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# A reliable reducing end assay for chito-oligosaccharides

Svein Jarle Horn*, Vincent G.H. Eijsink

*Department of Chemistry Biotechnology and Food Science, Agricultural University of Norway, P.O. Box 5040 1432 Ås, Norway*

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**Abstract**

The enzymatic degradation of chitin typically yields a mixture of chito-oligosaccharides of different size. This degradation process can be followed by measuring the production of reducing ends. We show that the widely used Schales' procedure does not permit accurate determination of reducing ends since the signal increases with the length of the chito-oligosaccharides. In contrast, the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method gives the same absorbance per reducing end, irrespective of the length of the chito-oligosaccharide. To our knowledge the MBTH method is the most sensitive reducing end assay for the quantification of chito-oligosaccharides, detecting concentrations down to 5 μM. This method was successfully applied to monitor the enzymatic degradation of chito-oligosaccharides and beta chitin.


*Keywords:* Reducing end; *N*-acetylchitooligosaccharides; Chitin; Schales'; 3-Methyl-2-benzothiazolinone hydrazone; Chitinase B; *Serratia marcescens*; Chitinase

## 1. Introduction

Enzymatic hydrolysis of polysaccharides like cellulose and chitin is a complex process requiring the concerted action of several enzymes. Generally, these enzymes can be divided into two groups: endo-enzymes that hydrolyse the polysaccharide chain in a random manner, and exo-enzymes that act near the chain end. Each new glycosidic bond that is hydrolysed yields a new reducing end-group. The course of the enzymatic reaction is typically followed by utilising one of several colorimetric assays, which measure the concentration of reducing end-groups. Most of these assays are based on redox reactions involving electron transfers from the aldehyde/hemiacetal to metal ions like copper. Changes in cation concentrations are monitored spectrophotometrically as complexes with various chelators.

Many authors have been criticizing the colorimetric methods used for measuring cellulase activity, mainly because of low sensitivity and non-stoichiometric product estimation (Sharrock, 1988). For instance, the Nelson-Somogyi (Somogyi, 1952) and 3,5-dinitrosalicylic acid (Miller, 1959) methods are not accurate bellow glucose concentrations of 100 and 1000 μM, respectively. In addition, the response per reducing group is dependent on the length of the oligosaccharide in both methods, which complicates quantitative determination of reducing groups in mixtures of different oligosaccharides (Vlasenko, Ryan, Shoemaker, & Shoemaker, 1998). The most sensitive copper reduction assay utilises bicinchonic acid (BCA) as the chromogen (Anthon & Barrett, 2002a). The BCA assay has been shown to give relatively uniform values when applied to oligosaccharides derived from starch, polygalacturonic acid and chitin, independent of the degree of polymerisation (Doner & Irwin, 1992). Unfortunately, the BCA assay is not specific for sugars; other compounds capable of reducing copper, most notably protein, also react. In fact, a BCA-based method is among one of the most common methods for protein concentration determination.

Recently a new reducing end assay was developed where 3-methyl-2-benzothiazolinone hydrazone (MBTH) was utilised as the electron acceptor (Anthon & Barrett, 2002a). The MBTH method was originally developed to determine aldehydes (Hauser & Cummins, 1964), but has been adapted to the quantification of reducing sugars, and was shown to be useful for determination of polygalacturonase activity (Anthon & Barret, 2002b; se below for further details).

Chitin is a linear polysaccharide of β(1,4)-linked *N*-acetylglucosamine (GlcNAc) residues, and is the second

* Corresponding author. Tel.: +47-64947703; fax: +47-64947720.
*E-mail address:* sevin.horn@ikbm.nlh.no (S.J. Horn).

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most abundant biopolymer in nature next to cellulose (Muzzarelli & Muzzarelli, 1998; Rudrapatnam, Tharanathan, & Kittur, 2003). Several plants, insects and microorganisms have chitinolytic enzyme systems capable of degrading chitin (Patil, Ghormade, & Deshpande, 2002). For instance, the enterobacterium *Serratia marcescens* produces at least three chitinases with complementary activities (ChiA, ChiB, ChiC1; Suzuki et al., 2002). Degradation of chitin results in a range of chito-oligosaccharides with different degree of polymerisation. Thus, quantitative determination of chitin degradation requires a reducing end assay that is independent of oligosaccharide length.

