Development of enzyme cocktails for complete saccharification of chitin using mono-component enzymes from *Serratia marcescens*

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ABSTRACT

One potential strategy for biorefining of chitin-rich biomass entails enzymatic saccharification, which, so far, has been scarcely explored. Here, saccharification of chitin was explored using response surface methodology available in the MODDE®10 software, to develop optimal cocktails of five mono-component enzymes from *Serratia marcescens*, three chitinases, *Sm*ChiA, *Sm*ChiB, *Sm*ChiC, a lytic polysaccharide monooxygenase, *Sm*LPMO10A (or "CBP21"), and a beta-*N*-acetylhexosaminidase, *Sm*CHB ("chitobiase"). These five enzymes were recombinantly produced in *Escherichia coli*. For both shrimp and crab chitins, *Sm*ChiA was the most abundant (40% and 38%, respectively) in the optimized cocktails, whereas *Sm*ChiB, *Sm*ChiC and *Sm*LPMO10A were present at 30% and 26%, 15% and 23%, and 3% and 2%, respectively. Saccharification yields were 70% - 75%, whereas a "minimal" cocktail of *Sm*ChiA and *Sm*CHB gave only 40% saccharification. These results show that enzymatic saccharification of chitin requires multiple enzyme activities applied at dosages similar to those used for saccharification of cellulose.

Keywords: Response surface methodology; *Serratia marcescens*; chitinases; chitin; saccharification

INTRODUCTION

The fishing industry disposes large amounts of biomass containing chitin, an insoluble polysaccharide composed of linear chains of β(1->4) linked *N*-acetylglucosamine (GlcNAc). In Nature, chitin is synthesized by organisms such as crustaceans, insects, yeasts and fungi [1]. The main commercial sources of chitin are derived from shells of marine crustaceans such as crabs and shrimps [2]. Chitin is found in three allomorphs α, β and γ. α-chitin and β-chitin are composed of layers of polysaccharide chains organized in an anti-parallel and parallel fashion, respectively, while γ-chitin contains parallel polysaccharide chains interspersed with anti-parallel single chains [3].

In Nature, chitin is readily converted to chito-oligosaccharides and GlcNAc. Chitinolytic enzymes are produced by a wide range of organisms including bacteria [4, 5], fungi [6], mammals [7], plants [8] and insects [9] for different purposes. Hydrolysis of chitin involves synergistic attacks of multiple chitinolytic enzymes including endo-acting (cleavage within the polysaccharide chain) and exo-acting (cleavage from the polysaccharide chain ends) chitinases that occur in the glycoside hydrolase (GH) families 18 and 19 of the Carbohydrate Active enZymes database [4, 10]. The primary product of these chitinases is chitobiose, which is converted to GlcNAc by a family GH20 beta-*N*-acetylhexosaminidase (known as “chitobiase” or CHB) [11]. In addition, chitinolytic enzyme systems tend to include copper-enzymes named lytic polysaccharide monooxygenases or LPMOs. These enzymes, occurring in CAZy families AA10 and AA11, use molecular oxygen and an external electron donor to cleave glycosidic bonds and are capable of acting on crystalline material. Thus, LPMOs

disrupt the crystalline surface of chitin, hence providing chitinases with better access to the substrate and boosting chitinase efficiency [4, 12, 13]. Organisms containing such chitinolytic machineries perform efficient depolymerization of chitin, one example being the Gram-negative soil bacterium *Serratia marcescens* [4].

Serratia marcescens is a well-known chitin-degrading bacterium. When grown on chitin, this bacterium produces a chito-oligosaccharide-attacking *N*-acetylhexosaminidase (Chitobiase; *Sm*CHB), two exo-processive chitinases known as *Sm*ChiA and *Sm*ChiB that cleave β-1,4 glycosidic bonds from the reducing and non-reducing ends, respectively, a non-processive endo-chitinase (*Sm*ChiC) and an LPMO (*Sm*LPMO10A; also known as “CBP21”) [4]. The genome of *S. marcescens* encodes one more GH18 enzyme (ChiD; Mekasha and Eijsink, unpublished observations; [14]), but the role of this enzyme in chitin conversion remains uncertain and it is not a prominent part of the secretome during growth on chitin [15].

Marine chitin-rich biomass is complex and co-polymeric, and direct enzymatic conversion of the chitin is challenging due to the association of the polysaccharide with other compounds such as structural proteins and minerals. Thus, pretreatment technologies have been established to obtain the chitin in a more pure form, which then is amenable to further processing [2]. Even when relatively pure, the crystalline nature of chitin limits the efficiency of enzymatic depolymerization, posing similar challenges as those met in the enzymatic conversion of cellulose, which, recently, has received massive attention [16-18]. The crystallinity and the lack of accessibility of the substrate

slow down the enzymatic degradation process and increase both the quantity and the cost of the enzymes required for complete saccharification. The chemical and physical composition of the chitin and, hence, its degradability, vary depending on the biomass source and the pretreatment method. Several studies have addressed the impact of chitin-pretreatment methods on enzymatic hydrolysis, as well as the impact of such methods on the efficiency of individual chitin attacking enzymes [19, 20]. However, so far, little research has been done on developing enzyme cocktails for complete saccharification of pretreated chitinous biomass.

In the current study, five mono-component chitin-specific enzymes from *Serratia marcescens*, *Sm*ChiA, *Sm*ChiB, *Sm*ChiC, *Sm*LPMO10A and *Sm*CHB, each of them produced recombinantly in *E. coli*, were used for designing optimal enzyme mixtures for efficient and complete saccharification of two chemically pretreated α-chitins. We used two different industrial chitin substrates to assess the versatility of our approach and to get an impression of the substrate-dependency of enzyme efficiency: one named Chitinor, obtained from Norwegian shrimp (*Pandalus borealis*), and one named PTS, obtained from Atlantic blue crab (*Callinectes sapidus*). The performance of a two-component cocktail comprising only *Sm*ChiA and *Sm*CHB was also investigated. The optimization processes were performed by generating cubic models, each containing 41 experiments, using the MODDE® 10 software. While response surface methodology and similar methods have previously been used to design enzyme cocktails for saccharification of lignocellulosic biomass [21-23], to our knowledge, the enzyme blends

developed in the present study are the first designed enzyme cocktails for saccharification of chitin.

METHODS

Substrates

Alkaline and acid pretreated commercial chitin from shrimp (*Pandalus borealis*) shell, named Chitinor, was purchased from Chitinor AS (Senjahopen, Norway). Atlantic blue crab shells (PTS) were obtained from PT Biotech Surindo (Cirebon, Indonesia).

