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Enhanced enzymatic hydrolysis of langostino shell chitin with mixtures of enzymes from bacterial and fungal sources

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Abstract

A combination of enzyme preparations from *Trichoderma atroviride* and *Serratia marcescens* was able to completely degrade high concentrations (100 g/L) of chitin from langostino crab shells to *N*-acetylglucosamine (78%), glucosamine (2%), and chitobiose (10%). The result was achieved at 32 °C in 12 days with no pre-treatment (size reduction or swelling) of the substrate and without removal of the inhibitory end-products from the mixture. Enzymatic degradation of three forms of chitin by *Serratia*/*Trichoderma* and *Streptomyces*/*Trichoderma* blends was carried out according to a simplex-lattice mixture design. Fitted polynomial models indicated that there was synergy between prokaryotic and fungal enzymes for both hydrolysis of crab chitin and reduction of turbidity of colloidal chitin (primarily endo-type activity). Prokaryotic/fungal enzymes were not synergistic in degrading chitosan. Enzymes from prokaryotic sources had much lower activity against chitosan than enzymes from *T. atroviride*.

Keywords: *N*-Acetylglucosamine; *Trichoderma*; *Serratia*; *Streptomyces*; Hydrolysis; Chitin

1. Introduction

N-Acetylglucosamine and glucosamine are amino sugars having therapeutic potential for the treatment of a variety of diseases, including arthritis,[1,2] inflammatory bowel disease,[3] and general inflammatory damage.[4] Commercial production of these amino sugars currently relies upon acid hydrolysis of de-proteinized and de-mineralized crustacean shells.[5] Acid hydrolysis is relatively efficient but involves strong acids (4–8 M HCl) at high temperatures and results in production of toxic wastes. Moreover, the extreme conditions used in the process may result in unwanted modifications to the hydrolysis products.

Conversely, enzymatic degradation of crustacean shells is environmentally friendly but is more complex since it involves both production of the enzyme and the digestion of the substrate. More importantly, enzymatic processes have been plagued by low yields and have resulted in incomplete conversion of chitin into its monomer.[6,7]

However, there has been substantial recent progress in studies of chitinases from both prokaryotic and eukaryotic sources.[8–10] Of these, the most widely examined enzyme sources for commercial chitin degradation are strains of *Serratia marcescens*.[8] However, its enzyme preparations do not completely convert chitin to GlcNAc but instead accumulate a blend of both monomers and chito-oligomers (mainly chitobiose).[11] The phenomenon is particularly evident when high concentrations of chitin (5–10% w/v) are digested.

Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; GlcN, glucosamine; MUA, 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide; MUB, 4-methylumbelliferyl-*N*,*N*′-diacetyl-β-chitobioside; nahase, *N*-acetylhexosaminidase (EC 3.2.1.52); endo, Endochitinase (EC 3.2.1.14).

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Chitinolytic enzymes from fungi in the genus *Trichoderma* have also been extensively studied.[12] Unlike their bacterial counterparts, *Trichoderma* chitinolytic preparations have a high ratio of exochitinase to endochitinase activity and release almost exclusively monomeric GlcNAc from chitin. Insofar as we are aware, enzymes from these fungi have not been examined for their abilities for commercial or pilot-scale production of GlcNAc from crustacean chitin, although enzymes from *T. reesei* are widely used for commercial hydrolysis of cellulose and other plant polysaccharides.

This study describes the efficacy and the synergy of mixtures of enzymes from *Trichoderma atroviride*, *S. marcescens* and *Streptomyces albidoflavus* in hydrolyzing several forms of chitin, including native chitin from langostino crab shells, colloidal chitin, and chitosan.

2. Results

2.1. Hydrolysis of langostino crab chitin

Enzyme preparations from the three organisms differed markedly in their endochitinase and nahase levels as measured by methylumbelliferyl substrates. The activity of the preparation from *S. marcescens* was several-fold higher than that from *S. albidoflavus* or *T. atroviride*. Further, the ratios of endochitinase to nahase activity from preparations from the various microbes differed by about an order of magnitude with the highest proportions of exochitinase activity in the enzymes from *T. atroviride* (Table 1).

As a first step, we examined the release of GlcNAc from chitin by enzyme blends from the most active sources, namely, from *S. marcescens*, and from the fungus *T. atroviride* (Fig. 1).

The lowest rate of release with single preparations was with the enzymes from *T. atroviride*. Enzymes from *S. marcescens* released GlcNAc rapidly through day 2 and then the rate slowed. After the second addition of chitin at day 7 the rate of GlcNAc release again increased. The mixtures of *S. marcescens* and *T. atroviride* enzyme preparations were more effective than any single enzyme preparation, with the greatest release occurring at a ratio of 75:25 *Serratia*/*Trichoderma* (Fig. 1). This mixture provided about 100 g/L of GlcNAc (measured by Elson–Morgan assay) from 100 g/L of total chitin added.