One of the most widely used reducing end assays for the quantification of chito-oligosaccharides is the so-called Schales' procedure (Imoto & Yagishita, 1971; Schales & Schales, 1945). It has been noted that chito-oligosaccharides of different lengths give different signals per reducing end (Imoto & Yagishita, 1971), but these potential problems are not well documented and the assay is still widely used. In the present study, we have quantified the oligosaccharide-length-dependency of the Schales' procedure and we present the MBTH method as an alternative. We have evaluated the usefulness of the MBTH method for quantitative determination of chito-oligosaccharides and chitinase activity.

## 2. Experimental

### 2.1. Chemicals

β-chitin from squid pen was supplied by France—Chitine, Marseille, France (size = 180 μm). *N*-acetyl-D-glucosamine (GlcNAc, minimum 99%), *N*,*N*-diacetylchitobiose ($GlcNAc_2$, minimum 96%), tetra-*N*-acetylchitotetraose ($GlcNAc_4$, minimum 95%), hexa-*N*-acetylchitohexaose ($GlcNAc_6$, minimum 95%) and all other chemicals were purchased from Sigma (St Louis, USA).

### 2.2. Enzyme

Chitinase B (ChiB) from *Serratia marcescens* was expressed and purified as described elsewhere (Brurberg, Nes, & Eijsink, 1996).

### 2.3. Schale's procedure

The assay was carried out as described by Imoto and Yagishita (1971). Briefly, 0.6 ml of a 0.5 M sodium carbonate solution containing 0.5 g/l potassium ferricyanide was mixed with 450 μl of the chito-oligosaccharide sample. The mixture was heated for 15 min at 100 °C and optical density was determined at 420 nm.

### 2.4. MBTH-method

Two-hundred microlitre samples containing 0–100 μM of chito-oligosaccharides were mixed with 200 μl 0.5N NaOH in reaction tubes equipped with a screw cap. Equal volumes of 3 mg/ml MBTH and 1 mg/ml DTT were mixed and 200 μl of this fresh mixture were added to the reaction tubes. The screw caps were sealed and the tubes were heated for 15 min at 80 °C in an aluminium heating block. Immediately after removal from the heating block 400 μl of a solution containing 0.5% $(FeNH_4(SO_4)2)\cdot 12H_2O$, 0.5% sulfamic acid and 0.25N HCL was added to the samples. The caps were fitted again and the tubes were inverted a few times and allowed to cool to room temperature. Finally absorbance was determined at 620 nm.

### 2.5. Enzymatic activity

Twenty-four micromolar $GlcNAc_4$ or $GlcNAc_6$ was incubated with 3.2 nM purified ChiB in 50 mM sodium acetate buffer, pH 6.1, containing 0.05 mg/ml BSA, at 37 °C. 1 mg/ml β-chitin was incubated with by 34 μg/ml ChiB (612 nM) in 50 mM sodium acetate buffer, pH 6.1. The reaction tubes were incubated at 37 °C in a reciprocal shaker to avoid settling of the β-chitin particles. 200 μl of sample was regularly taken and immediately mixed with 200 μl 0.5 M NaOH to stop the enzyme activity. The reducing ends were determined as described in the MBTH-method.

## 3. Results and discussion

### 3.1. Schales' procedure

The Schales' procedure was originally developed to determine glucose concentrations in blood (Schales et al., 1945). A modified Schales' procedure was later developed for the quantification of lysozyme activity (Imoto & Yagishita, 1971). This modified procedure is widely used to determine the formation of reducing ends during enzymatic degradation of chitin or modified chitins. The method is based on the disappearance of ferricyanide due to reducing groups, and increasing concentrations of reducing groups thus lead to a decrease in colour in the assay. Fig. 1 shows the standard curves for GlcNAc, $GlcNAc_2$, $GlcNAc_4$ and $GlcNAc_6$. The results clearly show that the size of the chito-oligosaccharides affects the signal from the reducing ends: the longer the chito-oligosaccharide, the stronger the signal. Thus, quantification of reducing ends in a mixture of chito-oligosaccharides with different lengths based on a standard curve of GlcNAc will result in severe over-estimation of reducing end formation. This problem is often neglected in literature on chitin degradation.