Demineralization of PTS was performed using a 10:1 (v/w) ratio of 1M hydrochloric acid (HCl) and incubation at room temperature for 2 h. After incubation, the supernatant was decanted and the demineralized shell wastes were washed to pH 7.0 initially using tap water, then de-ionized water. The demineralized shells were dried overnight at 55 ^{o}C. For deproteinisation, the dried demineralized shells were mixed with 1 M sodium hydroxide (NaOH) to obtain a 1:10 (w/v) ratio and subsequently placed in a preheated oven at 65 ^{o}C for 2 h, with shaking every 15 min. After this incubation, the chitin was recovered by decanting the supernatant and washing to pH 7, initially with tab water, then de-ionized water [24, 25]. The PTS chitin was dried overnight at 55 °C and stored until further use.

Both chemically pretreated chitins were size reduced to ~200 µm by milling using a Retsch® PM100 planetary ball mill with zirconium oxide vessels (500 ml) containing

zirconium oxide balls (10 x 10 mm) operated at 450 rpm. The milling conditions were set to reach a 200 µm particle size, according to the manufacturer's instructions. The milling period ranged from 15 to 30 min, with 5 min milling periods being interrupted by 2 min pauses, to avoid excess heating. To prevent microbial contamination in subsequent experiments, the milled chitins were autoclaved at 121 °C for 15 min and stored in sterile falcon tubes until compositional analysis and further use. The moisture contents of Chitinor and PTS were measured using a Karl-Fischer titrator (Mettler Toledo V20; Columbus, Ohio, USA). The ash contents were determined gravimetrically by burning the samples (~0.6 g) for 60 min in a muffle furnace pre-heated to 550 °C. Prior to weighing the ash, the samples were left for cooling in a desiccator [26].

Enzymes

Mono-component enzymes were produced in *E. coli* by overexpression of the following genes from *Serratia marcescens* BJL200: *chia* (Genebank ID: Z36294; [27]), *chib* (Z36295; [28]), *chic* (AJ630582; [29]), *cbp21* (AY665558; [30]) and *chb* (L43594; [31]). *Chia*, *chib* and *chic* were re-cloned in pET28b between *Nco*I and *Xho*I restriction sites using their Genebank database deposited sequence which includes the native signal peptide (applicable to *chia* only). For *cbp21* (with signal peptide and no tag) and *chb* (containing an N-terminal hexa-histidine tag), we used expression plasmids that had previously been generated in our laboratory, as described by Vaaje-Kolstad *et al* and Loose *et al* respectively [30, 31]. All constructs harboring the target genes were transformed to *E. coli* BL21 Star™ (DE3) cells (Invitrogen™, Carlsbad, CA, USA). LB medium (5.5 L), supplemented with 100 µg/ml of either ampicillin (for pRSETB/*cbp21*)

or kanamycin (for pET28b/*chia*, pET28b/*chib*, pET28b/*chic* and pET-30Xa/LIC-*chb*) was inoculated with 8 mL of overnight culture of *E. coli* BL21 Star™ (DE3) cells harboring the appropriate expression plasmid. Cells harbouring pET28b/*chia*, pET28b/*chib*, pET28b/*chic* were cultured at 37 °C, 210 rpm until the OD_{600} reached 0.4. Then, the temperature was reduced to 21 °C and the cells were further grown until OD_{600} reached 0.6. At this point gene expression was induced by IPTG to a final concentration of 0.2 mM and the cells were grown over night before harvesting. Cells harboring pRSETB/*cbp21* were incubated at 37 °C (210 rpm) for 16 h and the protein was expressed without induction [30]. Cells harboring pET-30Xa/LIC-*chb* were grown at 37 °C (210 rpm) until the OD_{600} reached 0.4, after which the temperature was reduced to 30 °C. When the OD_{600} reached 0.6, gene expression was induced by adding IPTG to a final concentration of 0.1 mM followed by incubation for another 3 h before the cells were harvested.

Cells were harvested by centrifugation. *Sm*ChiA and *Sm*LPMO10A were extracted using a periplasmic extraction method following the protocol described by Brurberg *et al* [28]. *Sm*ChiB, *Sm*ChiC and *Sm*CHB, were extracted from the cytoplasmic space by sonicating (57 Watt, amplitude of 5, 30s ON/1 min OFF) for six min using a Misonix Sonicator 3000 (Misonix Inc., Farmingdale, NY, USA) after re-suspending the cell pellet from 5.5 l culture in 250 ml of 20 mM Tris-HCl pH 8.0 containing 0.1 mg/ml lysozyme.

Crude extracts containing *Sm*ChiA, *Sm*ChiB, *Sm*ChiC or *Sm*LPMO10A were dialyzed against 40 mM ethanolamine (PENTA, Prague, Czech republic), pH 9.5 (= the loading

buffer for purification) before loading 250 ml onto a 5 ml Q-Sepharose column (GE Healthcare, Uppsala, Sweden) equilibrated with the same buffer. *Sm*ChiA and *Sm*ChiB were eluted in step-wise manner by washing the column with 4 column volumes of 40 mM ethanolamine, 50 mM NaCl, pH 9.4, and subsequently changing the elution buffer to 40 mM ethanolamine, 100 mM NaCl, pH 9.4. *Sm*ChiC and *Sm*LPMO10A were eluted by applying 4 column volumes of 40 mM ethanolamine, 50 mM NaCl, pH 9.4. The extract containing *Sm*CHB was dialyzed against 100 mM Tris, 20 mM imidazole, pH 8.0, and loaded onto a home-made column of Ni- Sepharose High Performance (GE Healthcare , Uppsala, Sweden) in a (1.5 x 12 cm) Econo-Pac column (Bio-Rad, Munich, Germany), equilibrated with 100 mM Tris, 20 mM imidazole, pH 8.0 (loading buffer). *Sm*CHB was eluted with 100 mM Tris, 100 mM imidazole, pH 8.0. The purified enzymes were concentrated and their buffer was exchanged to 20 mM Tris, pH 8.0) using Amicon Ultra centrifuge filters with 10 kDa cutoff (Millipore, Cork, Ireland). Enzyme solutions were sterilized by filtration using a 0.2 µm sterile filter and stored at 4 °C until further use.

