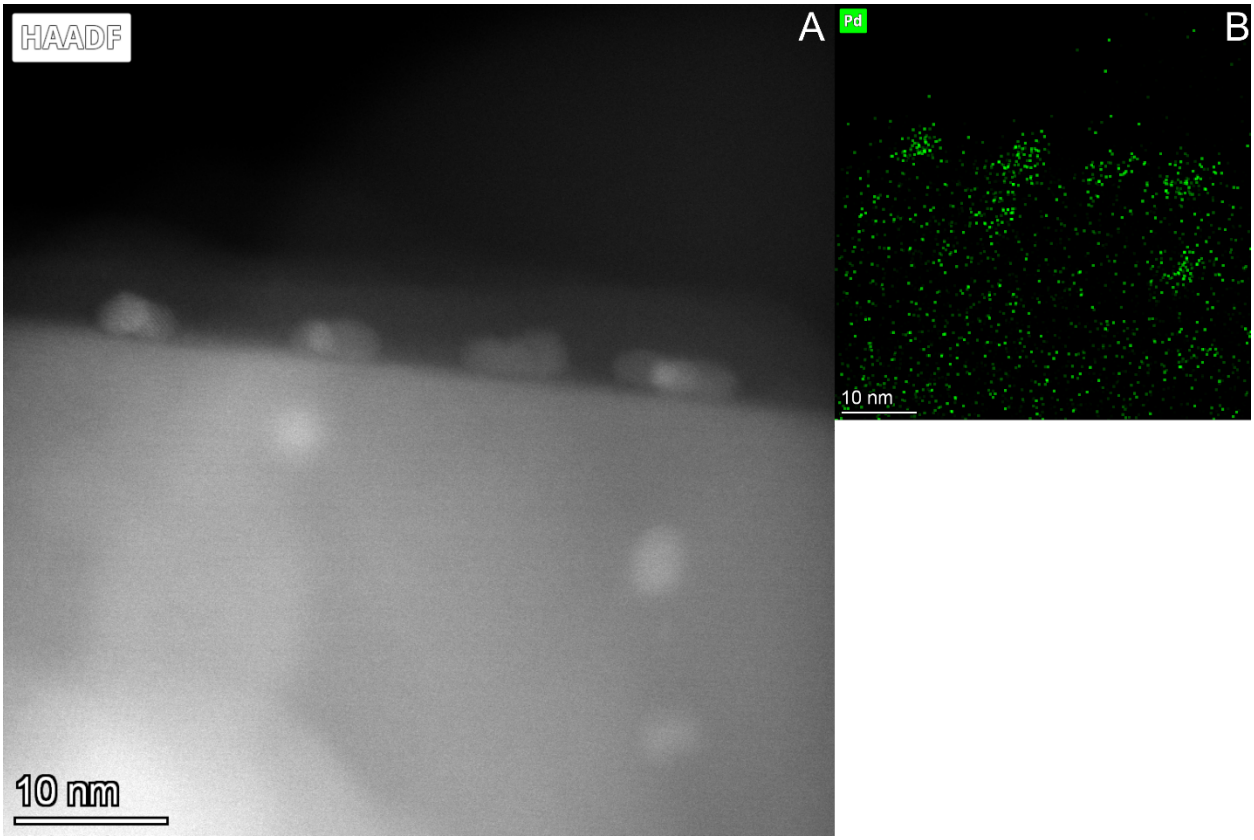



**Figure S17.** Catalyst surface structure and morphology of the 0.5%Au-0.5%Pd/$TiO_2$ catalyst, used for 2-hour reaction, using (**A**) (High-angle annular dark-field scanning transmission electron microscopy and paired (**B**) X-EDS imaging showing small (1-5 nm, Pd only) particles.