It should be noted that effects of oligosaccharide length on reducing end signal have been reported for several of the known reducing end assays. For example, the dinitrosalicylic

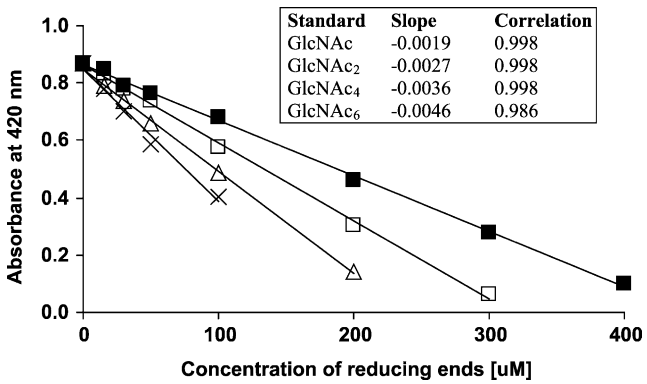


Fig. 1. Calibration of the Schales' procedure. The figure shows absorbance at 420 nm versus molar concentrations of GlcNAc (■), $GlcNAc_2$ (□), $GlcNAc_4$ (△) and $GlcNAc_6$ (×) in the 450 μl samples. The experiment was carried out in duplicate and the values represent the average. The insert shows the data for the regression lines.

acid method showed a similar response as the Schales' procedure: tests with xylose, xylobiose and xylotriose gave increasing responses per reducing end group with increasing oligosaccharide length (Jeffries, Yang, & Davis, 1988). The Nelson-Somogyi (arsenomolybdate) method gave the opposite response for the same oligosaccharides (Jeffries et al., 1988). The dinitrosalicylic acid and Nelson-Somogyi methods showed similar effects when used for measurement of cello-oligosaccharides (Vlasenko et al., 1998).

### 3.2. The MBTH method

Fig. 2 shows the standard curves for chito-oligosaccharides measured by the MBTH method. The results clearly show that signals obtained are independent of the length of the chito-oligosaccharide. Mixtures of different chito-oligosaccharides gave absorbances corresponding to the values that would be expected on the basis of the standard curves shown in Fig. 2 (results not shown). Interestingly, Fig. 2 also shows that the MBTH method is two to five times

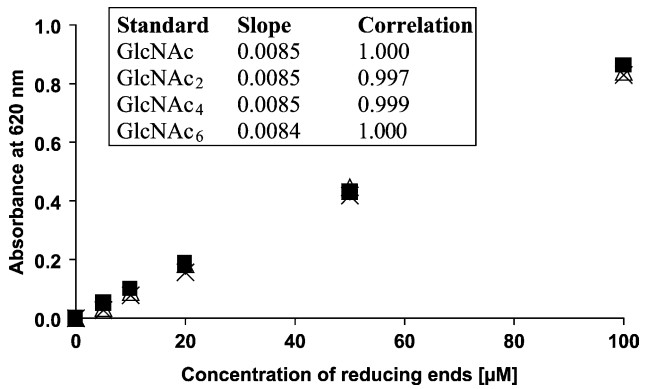


Fig. 2. Calibration of the MBTH method. The figure shows absorbance at 620 nm versus molar concentrations of GlcNAc (■), $GlcNAc_2$ (□), $GlcNAc_4$ (△) and $GlcNAc_6$ (×) in the 200 μl samples. The experiment was carried out in triplicate and the values represent the average. The insert shows the data for the regression lines.

more sensitive than the Schales' procedure (compare slopes in the inserts of Figs. 1 and 2). The MBTH method allows detection of reducing end concentrations down to 5 μM.

It has previously been reported that the MBTH method gives a slight increase in signal per reducing end when going from glucose to cellobiose to cellotetraose (Anthon & Barrett, 2002a). The fact that we do not see such an increase for the chito-oligosaccharides may mean that glucose and *N*-acetylglucosamine oligomers behave differently in the MBTH method. On the other hand, Anthon and Barrett (2002a) did not report the purity of the oligosaccharides used, meaning that it cannot be excluded that the increased signals observed for the longer glucose oligomers are due to contaminations with monomers or dimers. The chito-oligosaccharides used in the present study had a reported purity of 95–99% (according to the supplier). HPLC analyses conducted in our laboratory did not reveal significant amounts of impurities (results not shown).