Chitin hydrolysis

The hydrolysis experiments were conducted in 2.0 ml sample tubes containing 15 mg/ml Dry Matter (DM) in 10 mM BisTris buffer, pH 6.2, at 45 °C. The hydrolysis was initiated by adding chitinolytic enzymes (total enzyme dosage: 15 mg enzyme/g DM) to sample tubes containing the pre-heated chitin-buffer mixture and reactions were subsequently incubated at 45 °C in a pre-heated Eppendorf Comfort Thermomixer with a ThermoTop, with shaking at 800 rpm, for 24 h. To ensure activation of *Sm*LPMO10A,

ascorbic acid was added in all reactions to a final concentration of 1mM. After 24 h, 20 µl of reaction mixture was transferred to a 2 ml sample vial containing 20 µl 50 mM sulfuric acid, followed by incubation at room temperature for 10 min. After diluting 10-fold with Milli-Q water the samples were filtered using 96-well filter plates (Millipore, Cork, Ireland) operated with a Millipore vacuum manifold, to separate the un-degraded chitin from the soluble products. Hydrolysis products [GlcNAc to $(GlcNAc)_6$] were analyzed using a Dionex Ultimate 3000 UHPLC system (Dionex Corp., Sunnyvale, CA, USA), equipped with a Rezex RFQ-Fast acid H^+ (8%) 7.8 x 100 mm column (Phenomenex, Torrance, CA, USA) pre-heated to 85 °C, using 5 mM H_2SO_4 as mobile phase and a flow rate of 1 ml/min. Soluble products were separated isocratically and detected using UV absorption at 194 nm. The amount of GlcNAc was quantified using GlcNAc (Sigma, St. Louis, MO, USA) standards, which were regularly analyzed.

Experimental design, statistical analysis and verification of optimal enzyme cocktails

Optimization of a cocktail containing the five chitinolytic enzymes from *Serratia marcescens* for efficient and complete depolymerization of α-chitin to GlcNAc was carried out using the MODDE® 10 software (Umetrics, Umeå, Sweden) using a full cubic model and D-optimal design [32]. The experimental design comprised 41 hydrolysis experiments in total, with wide abundance ranges for the individual enzymes as summarized in Table 1 (see Tables S1 and S2 for details). The enzyme mixtures were dosed on the basis of protein weight fractions, with a fixed total enzyme dosage of 15 mg enzyme/g DM. To ensure production of monomeric GlcNAc, the minimum level of

231 *Sm*CHB was 10% (maximum 90%), whereas *Sm*ChiA, *Sm*ChiB, *Sm*ChiC and
232 *Sm*LPMO10A were ranged from 0 to 90%. For investigating experimental error and
233 reproducibility, three center points (= three identical runs) were included in the
234 experimental design (see Tables S1 and S2). In all enzyme reactions, the conditions
235 were as described above, under “Chitin hydrolysis”. Data analysis and model
236 evaluation were carried out using Partial Least Squares (PLS) regression. The model
237 was experimentally verified by running reactions with the predicted optimal cocktails
238 using the same experimental conditions as described above. The experimentally
239 obtained yields were then compared with the predicted yields.
240

241 **Solubilization using only *Sm*ChiA and *Sm*CHB**

242 Solubilization of Chitinor by a cocktail containing only *Sm*ChiA and *Sm*CHB was also
243 evaluated. To investigate the enzyme proportion that produces the maximum amount of
244 GlcNAc, the amount of *Sm*ChiA was ranged from 0-80% (0-12 mg) while keeping a
245 constant amount of *Sm*CHB (3 mg, or 20%). For reactions containing less than 12 mg of
246 *Sm*ChiA, the reduced protein amount was compensated by adding BSA (New England
247 BioLabs, Ipswich, MA, USA). The reactions were sampled at 1, 2, 6, 24 and 48 h, and
248 released GlcNAc was quantified by HPLC as described above.
249

250 **RESULTS AND DISCUSSION**
251

252 **Chitin pretreatment and compositional analyses**

253 Chitin was extracted from Norwegian shrimp (*Pandalus borealis*) shells and Atlantic
254 blue crab (*Callinectes sapidus*) shells by chemical pretreatment with acid, for
255 demineralization, and alkali, for deproteination. The resulting chitins were size reduced
256 to ~200 µm by milling using a Retsch® PM100 planetary ball mill. After the pretreatment
257 processes, the possible release of soluble sugars was investigated by HPLC, but no
258 soluble sugar [i.e. GlcNAc - $(GlcNAc)_6$] was detected.
259
260 Measurements of moisture and ash contents (Table 2) showed similar results for both
261 substrates. The moisture contents of Chitinor and PTS were 5.42 ± 0.13% and 6.02 ±
262 0.04% (w/w), respectively, whereas the ash contents (w/w) were 1.01 ± 0.06% and 1.05
263 ± 0.13%, respectively. The chitin contents (w/w) for Chitinor and PTS were calculated to
264 be 93.57±0.14% and 92.93±0.14%, respectively.
265
266 **Optimization of enzyme mixtures**
267 Enzyme cocktails comprised of the mono-component enzymes *Sm*ChiA, *Sm*ChiB,
268 *Sm*ChiC, *Sm*LPMO10A and *Sm*CHB (Fig. 1) were optimized for maximum
269 saccharification of Chitinor and PTS by response surface methodology using MODDE®
270 10 software. The optimization process involved 41 independent experimental runs for
271 each substrate (Table 1; Tables S1 & S2). The total enzyme dosage was held constant
272 at 15 mg/g DM. Model development was based on a PLS method where the numbers of
273 significant PLS components were automatically computed by MODDE by cross
274 validation. In order to avoid parameter over-fitness and computational complexity, two-
275 step manual hierarchical model reduction was performed on the “full models”, which