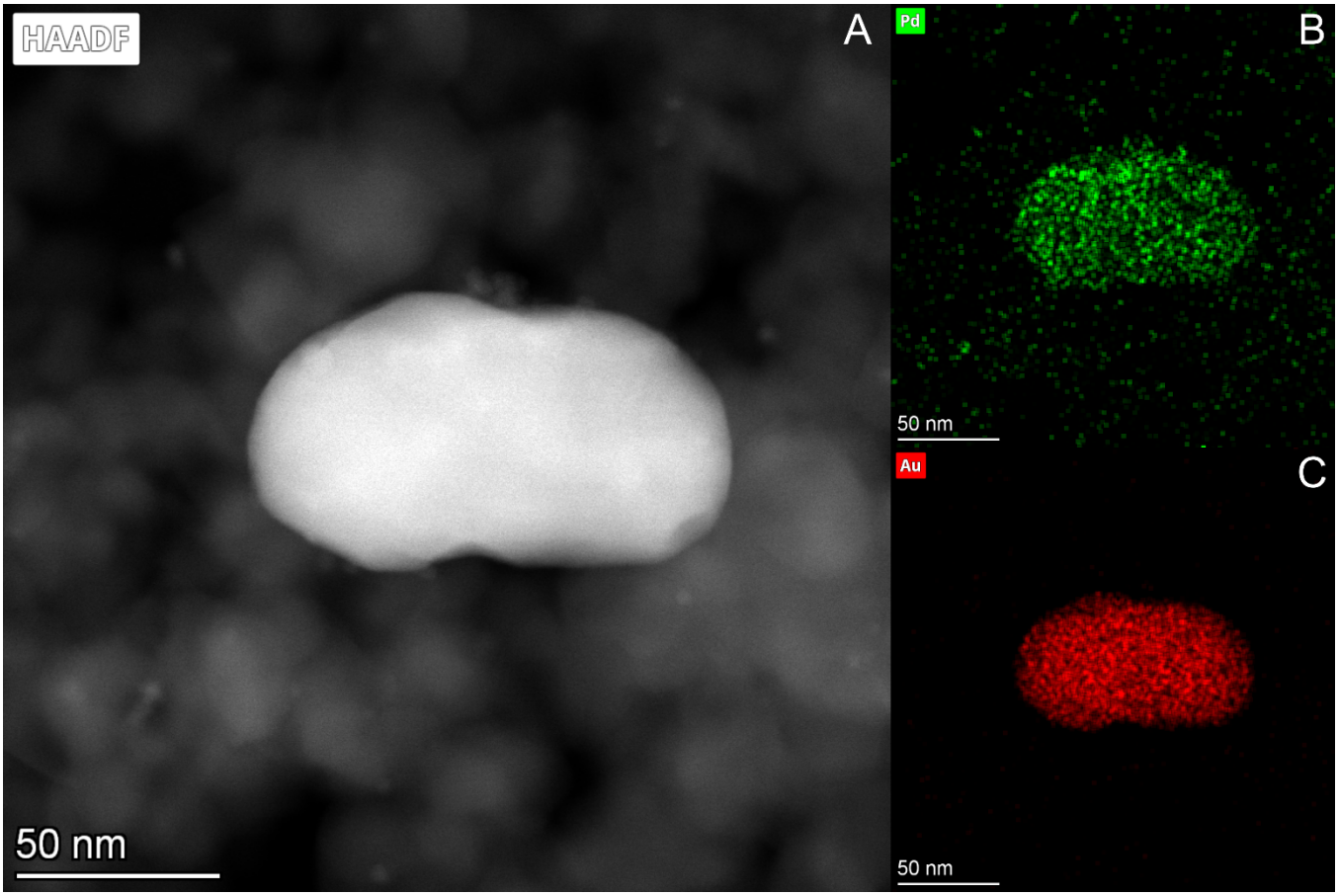



**Figure S18.** Catalyst surface structure and morphology changes of the 0.5%Au-0.5%Pd/$TiO_2$ catalyst, used for 96-hour reaction, using (**A**) (High-angle annular dark-field scanning transmission electron microscopy and paired (**B,C**) X-EDS imaging showing large (>50 nm, alloyed, Au and Pd containing) nanoparticles.