Doner and Irwin (1992) have reported excellent results for quantification of glucose oligomers (mono- to heptamer) using reducing end determination by the BCA assay. Others have confirmed that the BCA assay gives the same absorbance for glucose and cellooligosaccharides of different size (Garcia, Johnston, Whitaker, & Shoemaker, 1993). The BCA assay was also found to give oligosaccharide-length-independent results for $GlcNAc_{3-6}$. However, $GlcNAc_2$ gave a somewhat lower absorbance and GlcNAc a much higher absorbance than the higher chito-oliogosaccharides (Doner & Irwin, 1992). It should be noted that the BCA method is about six times more sensitive for cello-oligoaccharides than for chito-oliogosaccharides (Doner & Irwin, 1992). We have confirmed in our laboratory that the BCA assay yields a higher absorbance for GlcNAc compared to higher chito-oliogosaccharides (By approximately a factor of 3; results not shown). We have also observed that the MBTH method is about nine times more sensitive than the BCA assay for higher chito-oliogosaccharides (results not shown). Thus, the MBTH method is to our knowledge the most sensitive reducing end assay for quantification of chito-oliogosaccharides.

An additional advantage of the MBTH method compared to the BCA assay concerns interference by proteins. Anthon and Barrett (2002a) reported that 0.5 mg/ml BSA in the sample increased the absorbance by 0.12 in the MBTH method. The same BSA concentration increased the absorbance in the BCA assay by 1.5 (Anthon & Barrett, 2002a). BSA is commonly added to reaction mixtures with low enzyme concentrations, to avoid the enzymes to stick to the walls of the reaction tubes.

### 3.3. Monitoring chitinase activity by the MBTH method

It has previously been shown that ChiB, an exochitinase from *Serratia marcescens* (Aalten et al., 2000), converts $GlcNAc_4$ exclusively to $GlcNAc_2$, whereas $GlcNAc_6$ is converted to $GlcNAc_2$ and GlcNAc (via initial formation of

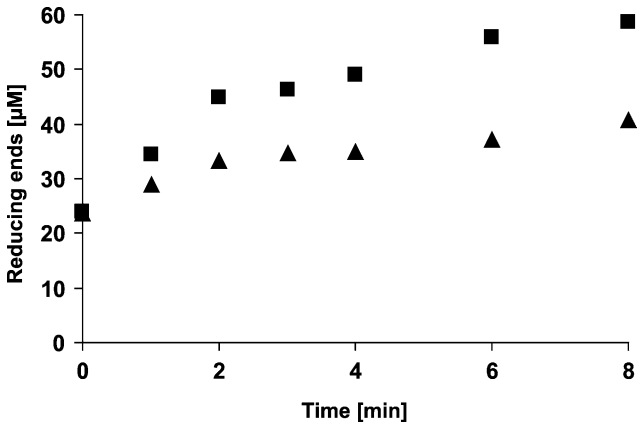


Fig. 3. Degradation of $GlcNAc_4$ (▲) and $GlcNAc_6$ (■) by ChiB. The enzyme concentration was 3.2 nM; the starting concentration for the substrates was 23.7 nM. The concentration of reducing ends was calculated by dividing the absorbance by 0.0085 (Insert in Fig. 2). The experiment was carried out in duplicate and the values represent the average.

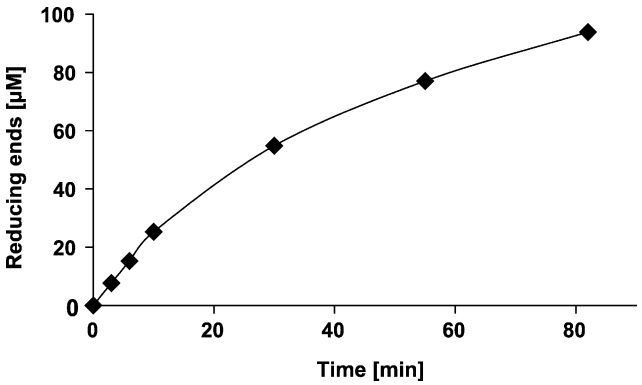


Fig. 4. Degradation of 1 mg/ml β-chitin by 34 µg/ml ChiB. The concentration of reducing ends was calculated by dividing the absorbance with 0.0085 (Insert in Fig. 2). The enzyme concentration in the reaction mixture corresponds to 612 nM; the amount of substrate corresponds to a dimer concentration of 2.36 mM. The experiment was carried out in duplicate and the values represent the average.