276 contained 45 parameters. Accordingly, the “full models” were first reduced to 16
277 parameter containing models (named “reduced model”) and further reduced to 12
278 parameter containing models (named “further reduced model”). A full overview is
279 provided in Table S3 and key results are discussed below.
280
281 For Chitinor, the “full model” predicted an optimized enzyme mixture containing 40%
282 *Sm*ChiA, 30% *Sm*ChiB, 15% *Sm*ChiC, 3.0% *Sm*LPMO10A and 12% *Sm*CHB with an
283 average predicted theoretical yield of 55% and average lower and upper theoretical
284 yield limits of 48% and 62% respectively. The “reduced model” predicted an optimized
285 enzyme mixture for Chitinor containing 40% *Sm*ChiA, 30% *Sm*ChiB, 15% *Sm*ChiC,
286 3.0% *Sm*LPMO10A and 12% *Sm*CHB with an average predicted theoretical yield of
287 71% and average lower and upper theoretical yield limits of 68% and 75% respectively.
288 Further reduction led to better model reliability index (Q^2 = 0.66 compared to 0.46 for
289 the “reduced model”) with similar R^2 (= Model fit; 0.84 and 0.87 respectively; Table S3).
290 However, the prediction derived from the “further reduced model” was very similar to
291 that of the “reduced model”: 38% *Sm*ChiA, 28% *Sm*ChiB, 17% *Sm*ChiC, 5.0%
292 *Sm*LPMO10A and 12% *Sm*CHB, with an average predicted theoretical yield of 71% and
293 average lower and upper theoretical yield limits of 68% and 74%, respectively.
294
295 For PTS, the “full model” predicted an optimized enzyme mixture containing 42%
296 *Sm*ChiA, 27% *Sm*ChiB, 21% *Sm*ChiC, 0% *Sm*LPMO10A and 10% *Sm*CHB with an
297 average predicted theoretical yield of 68% and average lower and upper theoretical
298 yield limits of 64% and 71%, respectively. The “reduced model” predicted an optimized

299 enzyme mixture containing 38% *Sm*ChiA, 23% *Sm*ChiB, 28% *Sm*ChiC, 0.3%
300 *Sm*LPMO10A and 10% *Sm*CHB with an average predicted theoretical yield of 76% and
301 average lower and upper theoretical yield limits of 74% and 77%, respectively. Only the
302 "further reduced" model yielded a Q^2 larger than 0.5 and predicted an enzyme mixture
303 containing 38% *Sm*ChiA, 26% *Sm*ChiB, 23% *Sm*ChiC, 2% *Sm*LPMO10A and 10%
304 *Sm*CHB, with an average predicted theoretical yield of 76% and average lower and
305 upper theoretical yield limits of 71% and 79%, respectively.
306
307 Figure 2 shows surface response plots and Table 3 shows the optimized cocktails for
308 the models that were primarily used in the rest of this study. Experimental validation of
309 the "reduced" model for Chitinor and the "further reduced" model for PTS gave yields of
310 74.8 ± 0.9% and 71.4 ± 2.2%, respectively, which is in good accordance with the model
311 predictions. Quality parameters for the experimentally validated models, R^2 & Q^2 (Table
312 3), showed acceptable values, although Q^2 for Chitinor was just below 0.5, which is
313 sometimes considered as a cut-off value. Experimental validation of the optimized
314 cocktail obtained from the "further reduced" model for Chitinor (Q^2 = 0.66) was also
315 performed, giving a yield of 72.4 ± 3.7% which is in good accordance with the model
316 prediction, but not significantly different from the value obtained for the "reduced model".
317
318 For all experimentally evaluated models, the "reproducibility value" calculated by
319 MODDE (i.e. comparison of the variation of the replicates with the total variation of the
320 data set) was greater than 98%, which indicates high reliability of the models.
321

For both Chitinor and PTS *Sm*ChiA seems to be the most important enzyme (40% for Chitinor and 38% for PTS). The fraction of *Sm*ChiB ranges from 26% (for PTS) to 30% (for Chitinor). *Sm*ChiC is required in higher amounts (23%) for PTS compared to Chitinor where 15% of *Sm*ChiC is needed. The dominance of *Sm*ChiA aligns with previous studies showing that this enzyme clearly is the most powerful of the three *Serratia* chitinases [19,33]. The variation in the fraction of *Sm*ChiC may relate to variation in substrate amorphousness, as discussed further below. *Sm*LPMO10A seems not to have a major impact on conversion of both Chitinor and PTS as only minor amounts (2 – 3%) are needed (Table 3). Notably, the fraction of *Sm*LPMO10A was somewhat higher (5%) in the optimized cocktail predicted by the "further reduced" model for Chitinor (Table S3). Additional experiments confirmed that, indeed the LPMO is of minor importance in the conversion processes studied here; its omission from the optimized cocktail reduced yields by less than 6% (Fig. S1).

The modest role of the LPMO may be explained by the substrate specificity of *Sm*LPMO10A. It has previously been reported that *Sm*LPMO10A attacks the crystalline regions of chitin [13]. Furthermore, while the enzyme is active on α-chitin [19], its preferred substrate seems to be β-chitin [13, 30]. Milling is known to reduce substrate crystallinity [19] and it is thus likely that the milled alpha-chitins used in the present study are not good substrates for the LPMO.

Dose response and progress curves of optimum enzyme mixtures

In subsequent experiments, dose-response curves for the optimized enzyme cocktails were determined by studying hydrolysis at four total enzyme dosages (2, 5, 10 and 15 mg enzyme/g DM). The results, depicted in Fig. 3, show clear dose-response effects and also reveal differences between the Chitinor and PTS substrates. Saccharification of PTS (Fig. 3B) was achieved faster and at lower enzyme dosage compared to Chitinor (Fig. 3A).

Fig. 3 clearly shows that the enzyme dosage needed for achieving maximum degradation of a specific chitin depends on the type of substrate. For PTS, almost maximum saccharification could be reached after 24 hours using a reduced (5 mg/g) enzyme loading, whereas this clearly is not the case for Chitinor. Interestingly, compared to Chitinor, the optimum enzyme mix for PTS contained more *Sm*ChiC, which is thought to act on more easily degradable amorphous regions, and less *Sm*LPMO10A, which is thought to act on crystalline regions. It may thus seem that the chemically pretreated crab chitin in PTS has reduced crystallinity compared to Chitinor. Indeed Nakagawa *et al* have previously reported that *Sm*ChiC activity benefits more from a low degree of chitin crystallinity than *Sm*ChiA and *Sm*ChiB [19].

Solubilization of Chitinor using *Sm*ChiA and *Sm*CHB

From the results described above one may conclude that *Sm*ChiA is the most important enzyme for solubilizing α-chitin. Indeed, in another study on hydrolysis of milled crab-derived chitin flakes, *Sm*ChiA was concluded to be the most powerful of the *Serratia marcescens* chitinases [19]. To further explore the potential of *Sm*ChiA, enzyme

cocktails containing varying amounts (0 – 12 mg/g) of *Sm*ChiA and a constant amount (3 mg/g) of *Sm*CHB were tested for their potential in saccharification of Chitinor. The presence of a constant amount of *Sm*CHB ensured that all products generated by *Sm*ChiA were converted to GlcNAc. Hence, no chitobiose (the major product of *Sm*ChiA) or longer chito-oligomers were detected at all reaction time points.