# Supplementary Tables

**Table S1.** Effect of varying the chemo-catalyst/CBP-21 cascade conditions on β-chitin degradation over a 2 h period.[a,b]

| AuPd[c] | $TiO_2$[c] | *sm*AA10A[d] | Ascorbate[e] | β-chitin[f] | $H_2$ | Air | $N_2$ | CBA[g] | TON[h] | $H_2O_2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | (μM) | | (μM) |
| + | - | + | + | + | + | + | - | 603 | 1206 | 0[i] |
| - | - | + | + | + | + | + | - | 114[j] | 228 | 0 |
| - | + | + | + | + | + | + | - | 109[j] | 218 | 0 |
| + | - | + | + | + | - | - | + | 98[j] | 196 | 0 |
| + | - | + | - | + | + | + | - | 0 | 0 | 300 |
| + | - | - | + | + | + | + | - | 0 | 0 | 250 |
| + | - | + | + | - | + | + | - | 0 | 0 | 530 |

**Footnotes:** (**a**) + and – denote the presence or absence of the reaction component, respectively; (**b**) All experiments were performed with stirring (250 rpm) in 100 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 6 (10 mL), under a $H_2$ (1.6 bar) and air (0.4 bar) gas mixture or $N_2$ (2.0 bar); (**c**) 1 mg of 0.5%Au-0.5%Pd/$TiO_2$ or $TiO_2$ added when present; (**d**) 0.5 mM when present; (**e**) 1 mM when present; (**f**) 10 mg when present; (**g**) Chitobionic acid (CBA) produced after 2 h as determined by HPLC; (**h**) TON calculated based on *sm*AA10A; (**i**) Amount of residual $H_2O_2$ after completion of the reaction. A zero value indicates that the amount was below the limit of detection (8.8 μM). (**j**) These residual activities arise from the aerobic activity of the enzyme using oxygen dissolved in the reaction buffer.

**Table S2.** Effect of catalyst, gas atmosphere and stirring rate on *sm*AA10A activity.[a,b,c]

| **0.5%Pd-0.5%Au/$TiO_2$** (mg) | **$TiO_2$** (mg) | **$H_2$** (bar) | **Air** (bar) | **$N_2$** (bar) | **Stirring Rate** (rpm) | **Activity Loss** (%) |
|---|---|---|---|---|---|---|
| | | | | | | |
| **0** | 0 | 0 | 1 | 0 | 0 | 7.9 ± 0.3 |
| **0** | 0 | 0 | 1 | 0 | 250 | 9.9 ± 0.2 |
| **0** | 1 | 0 | 1 | 0 | 250 | 11.1 ± 0.1 |
| **1** | 0 | 0 | 1 | 0 | 250 | 15.0 ±1.4 |
| **0** | 0 | 0 | 0 | 2 | 250 | 11.2 ± 0.3 |
| **0** | 0 | 0 | 2 | 0 | 250 | 11.2 ± 0.2 |
| **0** | 0 | 2 | 0 | 0 | 250 | 11.1 ± 0.3 |
| **0** | 0 | 1.6 | 0.4 | 0 | 250 | 11.7 ± 0.2 |
| **1** | 0 | 0 | 0 | 2 | 250 | 14.0 ± 0.05 |

**Footnotes:** (**a**) + and – denote the presence or absence of the reaction component, respectively; (**b**) All reaction mixtures contained *sm*AA10A (0.5 μM), dissolved in 50 mM $K_2HPO_4/KH_2PO_4$, pH 6 (10 mL). After incubation at 21 $^oC$ for 2 h, an aliquot (880 μL) of the resulting solution was mixed with 10 mM 2,6-DMP (100 μL) in a cuvette. After heating at 30°C for 10 min, 5 mM $H_2O_2$ (20 μL) was added and activity assayed by monitoring the adsorption at 469 nm over a period of 5 min; (**c**) Exposure to β-chitin gives no activity in the 2,6-DMP assay because *sm*AA10A binds tightly to β-chitin and the enzyme is therefore removed in the filtration step. In addition, this assay requires the use of well-defined amounts of $H_2O_2$, and so we were unable to use an $H_2$/air mixture in the presence of the 0.5%Au-0.5%Pd/$TiO_2$chemo-catalyst.