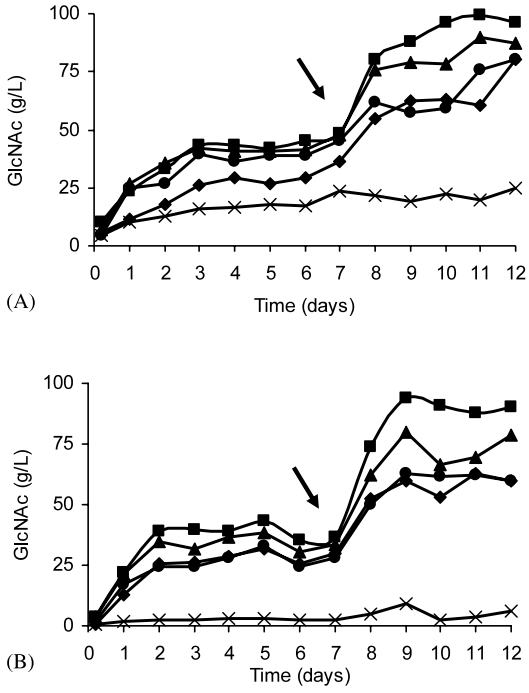

Fig. 1. Time-course of *N*-acetylglucosamine release from langostino chitin during incubation with *S. marcescens* and *T. atroviride* enzyme blends. (A) Stirred flasks, 32 °C in, 350 rpm; (B) shaken flasks, 37 °C, 220 rpm. *Serratia*/*Trichoderma* filtrate percent ratios: (-◆-) 100:0; (-■-) 75:25; (-▲-) 50:50; (-●-) 25:75; (- × -) 0:100. Fifty grams per litre of chitin were initially present in all the digestions; additional 50 g/L were added at the time indicated by the arrows.

These results largely were confirmed by HPLC analyses of the reaction mixtures. The 75:25 mixture released per liter 78 g of GlcNAc, 2 g of GlcN and 10 g of chitobiose at the termination of the test (Table 2). Unblended *S. marcescens* yielded 47 g/L of GlcNAc, and no detectable GlcN, while 37% of the total detected amino sugars was chitobiose. Larger oligomers were never detected, even though the technique was capable of resolving up to the 7-mer. Digestions carried out with *T. atroviride* enzymes alone released primarily GlcNAc

Table 1
Endochitinase (Endo) and *N*-acetylhexosaminidase (Nahase) activities of chitinolytic enzyme preparations used in this work

Enzyme source	Endo activity (U) [a]	Nahase activity (U) [b]	Ratio E/N
Serratia marcescens	822	193	4.3
Streptomyces albidoflavus	25	23	1.1
Trichoderma atroviride	17	39	0.43

[a] U = release of 1 nmol of methylumbelliferone/s mL from 4-methylumbelliferyl-D-*N*,*N*′-diacetyl-β-chitobioside.
[b] U = release of 1 nmol of methylumbelliferone/s mL from 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide.

Table 2
HPLC quantification of the hydrolysis products released from chitin digestion (stirring flasks) with enzyme mixtures from different organisms

Blend composition	GlcNAc (g/L)	GlcN (g/L)	$GlcNAc_2$ (g/L)	$GlcNAc_3$ (g/L)	Glucose (g/L)	Total (g/L)
SM 100%	47.10 (62.8%)	< 0.004 (0.0%)	27.90 (37.2%)	< 0.020 (0.0%)	< 0.005 (0.0%)	75.00
SM:TA 75%:25%	77.90 (86.0%)	2.17 (2.4%)	10.50 (11.6%)	< 0.020 (0.0%)	< 0.005 (0.0%)	90.57
TA 100%	26.90 (95.5%)	0.95 (3.4%)	0.32 (1.1%)	< 0.020 (0.0%)	< 0.005 (0.0%)	28.17

Analysis was performed by high-performance anion-exchange chromatography with pulsed amperometric detection on a Dionex DX 500 apparatus. Abbreviations are: SM, *Serratia marcescens*; TA, *Trichoderma atroviride*; GlcNAc, *N*-acetylglucosamine; GlcN, glucosamine; $GlcNAc_2$, chitobiose; $GlcNAc_3$, chitotriose.

(96% of the total detectable amino sugars); of the remainder, GlcN was more abundant than chitobiose. These results were confirmed in other independent replications. Thin layer chromatography (TLC) analyses of the reaction mixtures confirmed the results of the HPLC analysis (data not shown).

In order to describe better the *Serratia*/*Trichoderma* system, we repeated the experiment using a two component, five-level simplex-lattice mixture design (Table 3). We also included a second set of digestions with a different prokaryotic/fungal mixture (*Streptomyces*/*Trichoderma*). GlcNAc released at 72 h was used to fit quadratic equations. The models were statistically significant ($P = 0.0002$; adjusted $R^2 > 0.95$) and pointed to the presence of a strong positive interaction in both the prokaryotic/*Trichoderma* mixtures as compared to any enzyme used singly (Fig. 2). The interaction terms of the models explain up to 35% (*Serratia*/*Trichoderma*) and 48% (*Streptomyces*/*Trichoderma*) of the overall GlcNAc released (Table 4) with the best theoretical blend being 55:45 *Serratia*/*Trichoderma* and 45:55 *Streptomyces*/*Trichoderma*.

Product profiles from these partial digestions were analyzed by TLC (data not shown) and were consistent with product composition of the complete hydrolysis. Neither single nor blended chitinase preparations yielded chito-oligomers higher than chitotriose. The presence of *Trichoderma* chitinases in the mixtures increased GlcNAc yield relative to *Serratia* and *Streptomyces* enzymes used singly.

Table 3
Runs and corresponding results of the simplex-lattice mixture designs presented in this paper: GlcNAc release from langostino crab chitin; turbidity reduction of colloidal chitin; reducing sugar release from chitosan

Run #	SM (%)	TA (%)	GlcNAc from langostino chitin (g/L)	Colloidal chitin turbidity red. (%)	Reducing sugars from chitosan (GlcN g/L)
1	100	0	20.9	63	0.034
2	100	0	21.7	63	0.015
3	75	25	30.7	66	0.078
4	50	50	28.9	66	0.135
5	50	50	30.5	70	0.126
6	25	75	27.2	58	0.197
7	0	100	18.2	25	0.252
8	0	100	17.8	18	0.264
Run #	SA (%)	TA (%)	GlcNAc from langostino chitin (g/L)	Colloidal chitin turbidity red. (%)	Reducing sugars from chitosan (GlcN g/L)
1	100	0	12.3	12	0.158
2	100	0	12.4	11	0.182
3	75	25	25.8	21	0.180
4	50	50	30.1	23	0.231
5	50	50	28.9	23	0.221
6	25	75	27.2	30	0.254
7	0	100	18.3	25	0.271
8	0	100	18.5	23	0.274

SM, *S. marcescens*; SA, *S. albidoflavus*; TA, *T. atroviride*.