Fig. 4 shows that the combination of *Sm*ChiA and *Sm*CHB performed worse than the optimized cocktail. Combining 6 mg/g DM of *Sm*ChiA with 3 mg/g DM *Sm*CHB yielded only 34% solubilization after 24 hours, as compared to 75% for the optimized cocktail. Extending the incubation period by an additional 24h hardly promoted further cleavage, the yield increase at all *Sm*ChiA dosages being in the order of 5%. Increasing the amount of *Sm*ChiA to 12 mg, meaning a total enzyme loading of 15 mg/g DM, increased the yield after 24 hours to 40%, i.e. still much lower than the yield obtained with the optimized cocktail.

*Sm*ChiA is the dominant enzyme in the optimized enzyme cocktails for both substrates. Yet, the hydrolysis of Chitinor by minimal cocktails containing only *Sm*ChiA and *Sm*CHB reveals the importance of synergistic actions with the other chitinolytic enzymes for hydrolysis of chitin. One of the other important enzymes is *Sm*ChiB which needs to be present in a 26-30% range. It is interesting to note that the chitobiohydrolases *Sm*ChiA and *Sm*ChiB together make up almost 70% of the optimized cocktails. *Trichoderma* based commercial cellulase cocktails are thought to contain up to 60% of a Cel7A type enzyme [34], the functional analogue of *Sm*Chi18A (i.e. a reducing-end specific

processive exo-acting enzyme) and up to 25% of a Cel6A type enzyme, the functional analogue of *Sm*ChiB.

Conclusions

This study provides the first example of successful development of enzyme cocktails that allow saccharification of chitin with reasonable yields and using enzyme dosages similar to those used in lignocellulose processing. While the present study represents a major step forward in saccharification of chitin, it also shows that enzymatic saccharification of alpha-chitin is at least as challenging as saccharification of cellulose. Further improvement of the enzyme cocktails may be possible, e.g. by including other hydrolases or LPMOs with higher activity on α-chitin (e.g. [35]). Alternative pretreatment methods may need to be developed. Combined further optimization of pretreatment methods and enzyme cocktails may yield efficient chitin hydrolysis processes, alike what has been achieved for lignocellulosic biomass in the past decade.

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513

Figure Legends

Fig. 1. Purified mono-component enzymes from *Serratia marcescens*. Lane 1, Marker proteins; lane 2, *Sm*CHB; lane 3, *Sm*ChiA, lane 4, *Sm*ChiB; lane 5, *Sm*ChiC and lane 6, *Sm*LPMO10A. The molecular masses of the marker proteins are indicated on both the left and right sides of the SDS-PAGE gel, in kDa.

Fig. 2. Response contour plots for the three GH18 chitinases for optimization of the conversion of Chitinor (A; “reduced” model) and PTS (B; “further reduced” model) to GlcNAc. The substrate concentration was 1.5% (w/v) and the total enzyme dosage was 15 mg/g substrate. Saccharification yields after 24 hours, expressed as percentage of the theoretical maximum, are shown in white boxes in the plots (in all reactions, GlcNAc was the only soluble product). The optimized fractions of *Sm*CHB and *Sm*LPMO10A are indicated below the legend bar.

Fig. 3. Dose response curves for the optimal enzyme cocktails developed for Chitinor (A, “reduced” model) and PTS (B, “further reduced” model). Yields were calculated as percentage of the theoretical maximum and the values presented are the average of three independent reactions containing 15 mg/ml chitin in 10 mM BisTris pH 6.2, incubated at 45 ^{o}C. At all time points, in all reactions, GlcNAc was the only detected soluble product. For Chitinor, similar experiments were carried out with the cocktail predicted by the “further reduced” model, with similar results (Fig. S2).

Fig. 4. Conversion of Chitinor with an enzyme cocktail containing 0-12 mg of *Sm*ChiA and 3 mg of *Sm*CHB per gram of substrate. GlcNAc, the only detected soluble sugar, was analyzed by HPLC and quantified. (A) Conversion by various cocktails at various time points. The red arrow indicates the yield after 24 hours obtained in the reaction with 6 mg (= 40% of 15 mg) *Sm*ChiA (i.e. the same amount of *Sm*ChiA as in the complete minimal cocktail). Panel B shows progress curves for selected reactions. All reactions contained 1.5% substrate (w/v) in 10 mM BisTris pH 6.2 and were incubated at 45 °C. In reaction mixtures containing less than 12 mg *Sm*ChiA, i.e. with an enzyme dosage lower than 15 mg/g, the reduced enzyme amount was compensated by adding bovine serum albumin (BSA). All results are expressed as the mean of three independent experiments ± SD.

560 **Tables**

561

562 **Table 1**. Constraints in the modelling procedure. The table shows the ranges for the
563 various enzyme components included in the modelling. The total enzyme amount was
564 set at 15 mg/g substrate.

Enzyme name	Abbreviation	Enzyme dosage ranges (%, w/w)
Serratia marcescens ChiA	*Sm*ChiA	0-90
Serratia marcescens ChiB	*Sm*ChiB	0-90
Serratia marcescens ChiC	*Sm*ChiC	0-90
Serratia marcescens LPMO10A ("CBP21")	*Sm*LPMO10A	0-90
Serratia marcescens CHB	*Sm*CHB	10-90

565

566 **Table 2.** Composition of Chitinor and PTS.

Composition	**Chitinor (%, w/w)**	**PTS (%, w/w)**
Chitin[a]	93.57 ± 0.14	92.93 ± 0.14
Moisture[b]	5.42 ± 0.13	6.02 ± 0.04
Ash[c]	1.01 ± 0.06	1.05 ± 0.13

567 [a] Calculated by subtracting ash + moisture content.

568 [b] Measured by Karl Fischer titration [26].

569 [c] Determined by burning the samples at 550 °C in a muffle furnace.

570 **Table 3.** Optimized enzyme mixtures for hydrolysis of chemically pre-treated chitin. Data for Chitinor refer to the
571 "reduced" model; see Table S3 for data for the "further reduced" model. Data for PTS refer to the "further reduced"
572 model. The lower and upper limit ranges were determined by MODDE based on the 95% confidence level.