**Table S3.** Deactivation of *sm*AA10A when exposed to the 0.5%Au-0.5%Pd/$TiO_2$ chemo-catalyst.[a]

| **0.5%Pd-0.5%Au/$TiO_2$** (mg $mL^{-1}$) | **Exposure Time** (min) | **Activity Loss** (%) |
|---|---|---|
| 0.05 | 120 | 10 ± 1.4 |
| 0.1 | 5 | 11.6 ± 0.1 |
| 0.1 | 15 | 14 ± 1 |
| 0.1 | 30 | 13.5 ± 0.2 |
| 0.1 | 45 | 15 ± 2.1 |
| 0.1 | 60 | 16 ± 1 |
| 0.1 | 120 | 15 ± 1.4 |
| 0.1 | 240 | 15.8 ± 0.8 |
| 0.2 | 120 | 19 ± 1.2 |
| 0.3 | 120 | 25.2 ± 0.8 |

**Footnote:** (**a**) The 0.5%Au-0.5%Pd/$TiO_2$ catalyst was added to a solution of *sm*AA10A (0.5 μM), in 50 mM $K_2HPO_4/KH_2PO_4$, pH 6 (10 mL). After incubation at 21 °C for a given time, the catalyst was removed by filtration. An aliquot (880 μL) of the filtrate was mixed with 10 mM 2,6-DMP (100 μL) in a cuvette. After heating at 30°C for 10 min, 5 mM $H_2O_2$ (20 μL) was added and activity assayed by monitoring the adsorption at 469 nm over a period of 5 min.

**Table S4.** Effect of varying the gas mixture on β-chitin degradation by the chemo-catalyst/CBP-21 cascade over a 2 h period with stirring (250 rpm).[a,b]

| AuPd | *sm*AA10A[c] | Ascorbate[d] | β-chitin | $H_2$ | Air | $N_2$ | TON[e] | $H_2O_2$ / μM |
|---|---|---|---|---|---|---|---|---|
| + | - | - | + | - | + | + | 0 | 0[f] |
| + | - | - | + | + | - | + | 0 | 170 |
| + | - | - | + | - | + | - | 0 | 600 |
| + | + | + | + | - | + | + | 204[g] | 0 |
| + | + | + | + | + | - | + | 168[g] | 0 |

**Footnotes:** (**a**) + and – denote the presence or absence of the reaction component, respectively; (**b**) All experiments were performed with β-chitin (100 mg) and 0.5%Au-0.5%Pd/$TiO_2$ (1 mg) in 100 mM $K_2HPO_4$/$KH_2PO_4$ buffer, pH 6 (10 mL) and charged with gas ($H_2$ (1.6 bar)/air (0.4 bar), $H_2$ (1.6 bar)/$N_2$ (0.4 bar), or air(0.4 bar)/$N_2$(1.6 bar)); (**c**) 0.5 mM when present; (**d**) 1 mM when present; (**e**) TON calculated based on chitobionic acid (CBA) produced after 2 h, as determined by HPLC, and the *sm*AA10A concentration; (**f**) Amount of residual $H_2O_2$ after completion of the reaction. A zero value indicates that the amount was below the limit of detection (8.8 mM). (**g**) These residual activities arise from the aerobic activity of the enzyme using oxygen dissolved in the reaction buffer.