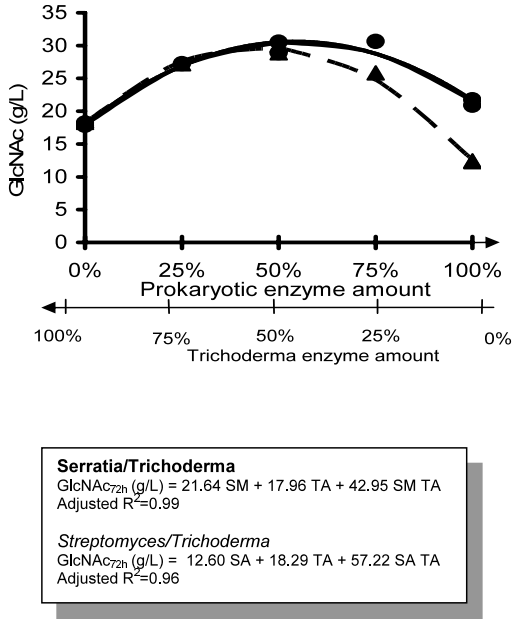

Fig. 2. Plot of the fitted models estimated for NAG release from langostino chitin after 72 h of incubation with *Serratia*/*Trichoderma* (●) and *Streptomyces*/*Trichoderma* (▲) mixtures. Corresponding equations are also reported. $GlcNAc_{72h}$ (g/L): GlcNAc released after 72 h of digestion; SA, SM, and TA represent, respectively the percentages of *Streptomyces*, *Serratia*, and *Trichoderma* enzyme in the mixtures.

2.2. Prokaryotic and fungal enzyme preps are synergistic in degrading colloidal chitin

The ability of *Serratia*/*Trichoderma* and *Streptomyces*/*Trichoderma* to hydrolyze colloidal chitin was also modeled using a similar simplex-lattice mixture design (Table 3 and Fig. 3). Both *Serratia* and *Streptomyces* enzymatic preparations were synergistic with *Trichoderma* enzymes in reducing turbidity of colloidal chitin suspensions (significant at $P = 0.0002$; adjusted $R^2 > 0.94$). The interaction between enzyme preparations was responsible for about 45 and 26% of the total turbidity reduction for *Serratia*/*Trichoderma* and *Streptomyces*/*Trichoderma*, respectively (Table 4). The only reaction product from colloidal chitin detected with TLC with any enzyme or mixtures of enzymes was GlcNAc (Fig. 4).

2.3. *Trichoderma* enzymes are more active on chitosan than their prokaryotic counterparts

When chitosan was used in another simplex-lattice design experiment, the quantity of reducing sugars released by enzymes from *T. atroviride* was 10- and 1.6-fold higher than *Serratia* or *Streptomyces* enzymes, respectively (significant at $P = 0.0001$; adjusted $R^2 > 0.94$), but the model gave no evidence for synergy between different enzyme sources (Fig. 5). TLC analysis of the digestion products showed that *Trichoderma* chitinolytic preparations accumulate both GlcN and a range of oligomers, while prokaryotic preps released mainly the dimer and oligomers larger than the tetramer (Fig. 6).

2.4. Both *Trichoderma* and prokaryotic chitinase preparations are differently inhibited by hexosamines

We hypothesized that a possible explanation for the prokaryotic/*Trichoderma* synergism could be related to lower enzyme inhibition by hexosamines as compared to the unblended preparations.[13] To test this hypothesis, we measured the decrease in hydrolytic activity against MUA or MUB of prokaryotic/*Trichoderma* enzymes blended according to a simplex-lattice mixture design in the presence of high concentrations of either GlcNAc or GlcN. GlcNAc or GlcN was added to give a range of about five- to 1400-fold excesses compared to the substrates. The highest concentrations of GlcNAc were severely inhibitory to nahase activity for all of the enzymes and enzyme mixtures, but the enzyme mixtures from *T. atroviride* or *S. albidoflavus* were somewhat less inhibited than those from *S. marcescens*. Conversely, the presence of GlcN was more inhibitory to the enzymes from *T. atroviride* than from the bacterial sources. Mixtures of prokaryotic/*Trichoderma* enzymes were not significantly interactive in degrading MUA in the presence of GlcNAc and GlcN compared with single enzyme sources (Fig. 7).

The activity of any of the enzyme mixtures against MUB was inhibited less by either GlcNAc or GlcN than was activity against MUA. Generally, the bacterial sources were less affected by GlcNAc or GlcN than the enzymes from *T. atroviride*. Activity of the enzymes from *S. albidoflavus* against MUB was increased at concentrations of GlcNAc above 5 g/L. However, unlike activity against MUA, blends displayed significant interaction in the hydrolysis of MUB. *Serratia*/*Trichoderma* mixtures were less inhibited by GlcN than were the *Streptomyces*/*Trichoderma* blends (Fig. 8).

3. Discussion

Chitin, one of the most abundant organic polymers on Earth, is a heterogeneous polysaccharide with different crystalline forms (α, β and γ) and characterized by variable levels and patterns of acetylation. In nature, degradation of all these forms of chitin is carried out efficiently by the combined activity of the enzymatic systems of a multitude of microorganisms.