$GlcNAc_4$ and $GlcNAc_3$) (Brurberg et al., 1996; Suzuki et al., 2002). The degradation of $GlcNAc_4$ and $GlcNAc_6$ by ChiB was monitored using the MBTH method, as depicted in Fig. 3. During the first 2 min of the degradation, product formation was linear, indicating that the initial substrate concentrations were considerably higher that the $K_m$ values for the substrates. The initial, linear parts of the curves permit the determination of specific activities towards various substrates. In the case of ChiB, these specific activities are 12.3 mol reducing ends/s/mol enzyme and 27.0 mol reducing ends/s/mol enzyme for the tetramer and the hexamer, respectively.

The non-linear character of product formation after the initial 2 min of incubation illustrates an intrinsic problem of this type of assay: because the substrate contains a reducing end and because the maximum increase in the number of reducing ends is no larger than a factor 2–3, significant signals can only be obtained upon cleavage of a considerable portion of the substrate. Thus, during the assay significant changes in substrate concentration occur, meaning that enzyme velocity decreases and product formation becomes non-linear. Another limitation comes from the fact that the $K_m$ for $GlcNAc_4$ and $GlcNAc_6$ is likely to be in the low micromolar range (Bokma, Barends, van Scheltinga, Dijkstra, & Beintema, 2000; Brurberg et al., 1996; B. Synstad and V.G.H. Eijsink, unpublished observations). Even the MBTH method is not sensitive enough to produce accurate results in such low concentration range. Thus accurate determination of kinetic parameters such as $k_{cat}$ and $K_m$ (which requires measurements of intial rates at substrate concentrations around $K_m$) is not possible with the MBTH method.

It should be noted that determination of tetramer degradation with the Schales's procedure is essentially impossible because the reducing ends on the product ($GlcNAc_2$) give lower signals than the reducing ends on the substrate ($GlcNAc_4$) (Fig. 1; S. Gåseidnes and V.G.H. Eijsink, unpublished observations).

Beta-chitin is an insoluble substrate for ChiB that gave no background signal in the MBTH method. The degradation of β-chitin by ChiB is shown in Fig. 4. Initially, product formation was linear with time but decreased gradually afterwards. A degradation curve of this shape is typical for insoluble substrates such as chitin (Kurita, Kaji, & Nishiyama, 2000; Shigemasa, Saito, Sashiwa, & Saimoto, 1994) or cellulose (Johnston, Shoemaker, Smith, & Whitaker, 1998; Lee & Fan, 1982). It has been suggested that this is due to substrate depletion caused by conversion of the substrate into a structurally more resistant form during the reaction (Lee & Fan, 1982). Another explanation may be that the amount of substrate that is actually accessible to the enzyme is much lower than the total amount of β-chitin in the reactions. Note that the concentration of reducing ends at the end of the linear phase of the reaction is only 25 µM. Thus, the initial linear phase of the reaction can only be detected with a method as sensitive as the MBTH method.

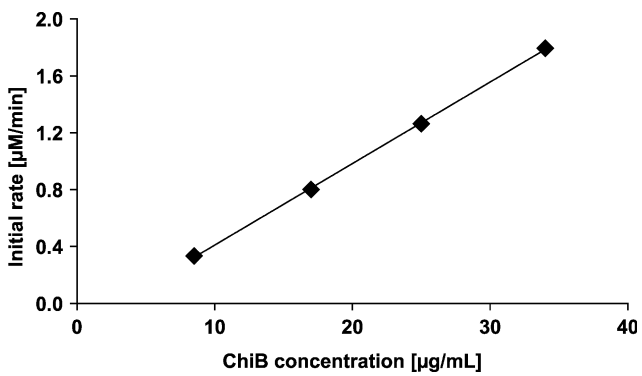


Fig. 5. Initial rates (reducing ends produced per minute) versus ChiB concentration. Data were collected by conducting the experiment depicted in Fig. 4 with various ChiB concentrations. Initial rates were determined by linear regression, using measurements collected before the concentration of reducing ends reached 30 µM.