**Table S5.** Time-dependence of metal leaching in the absence of β-chitin, as determined by ICP-MS analysis of the post-reaction solution.[a]

| Reaction time (h) | Pd loss (%) | Au loss (%) |
|---|---|---|
| 0.083 | 0.02 | 0.00 |
| 0.5 | 0.02 | 0.00 |
| 2 | 0.03 | 0.01 |
| 96 | 0.02 | 0.01 |

**Footnote:** (**a**) Experiments used 0.5%Au-0.5%Pd/$TiO_2$ (10 mg), *sm*AA10A (0.5 μM), Ascorbic acid (1 mM) dissolved in 50 mM $K_2HPO_4$/$KH_2PO_4$ buffer, pH 6 (10 mL) under a $H_2$ (1.6 bar) and air (0.4 bar) gas mixture. with stirring (250 rpm) at 21 °C,

**Table S6.** Impact of adding metal precursors on *sm*AA10A-catalyzed β-chitin saccharification.[a,b]

| Metal Salt | CBA (μM) | Residual $H_2O_2$ (μM) |
|---|---|---|
| None | 109 ± 5 | 0[e] |
| [c]$PdCl_2$/ $HAuCl_4$ | 102 ± 6 | 0[e] |
| [d]NaCl | 120 ± 15 | 0[e] |

**Footnotes:** (**a**) All experiments used β-chitin (100 mg), *sm*AA10A (0.5 μM) and ascorbate (1 mM) dissolved in 50 mM $K_2HPO_4/KH_2PO_4$, pH 6 (10 mL total volume) under a $H_2$ (1.6 bar) and air (0.4 bar) gas mixture. (**b**) Reaction mixtures were stirred (250 rpm) at 21 °C for 2 h, and the concentrations of CBA and residual $H_2O_2$ determined as described elsewhere. Metal salts were added at the following concentrations: (**c**) 0.83 μM $PdCl_2$ and 0.17 μM $HAuCl_4$;(**d**) 2.33 μM NaCl. (**e**) Zero value indicates that the amount was below the limit of detection (8.8 μM). **Note:** concentrations of $PdCl_2$ and $HAuCl_4$ based on model leaching experiments conducted over 2 h, as outlined in **table S.5**. NaCl concentration utilised is equivelant to Cl present from metal precursors used in (**c**).

**Table S7.** Effect of reusing 0.5%Au-0.5%Pd/$TiO_2$ on *sm*AA10A-catalyzed β-chitin saccharification.[a]

| Number of Uses | CBA (μM) | TON |
|---|---|---|
| 1 | 600 ± 27 | 1206 |
| 2 | 505 ± 6 | 1008 |
| 3 | 460 ± 36 | 912 |

**Footnotes:** (**a**) Reaction mixtures contained 0.5%Au-0.5%Pd/$TiO_2$ (1 mg) mixed with β-chitin (100 mg) and 0.5 μM *sm*AA10A and 1 mM ascorbate dissolved in 50 mM $K_2HPO_4/KH_2PO_4$ buffer, pH 6 (10 mL total volume). After stirring (250 rpm) under an atmosphere of $H_2$ (1.6 bar) and compressed air (0.4 bar) at 21 $^oC$ for 2 h, the reaction was depressurized and filtered. CBA concentrations in the filtrate were determined as outlined above. The solid was then washed and dried before being re-used twice (Materials and Methods).

**Table S8.** Comparison of the chemoenzymatic depolymerization of chitin with enzymatic approaches reported in the literature.