Heretofore, complete enzymatic degradation of complex native chitin has not been reported. Instead, studies of enzymatic hydrolysis of chitin exploited single

Table 4
Weight of both direct (D) and interaction (I) components to the overall hydrolytic activity predicted by the models describing the release of GlcNAc from langostino shell chitin and the reduction of turbidity in colloidal chitin suspensions for *Serratia*/*Trichoderma* (A) and *Streptomyces*/*Trichoderma* (B) mixtures

(A) *Serratia*/*Trichoderma*

Mixture composition (%)		Langostino shell (GlcNAc release)		Colloidal chitin (Turbidity Red.)	
Serratia	*Trichoderma*	*D*	*I*	*D*	*I*
100	0	100	0	100	0
90	10	85	15	93	7
80	20	75	25	85	15
70	30	69	31	76	24
60	40	66	34	68	32
50	50	65	35	62	38
40	60	65	35	57	43
30	70	68	32	55	45
20	80	73	27	56	44
10	90	83	17	64	36
0	100	100	0	100	0

(B) *Streptomyces*/*Trichoderma*

Streptomyces	*Trichoderma*	*D*	*I*	*D*	*I*
100	0	100	0	100	0
90	10	72	28	85	15
80	20	60	40	78	22
70	30	54	46	75	25
60	40	52	48	74	26
50	50	52	48	75	25
40	60	54	46	77	23
30	70	58	42	80	20
20	80	65	35	85	15
10	90	77	23	91	9
0	100	100	0	100	0

microbial sources and were effective only on easily degradable forms of the polymer such as β-chitin[7] or on relatively low concentrations of α-chitin.[7,14] Typically, the substrate was either physically (size reduction, heat-swelling) or chemically pre-treated in order to increase enzyme action.[6,14,15] In other cases, the efficiency of the process was increased by continuously removing end-products.[16]

In this work, we chose to examine synergistic mixtures of enzymes that more closely approximate those that occur in nature. However, we used organisms whose complexes of chitinolytic enzymes are well studied, produce a variety of activity types and possess enzymes with diverse abilities to interact with the substrate. *T. atroviride* strain P1 produces CHIT42, which is an endochitinase;[17] CHIT73, which is an exochitinase,[18] and CHIT38, which is a chitobiosidase (which requires at the least the trimer for activity),[12] as well as several larger molecular weight exochitinases. *S. marcescens* produces at least three chitinolytic enzymes, and again the enzymes and genes are well known.[19,20] The chitinolytic system of *S. albidoflavus* is not as well characterized as the other two, but multiple chitinolytic enzymes are known to be produced and the enzyme complexes from closely related species have been thoroughly investigated.[9,21]

The primary goal of the current research was to discover enzyme mixtures that were capable of relatively complete release of hexosamines even from complex chitin, such as the langostino shells used in this study. We hypothesized: (a) that blends of enzymes with different types of activities were more likely to be effective than single enzymes; and (b) that highly diverse mixtures were more likely to be effective than enzymes from a single source. These hypotheses were proven correct by the current study. We view as unlikely the discovery of any single enzyme, or even enzymes from a single organism, that will provide complete degradation of crustacean chitin.

We demonstrated that it is possible to significantly increase enzyme-mediated hexosamine production by exploiting synergism among enzyme preparations ob-

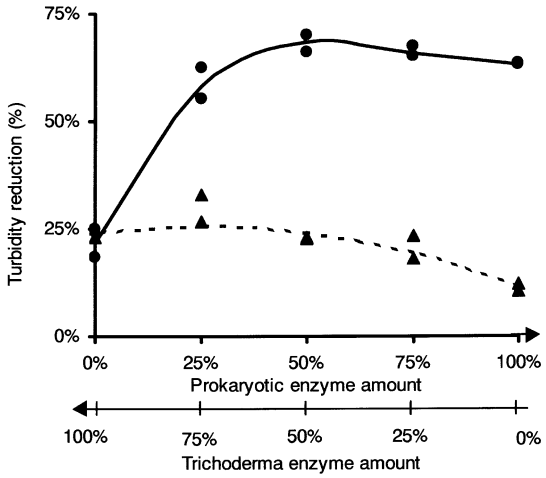

Fig. 3. Plot of the fitted model estimated for turbidity reduction of colloidal chitin from langostino chitin by *Serratia*/*Trichoderma* (●) and *Streptomyces*/*Trichoderma* (▲) mixtures. Corresponding equations are also reported. CC turbidity red (%): percent reduction of turbidity in chitin suspension; SA, SM, and TA represent, respectively the percentages of *Streptomyces*, *Serratia*, and *Trichoderma* enzymes in the mixtures.

tained from taxonomically distant sources. The process described is simple, since it involves the incubation of de-proteinized and de-mineralized crab shells in the presence of an appropriate enzyme mixture with continuous and vigorous stirring. At the same time, the process is efficient because it allows the complete saccharification of langostino crab shells.