Chitin	**Enzyme fractions (%; w/w)**					**Hydrolysis of *N*-acetylglucosamine, yield in % of theoretical maximum (at 24 h)**				**Model fit (R^2)**	**Model reliability (Q^2)**
						Model predicted			Experimental		
	*Sm*ChiA	*Sm*ChiB	*Sm*ChiC	*Sm* LPMO10A	*Sm*CHB	Yield (%)	Lower limit	Upper limit	Yield (%)		
Chitinor	40	30	15	3.0	12	71.2	67.8	74.6	74.8 ± 0.9	0.87	0.46
PTS	38	26	23	2.1	10	75.8	71.6	79.4	71.4 ± 2.2	0.86	0.61

573

Figures

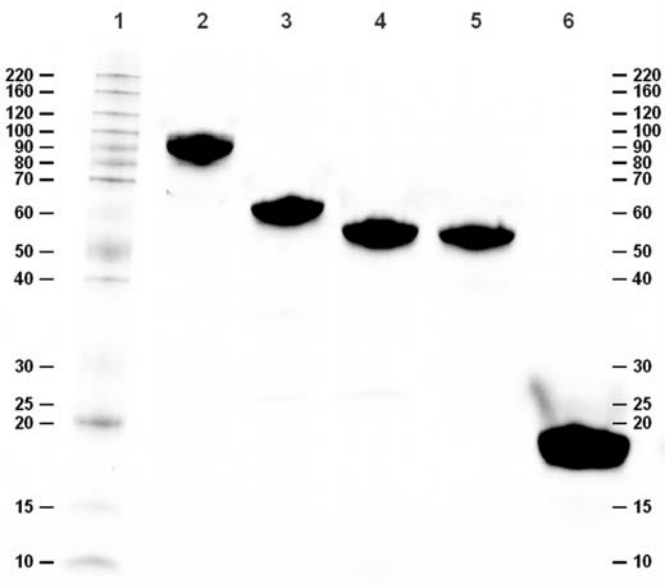


Figure 1

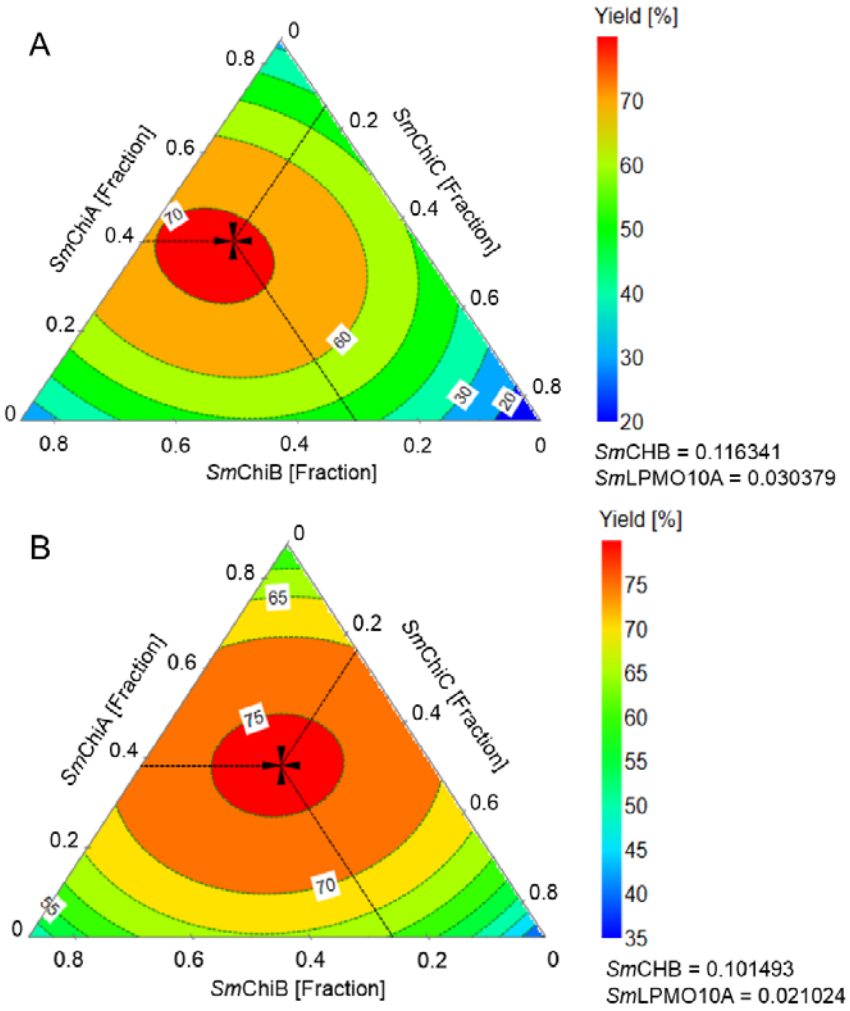
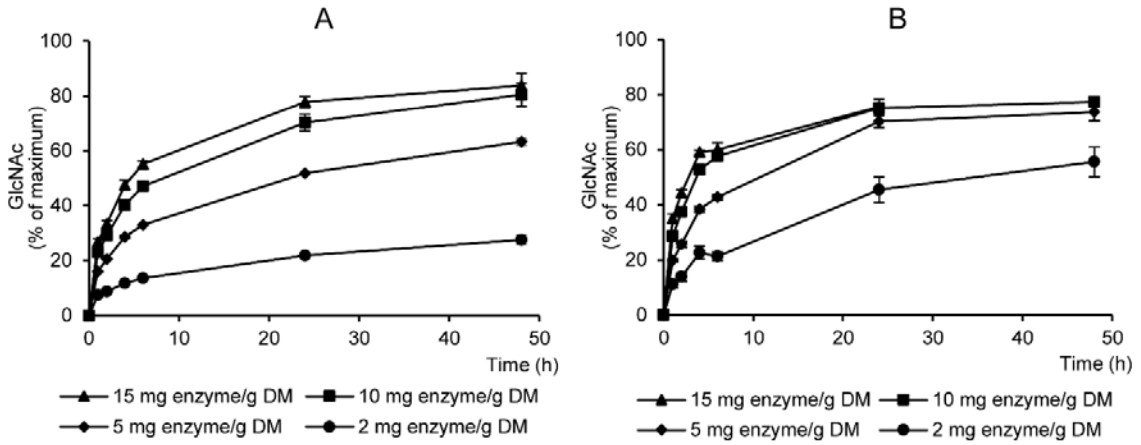