| $H_2O_2$ Delivery Method | LPMO | Substrate | Conditions | Reaction time / h | TON | Saccharification / % | Reference |
|---|---|---|---|---|---|---|---|
| 0.5%Au-0.5%Pd/$TiO_2$ | smAA10A | β-chitin | LPMO (0.5 μM), substate (10 mg $mL^{-1}$), Ascorbic acid (1 mM), potassium phosphate buffer (50 mM, pH 6, 10 mL), compressed air (0.4 bar), $H_2$ (1.6 bar), 21°C, stirring (250 rpm). | 96 | 6372 | 36.0 | This work |
| AgChOx/ChCl | smAA10A | β-chitin | LPMO (1 μM), substate (10 mg $mL^{-1}$), Ascorbic acid (0.1 mM), ChCl (2.5 mM), AgChOx (100 nM), sodium phosphate buffer (50 mM, pH 7, 2 mL), 37 °C, shaking (850 rpm). | 25 | 950 | N.D | 7 |
| AgChOx/ChCl | smAA10A | α-chitin | LPMO (1 μM), substate (10 mg $mL^{-1}$), Ascorbic acid (0.1 mM), ChCl (1 mM), AgChOx (100 nM), sodium phosphate buffer (50 mM, pH 7, 2 mL), 37 °C, shaking (850 rpm). | 6 | 150 | N.D | 7 |
| two-electron reduction of $O_2$ | smAA10A | β-chitin | LPMO (1 μM), substate (10 mg $mL^{-1}$), Ascorbic acid (1 mM), sodium phosphate buffer (50 mM, pH 7, 2 mL), 37 °C, shaking (850 rpm). | 24 | 910 | N.D | 8 |
| two-electron reduction of $O_2$ | smAA10A | β-chitin | LPMO (1 μM), substate (10 mg $mL^{-1}$), Gallic acid (1 mM), Catalase (100 ug $mL^{-1}$), Tris-HCl (50 mM, pH 8, 2 mL), 37 °C, shaking (850 rpm). | 24 | 1050 | N.D | 8 |
| two-electron reduction of $O_2$ | smChiA (40 %) smChiB (30 %) smChiC (15 %) smAA10A (3 %) smCHB (12 %) | Alkaline and acid pre-treated shimp shell | LPMO cocktail (15 $mg_{cocktail}$ $g^{-1}_{substrate}$), substate (15 mg $mL^{-1}$), Bistris (10 mM, pH 6.2, 2 mL), 45 °C, shaking (800 rpm). | 50 | N.D | 74.8 | 9 |

**Table S9.** Comparison of the chemoenzymatic depolymerization of chitin with chemical approaches reported in the literature.

| Strategy | LMPO | Substrate | Conditions | Reaction time / h | TON | Saccharification / % | Reference |
|---|---|---|---|---|---|---|---|
| 0.5%Au-0.5%Pd/$TiO_2$ | smAA10A | β-chitin | LPMO (0.5 μM), substate (10 mg $mL^{-1}$), Ascorbic acid (1 mM), potassium phosphate buffer (50 mM, pH 6, 10 mL), compressed air (0.4 bar), $H_2$ (1.6 bar), 21°C, stirring (250 rpm). | 96 | 6372 | 36.0 | This work |
| Acid hydrolysis (Microwave assisted) | N.A | chitin | HCl (8 mol/L ); chitin: HCl at 1: 12 (w/v), 400 watts | 0.42 | N.A | 67.1 | 10 |
| Low- acid hydrolysis (Microwave assisted) | N.A | α-chitin | HCl (0.12 mol/L ), 160 °C for 25 min, then 150 °C for 20 min | 0.75 | N.A | 27.0 | 11 |
| Acid Hydrolysis (Co-solvent Promoted) | N.A | ball-mill treated chitin | 4/1 (v/v) mixture of diethylene glycol diethyl ether and water, $H_2SO_4$ (100 mM ), 175 °C | 1 | N.A | 80.0 | 12 |
| Acid hydrolysis (sonication-assisted) | N.A | chitin | Phosphoric acid (85 wt%) and 40 kHz, 130 W ultrasonic; 50 °C | 2 | N.A | 28.9 | 13 |
| Acid hydrolysis (using acidified molten salts) | N.A | chitin | 60% LiBr AMSH (containing 40 mmol/L HCl), 120 °C | 0.5 | N.A | 71.5 | 14 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Acid hydrolysis (using acidified molten salts) | N.A | chitin | chitin (100 mg), NS-0.1 (80 mg), and LiBr MSH (4 mL) at 130 °C for 120 min. | 2 | N.A | 63.0 | 15 |
| Acid hydrolysis | N.A | α-chitin + β-chitin | Conc Hydrochloric acid at 40 °C | 1 | N.A | 17.0 | 16 |

N.A: Not applicable. N.D: Not determined

## Supplementary References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