The origin of synergism showed by both *Serratia*/*Trichoderma* and *Streptomyces*/*Trichoderma* mixtures is unknown but it is likely to derive from the differential ability of the single preparation in: (a) accessing chitin crystalline structure; (b) removing impurities such as proteins and lipids;[22] and (c) providing non-chitinolytic proteins with activity against chitin.[15] The enzymes produced by these organisms, and the genes that encode them, are dissimilar.[23,24,17,18] This difference is especially pronounced between prokaryotic and eukaryotic sources.[25] Synergism could be due to an increased efficiency of the blends in accessing/de-crystallizing the polymer, a feature considered of extreme importance for the hydrolysis of ligno-cellulosic substrates.[26] However, in our experiments with suspensions of colloidal chitin, there was also synergy between bacterial and fungal-source enzymes. Rapid decreases in turbidity are associated primarily with endo-type enzymes, indicating that enzymes of this class from different sources are synergistic in reduction of chain length in the amorphous substrate. Synergy may also have been enhanced by the activity of the enzymes from *T. atroviride* on deacetylated regions of the crystalline polymer, since low levels of glucosamine in chitin molecules might block activity of prokaryotic enzymes with low chitosanase activity. Similarly to other *Trichoderma* isolates, *T. atroviride* strain P1 displays high chitosanase activity.[27,28]

Synergism shown by bacterial/fungal mixtures could not be explained by a relief of hexosamine inhibition. Only *Serratia*/*Trichoderma* blends performed better

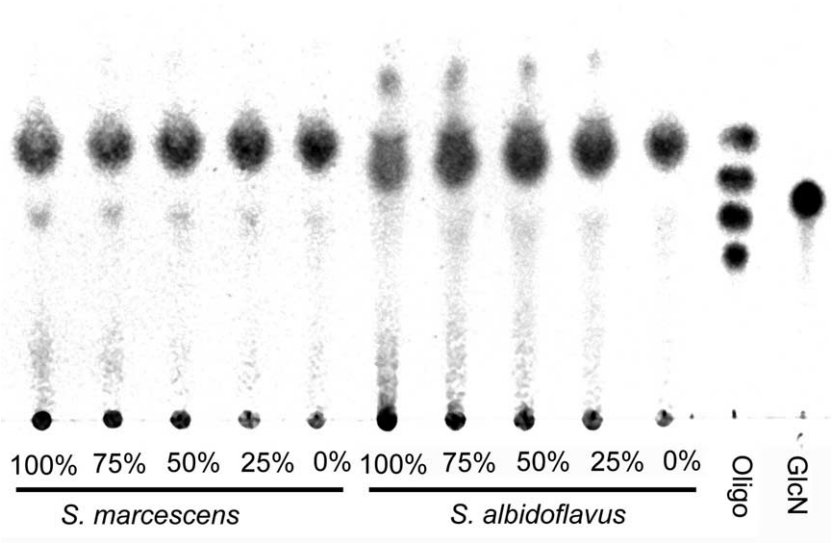

Fig. 4. TLC separation of the hydrolysis products from colloidal chitin after incubation with either *Serratia*/*Trichoderma* or *Streptomyces*/*Trichoderma* enzyme preparation blends. For each sample *Trichoderma* component is the complement to the percentage reported in the figure. Reactions were concentrated 5.5 × in Speedvac and 3 μL/lane were loaded. Oligo: *N*-acetylglucosamine (50 μg), chitobiose (25 μg), chitotriose (25 μg) and chitotetraose (25 μg). GlcN: glucosamine (50 μg). The image was digitally enhanced by uniformly replacing the background color (dark yellow) with white color.

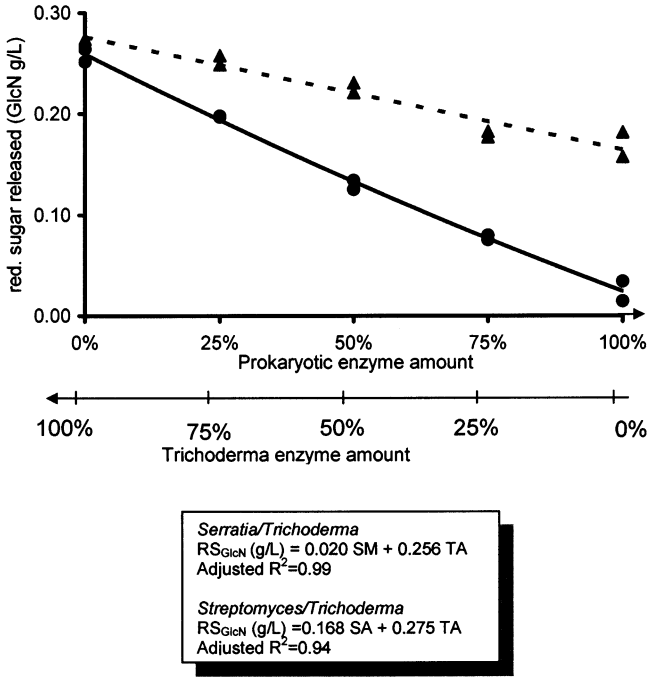

Fig. 5. Plot of the fitted models estimated for the release of reducing sugars (expressed as GlcN) from chitosan by *Serratia*/*Trichoderma* (●) and *Streptomyces*/*Trichoderma* (▲) culture filtrate blends. Corresponding equations are also reported. RS_{GlcN} (g/L): reducing sugars released after 4 h of digestion and expressed as GlcN. SA, SM, and TA represent, respectively, the percentage of *Streptomyces*, *Serratia*, and *Trichoderma* enzymes in the mixture.

against MUB in the presence of GlcN, compared to the unblended preparations.

The hydrolytic process illustrated in this paper can be further improved. A possible strategy is expanding the range of organisms contributing to the chitinolytic blend. Synergism among hydrolases of different origin seems a general phenomenon and the test of mixtures including two or more chitinolytic preparations from a wider array of sources is likely to provide blends with better performances.[29,30]

4. Experimental

4.1. Chitinolytic enzyme production

Chitinolytic enzymes from *S. marcescens* QMB1466 (ATCC 990) used for this work was a prototype commercial preparation provided by Biopolymer Engineering (Eagan, MN).