A
SmChiA [Fraction]
SmChiC [Fraction]
SmChiB [Fraction]
Yield [%]
70
60
50
40
30
20
SmCHB = 0.116341
SmLPMO10A = 0.030379
B
SmChiA [Fraction]
SmChiC [Fraction]
SmChiB [Fraction]
Yield [%]
75
70
65
60
55
50
45
40
35
SmCHB = 0.101493
SmLPMO10A = 0.021024

Figure 2



584

585 **Figure 3**

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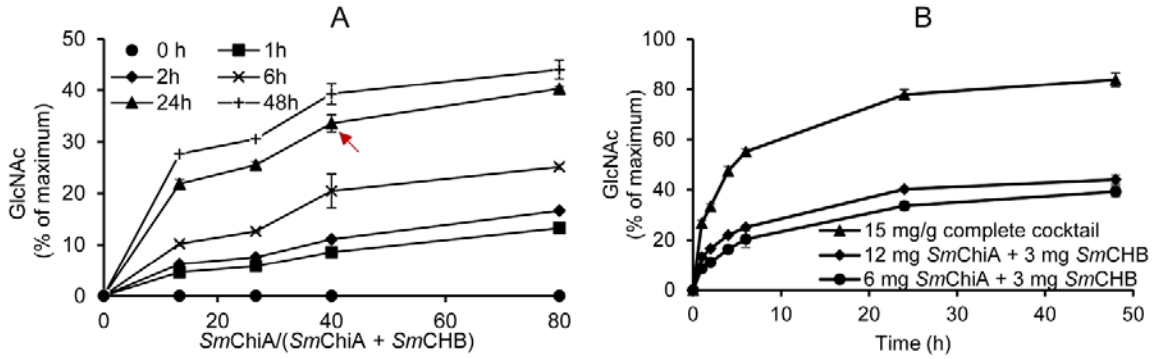

587

588 **Figure 4**

SUPPLEMENTARY MATERIAL

Development of enzyme cocktails for complete saccharification of chitin using mono-component enzymes from *Serratia marcescens*

Sophanit Mekasha[1], Ida Roksvåg Byman[1], Catherine Lynch[2,5], Hana Toupalová[3], Ladislav Anděra[3], Tormod Næs[4], Gustav Vaaje-Kolstad[1] and Vincent G.H. Eijsink[1]*

Table S1. Experimental setup for optimizing the enzyme cocktail for Chitinor. The Table shows the fractions of enzyme (w/w) used and the yield of the reaction, expressed as percentage of the theoretical maximum (= all chitin converted to GlcNAc). The total amount of enzyme added was 15 mg/g substrate. The Centre points are experiments 39 – 41.

Exp No	*Sm*ChiA	*Sm*ChiB	*Sm*ChiC	*Sm*CHB	*Sm*LPMO10A	Yield
1	0.9	0	0	0.1	0	28.703
2	0	0.9	0	0.1	0	26.043
3	0	0	0.9	0.1	0	12.024
4	0	0	0	0.1	0.9	5.249
5	0.1	0	0	0.9	0	18.725
6	0	0.1	0	0.9	0	12.953
7	0	0	0.1	0.9	0	10.297
8	0	0	0	0.9	0.1	4.561
9	0	0	0	0.367	0.633	5.443
10	0	0	0	0.633	0.367	5.188
11	0	0	0.633	0.367	0	18.310
12	0	0	0.367	0.633	0	13.467
13	0	0	0.6	0.1	0.3	15.313
14	0	0	0.3	0.1	0.6	16.163
15	0	0.633	0	0.367	0	21.793
16	0	0.367	0	0.633	0	22.199
17	0	0.6	0	0.1	0.3	27.838
18	0	0.3	0	0.1	0.6	18.570
19	0	0.6	0.3	0.1	0	46.107
20	0	0.3	0.6	0.1	0	40.515
21	0.633	0	0	0.367	0	36.132
22	0.367	0	0	0.633	0	31.475
23	0.6	0	0	0.1	0.3	37.729
24	0.3	0	0	0.1	0.6	30.405
25	0.6	0	0.3	0.1	0	45.102
26	0.3	0	0.6	0.1	0	42.707
27	0.6	0.3	0	0.1	0	68.145
28	0.3	0.6	0	0.1	0	51.341
29	0	0	0.25	0.5	0.25	26.860
30	0	0.25	0	0.5	0.25	38.794
31	0	0.25	0.25	0.5	0	34.295
32	0	0.3	0.3	0.1	0.3	48.790
33	0.25	0	0	0.5	0.25	32.242
34	0.25	0	0.25	0.5	0	46.129
35	0.3	0	0.3	0.1	0.3	41.424
36	0.25	0.25	0	0.5	0	63.176
37	0.3	0.3	0	0.1	0.3	68.439
38	0.3	0.3	0.3	0.1	0	73.349
39	0.125	0.125	0.125	0.5	0.125	55.296
40	0.125	0.125	0.125	0.5	0.125	61.594
41	0.125	0.125	0.125	0.5	0.125	60.788

Table S2. Experimental setup for optimizing the enzyme cocktail for PTS. The Table shows the fractions of enzyme (w/w) used and the yield of the reaction, expressed as percentage of the theoretical maximum (= all chitin converted to GlcNAc). The total amount of enzyme added was 15 mg/g substrate. The Centre points are experiments 39 – 41.