Culture filtrates from *T. atroviride* strain P1 (ATCC 74058) were prepared by inoculating 250 mL Erlenmeyer flasks containing 100 mL of medium with conidia (5×10^6/mL final concentration) harvested from colonies grown on potato dextrose agar (Difco Laboratories, Detroit, MI). The medium contained 15 g/L KH_2PO_4, 2 g/L $(NH_4)_2HPO_4$, 1.6 g/L $MgSO_4$, 0.6 g/L $CaCl_2$, 2.5 mg/L $FeSO_4 \cdot 7H_2O$, 20 mg/L $FeCl_3$, 1.6 mg/L $MnSO_4 \cdot H_2O$, 1.0 mg/L $ZnSO_4 \cdot 7H_2O$, 1 mg/L $CoCl_2$, 0.04 mg/L $CuSO_4 \cdot 5H_2O$, 0.013 mg/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1 g/L glucose, 5 g/L lactose, 8 g/L chitin from crab shells (Sigma Chemical Co., St. Louis, MO) and 1 g/L

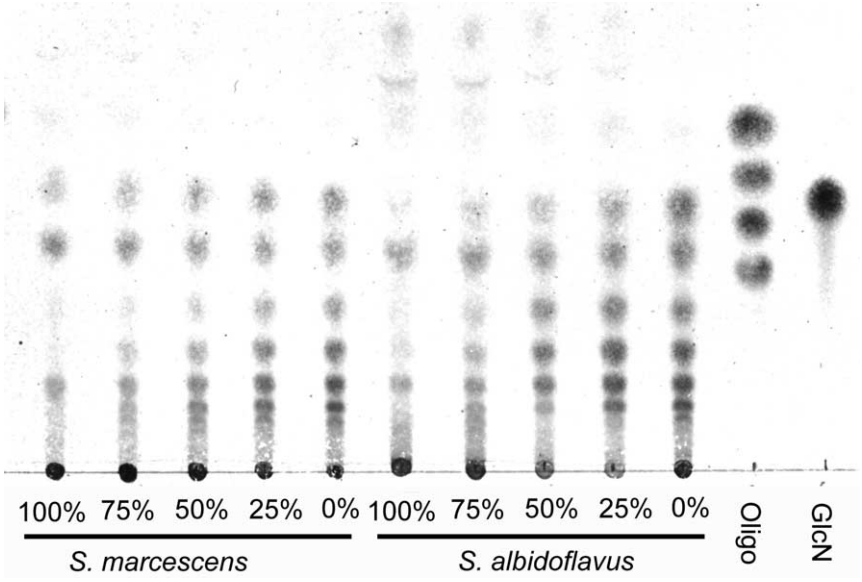

Fig. 6. TLC separation of the hydrolysis products from chitosan after incubation with either *Serratia*/*Trichoderma* or *Streptomyces*/*Trichoderma* enzyme preparation blends. For each sample, *Trichoderma* component is the complement to the percentage reported in the figure. Reactions were concentrated 5.5 × in Speedvac and 3 μL/lane were loaded. Oligo: *N*-acetylglucosamine (50 μg), chitobiose (25 μg), chitotriose (25 μg), and chitotetraose (25 μg). GlcN: glucosamine (50 μg). The image was digitally enhanced by uniformly replacing the background color (dark yellow) with white color.

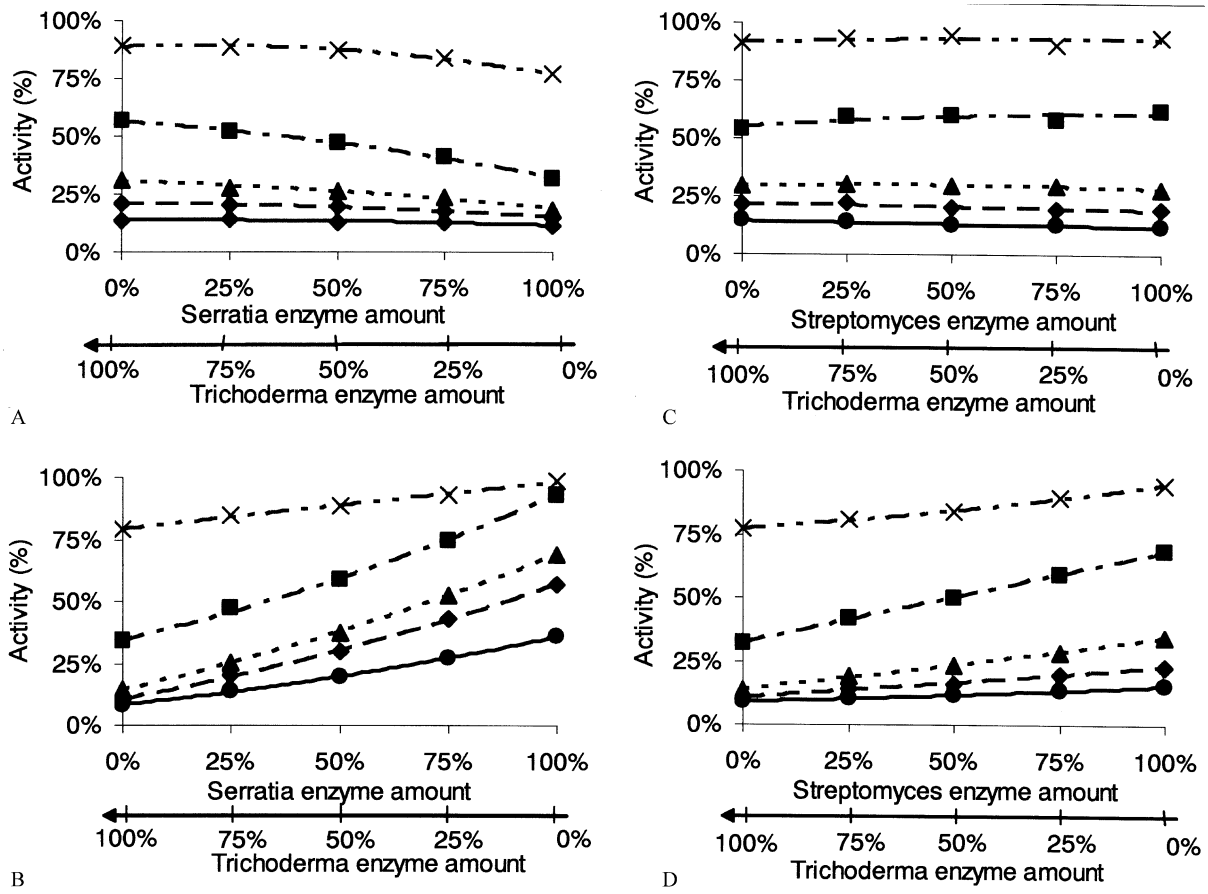

Fig. 7. Effect of hexosamine concentration on nahase activity (MUA) of blends containing (as percentage) 0/100, 25/75, 50/50, 75/25 or 100/0 of either *Serratia*/*Trichoderma* (A and B) or *Streptomyces*/*Trichoderma* (C and D) enzymes. Each blend was incubated in the presence of 25 (-●-), 10 (-◆-), 5 (-▲-), 1 (-■-), and 0.1 (- × -) g/L of either GlcNAc (A and C) or GlcN (B and D). Activity levels are expressed as the percent of the activity that a particular bend showed in the absence of hexosamine. The experiment was executed following a simplex-lattice mixture design.

scleroglucan (Carbomer, Westborough, MA). The pH was adjusted to 6.5. Flasks were incubated on a rotary shaker (150 rpm) at 25 °C for 9 days.

Culture filtrates from *S. albidoflavus* strain NRRL B-16746 were prepared as previously described;[31] however, the medium contained 2.5 g/L of casein (Fisher Scientific, Fair Lawn, NJ) and the growth time was 6 days.

Both enzyme preparations produced for this study were culture filtrates harvested by centrifugation and filtration to remove the producing organism.

4.2. Hexosamine production from langostino crab chitin

Deproteinized and demineralized langostino shell flakes (>90% chitin with a degree of acetylation >85%; caustic solubles, 1%; moisture, 4.6%; ashes, 1%) were provided by Biopolymer Engineering and used without any further treatment. Particle size was variable (up to 3 mm). Enzymatic reactions contained 50 g shells/L in presence of 100 mM NaOAc buffer, pH 5.0, and included blends of *S. marcescens* and *T. atroviride* at the following percentage ratios: 100/0; 75/25; 50/50; 25/75 and 0/100. Digestions were incubated in 25 mL Erlenmeyer flasks either at 32 °C on magnetic stirrers (350 rpm) or at 37 °C on an orbital shaker (220 rpm). After 7 days of incubation, the concentration of langostino shells was brought to 100 g/L. Reactions were incubated for 7 more days under the same conditions. Samples were taken every 24 h.

4.3. Synergism in the hydrolysis of chitin substrates by prokaryotic/Trichoderma mixtures

The following assays were conducted on *Serratia*/*Trichoderma* and *Streptomyces*/*Trichoderma* chitinase blends according to a two-component, five-level simplex-lattice mixture design that measured, in separate experiments: (a) release of GlcNAc from langostino shell chitin; (b) reduction of turbidity of colloidal chitin suspensions; or (c) release of reducing sugars (expressed as GlcN) from chitosan. Results were fitted by multiple linear regression using the program Design-Expert 6.0 (Stat-Ease Inc., Minneapolis MN).