Exp No	*Sm*ChiA	*Sm*ChiB	*Sm*ChiC	*Sm*CHB	*Sm*LPMO10A	Yield
1	0.9	0	0	0.1	0	56.127
2	0	0.9	0	0.1	0	53.658
3	0	0	0.9	0.1	0	37.13
4	0	0	0	0.1	0.9	9.715
5	0.1	0	0	0.9	0	34.439
6	0	0.1	0	0.9	0	26.069
7	0	0	0.1	0.9	0	18.669
8	0	0	0	0.9	0.1	5.3447
9	0	0	0	0.367	0.633	8.252
10	0	0	0	0.633	0.367	6.780
11	0	0	0.633	0.367	0	29.656
12	0	0	0.367	0.633	0	27.599
13	0	0	0.6	0.1	0.3	42.220
14	0	0	0.3	0.1	0.6	39.944
15	0	0.633	0	0.367	0	50.680
16	0	0.367	0	0.633	0	43.690
17	0	0.6	0	0.1	0.3	54.963
18	0	0.3	0	0.1	0.6	45.203
19	0	0.6	0.3	0.1	0	58.750
20	0	0.3	0.6	0.1	0	60.056
21	0.633	0	0	0.367	0	57.359
22	0.367	0	0	0.633	0	52.367
23	0.6	0	0	0.1	0.3	64.942
24	0.3	0	0	0.1	0.6	57.578
25	0.6	0	0.3	0.1	0	70.353
26	0.3	0	0.6	0.1	0	68.546
27	0.6	0.3	0	0.1	0	75.417
28	0.3	0.6	0	0.1	0	62.586
29	0	0	0.25	0.5	0.25	40.797
30	0	0.25	0	0.5	0.25	44.144
31	0	0.25	0.25	0.5	0	56.144
32	0	0.3	0.3	0.1	0.3	61.061
33	0.25	0	0	0.5	0.25	57.074
34	0.25	0	0.25	0.5	0	66.071
35	0.3	0	0.3	0.1	0.3	66.868
36	0.25	0.25	0	0.5	0	69.606
37	0.3	0.3	0	0.1	0.3	71.903
38	0.3	0.3	0.3	0.1	0	74.414
39	0.125	0.125	0.125	0.5	0.125	72.627
40	0.125	0.125	0.125	0.5	0.125	71.000
41	0.125	0.125	0.125	0.5	0.125	72.901

Table S3. Overview of the three types of models built by MODDE software. Abbreviations: NA, not available; R^2, statistical parameter indicating the model fit; Q^2, statistical parameter indicating the models' further prediction precision.

Model type	*Sm*ChiA (%)	*Sm*ChiB (%)	*Sm*ChiC (%)	*Sm*LPMO10A (%)	*Sm*CHB (%)	Predicted yield (%)	Lower limit	Upper limit	Experimental	R^2	Q^2
Chitinor full	40	30	15	3	12	54.8	48.0	61.5	NA	0.73	0.35
Chitinor reduced	40	30	15	3	12	71.2	67.8	74.5	74.8 ± 0.9	0.87	0.46
Chitinor further reduced	38	28	17	5	12	71.3	67.8	74.5	72.4 ± 3.7	0.84	0.66
PTS full	42	27	21	0	10	68.2	64.6	71.3	NA	0.71	0.38
PTS reduced	38	23	28	0.3	10	75.8	74.2	77.4	NA	0.84	0.45
PTS further reduced	38	26	23	2	10	75.8	71.6	79.4	71.4 ± 2.2	0.86	0.61

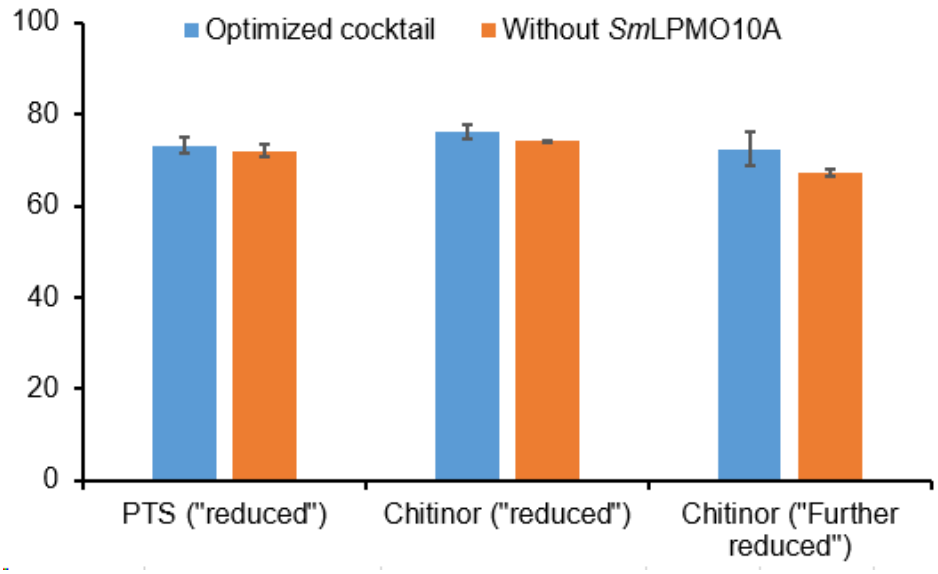

Fig. S1. Experimental validation of the PTS "reduced" and Chitinor "reduced" and "further reduced" models and the effect of *Sm*LPMO10A. The graph shows the yields from hydrolysis experiments carried out with the predicted optimized enzyme cocktails and these same optimized cocktails lacking *Sm*LPMO10A, where the LPMO was replaced with corresponding amounts of BSA. All reactions contained 15 mg enzyme/g DM and were incubated in 10 mM BisTris pH 6.2 containing 1 mM ascorbic acid at 45 °C. The values presented are the average of three independent reactions +/- SD.

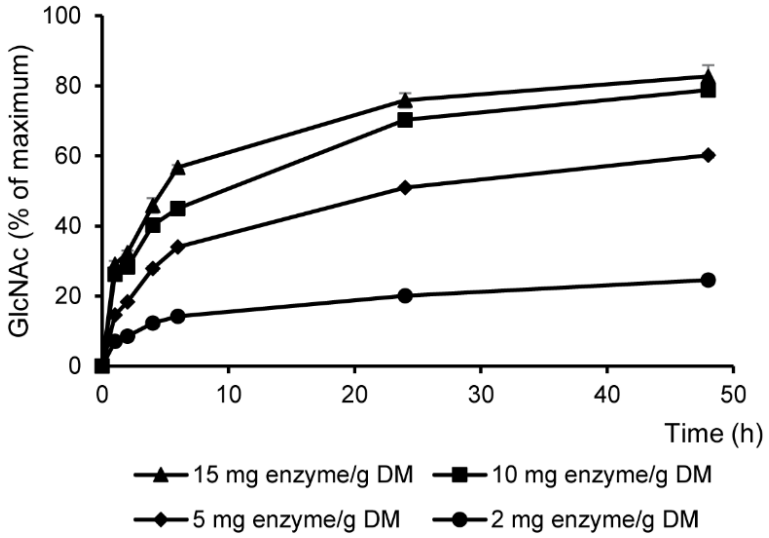

Fig S2. Dose response curves for the optimal enzyme cocktails predicted by the "further reduced" model for Chitinor. Yields were calculated as percentage of the theoretical maximum and the values presented are the average of three independent reactions containing 15 mg/ml chitin in 10 mM BisTris pH 6.2, incubated at 45 °C. At all time points, in all reactions, GlcNAc was the only detected soluble product.