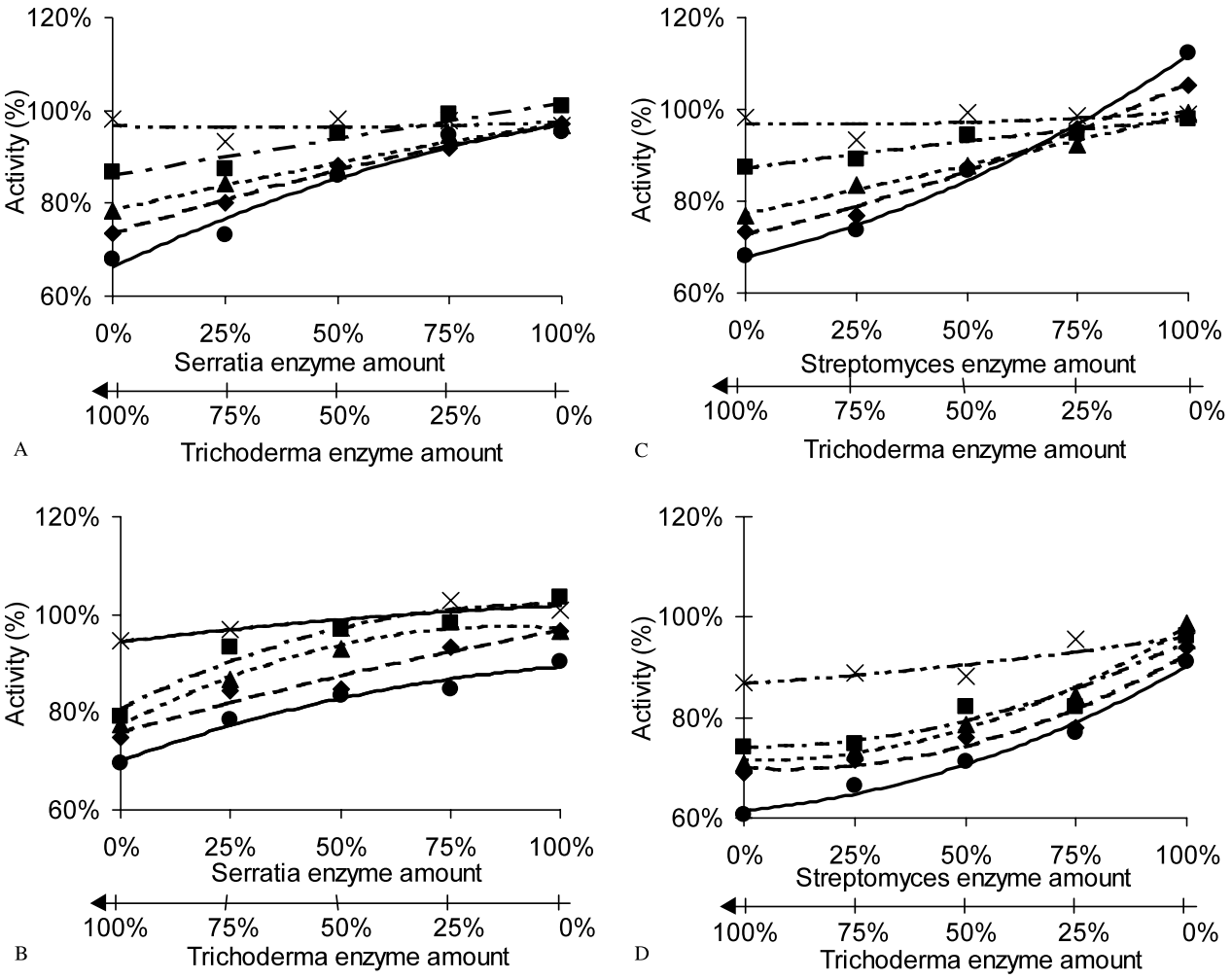

Fig. 8. Effect of hexosamine concentration on hydrolytic activity against MUB of blends containing (as percentage) 0/100, 25/75, 50/50, 75/25 or 100/0 of either *Serratia*/*Trichoderma* (A and B) or *Streptomyces*/*Trichoderma* (C and D) enzymes. Each blend was incubated in the presence of 25 (-●-), 10 (-◆-), 5 (-▲-), 1 (-■-), and 0.1 (- × -) g/L of either GlcNAc (A and C) and GlcN (B and D). Activity levels are expressed as the percent of the activity that a particular blend showed in the absence of hexosamine. The experiment was executed following a simplex-lattice mixture design.

4.4. Release of GlcNAc from langostino shell chitin

Fifty g/L of langostino shell fragments were incubated with prokaryotic/*Trichoderma* mixtures. Digestions were buffered at pH 5.0 with 100 mM NaOAc and incubated at 32 °C on magnetic stirrers (350 rpm). After 72 h, solids were removed by centrifugation and GlcNAc concentration was measured.

4.5. Turbidity reduction of colloidal chitin suspensions

Colloidal chitin turbidity reduction, which results primarily from endochitinase activity,[32] was measured in 96-well microtiter plates using 50 μL of colloidal chitin suspension ($OD_{600} = 0.6$) as the substrate. Fifty microliters of either *Serratia*/*Trichoderma* or *Streptomyces*/*Trichoderma* enzyme blends diluted 1:10 were added to the substrate and incubated for 3 h at 37 °C. Reduction of turbidity was calculated as the percent decrease in absorbance measured at 600 nm.

4.6. Release of reducing sugars from chitosan

Chitosan was prepared as previously described.[33] One volume of enzyme solutions or blends (500 μL) was incubated with one volume of 5% chitosan (w/v) suspended in 200 mM NaOAc, pH 5.0. Reactions were incubated at 37 °C for 4 h on an orbital shaker (215 rpm). Solids were removed by centrifuging the digestion for 10 min at 14,000*g*. Supernatants were diluted 5 × in water and reducing sugar release was assayed using GlcN as the standard.

4.7. Chitinase activity measurement

Exo- and endo-chitinase activities were measured by the release of 4-methylumbelliferone from either 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (MUA, Sigma) or 4-methylumbelliferyl-*N*,*N*′-diacetyl-β-chitobioside (MUB, Sigma), respectively. Both substrates were used at 0.1 mM final concentration in the presence of 50 mM NaOAc buffer at pH 5.0 and 1% BSA fraction V (Fisher Scientific). One unit of nahase or endochitinase activity was defined as the amount of enzyme required to release 1 nmol of methylumbelliferone $\times$ s^{-1} from MUA or MUB in 1 mL reaction volume at 37 °C.

4.8. Chitinase inhibition activity by GlcNAc and GlcN

In order to investigate whether prokaryotic/*Trichoderma* enzyme blends had reduced sensitivity to GlcNAc and GlcN compared to unblended preparations, we measured percentage variation in the chitinolytic activity against both MUA and MUB in the presence of 0, 0.1, 1, 5, 10, 25 g/L of either GlcNAc or GlcN. Assays were carried out following a two component, five-level simplex-lattice mixture design.

4.9. Amino sugar detection and measurement

GlcNAc release was measured using the Elson–Morgan assay.[34] Reducing sugar release was measured using the Somogyi assay.[35] TLC was carried out as described previously using pre-coated silica gel plates (Sigma, cat. # Z122785).[36] After separation, plates were air dried for 1 h, spayed with aniline–diphenylamine reagent (Sigma, cat. # A8142) and baked at 140 °C for 4 min. In addition, sugars were quantitated at Econotech (British Columbia, Canada) by high-performance anion-exchange chromatography with pulsed amperometric detection on a Dionex DX 500 apparatus as described.[37]

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