In order to test the applicability of the MBTH method for quantitative determination of chitinase activity, the initial rate of β-chitin degradation was determined for several enzyme concentrations. Fig. 5 shows that there is a linear relationship between the slope of the linear part of the degradation curve (Fig. 4) and the enzyme concentration. From the slope of the curve in Fig. 5 one may calculate the specific activity of ChiB for β-chitin to be 0.053 mol reducing ends/s/mol enzyme.

## 4. Conclusions

This work shows that the MBTH method (Anthon & Barrett, 2002a) is a sensitive and accurate alternative for the Schales' procedure that is commonly used in chitin research. Although the MBTH method cannot be used for determination of kinetic parameters, it can be used for accurate determination of specific activities towards chito-oligosaccharides and β-chitin.

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## References

Aalten, D. M. F. van, Synstad, B., Brurberg, M. B., Hough, E., Riise, B. W., Eijsink, V. G. H., & Wierenga, R. K. (2000). Structure of a two-domain chitotriosidase from Serratia marcescens at 1.9-angstrom resolution. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(11), 5842–5847.

Anthon, G. E., & Barrett, D. M. (2002a). Determination of reducing sugars with 3-methyl-2-benzothiazolinonehydrazone. *Analytical Biochemistry*, *305*(2), 287–289.

Anthon, G. E., & Barrett, D. M. (2002b). Kinetic parameters for the thermal inactivation of quality-related enzymes in carrots and potatoes. *Journal of Agricultural and Food Chemistry*, *50*(14), 4119–4125.

Bokma, E., Barends, T., van Scheltinga, A. C. T., Dijkstra, B. W., & Beintema, J. J. (2000). Enzyme kinetics of hevamine, a chitinase from the rubber tree *Hevea brasiliensis*. *FEBS Letters*, *478*(1–2), 119–122.

Brurberg, M. B., Nes, I. F., & Eijsink, V. G. H. (1996). Comparative studies of chitinases A and B from *Serratia marcescens*. *Microbiology*, *142*, 1581–1589.

Doner, L. W., & Irwin, P. (1992). L Assay of reducing end groups in oligosaccharides homologues with 2,2′-bicinchoninate. *Analytical Biochemistry*, *202*(1), 50–53.

Garcia, E., Johnston, D., Whitaker, J. R., & Shoemaker, S. P. (1993). Assessment of endo-1,4-beta-D-glucanase activity by a rapid colorimetric assay using disodium 2,2′-bicinchoninate. *Journal of Food Biochemistry*, *17*(3), 135–145.

Hauser, T. R., & Cummins, R. L. (1964). Increasing sensitivity of 3-methyl-2-benzothiazolone hydrazone test for analysis of aliphatic aldehydes in air. *Analytical Chemistry*, *36*(3), 679–681.

Imoto, T., & Yagishita, K. (1971). A simple activity measurement of lysozyme. *Agricultural and Biological Chemistry*, *35*(7), 1154–1156.

Jeffries, T. W., Yang, V. W., & Davis, M. W. (1988). Comparative study of xylanase kinetics using dinitrosalicylic, arsenomolybdate, and ion chromatographic assays. *Applied Biochemistry and Biotechnology*, *70–72*, 257–265.

Johnston, D. B., Shoemaker, S. P., Smith, G. M., & Whitaker, J. R. (1998). Kinetic measurements of cellulase activity on insoluble substrates using disodium 2,2′ bicinchoninate. *Journal of Food Biochemistry*, *22*(4), 301–319.

Kurita, K., Kaji, Y., Mori, T., & Nishiyama, Y. (2000). Enzymatic degradation of beta-chitin: susceptibility and the influence of deacetylation. *Carbohydrate Polymers*, *42*(1), 19–21.

Lee, Y. H., & Fan, L. T. (1982). Kinetic studies of enzymatic hydrolysis of insoluble cellulose: analysis of the initial rates. *Biotechnology and Bioengineering*, *24*(11), 2383–2406.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry*, *31*, 426–428.

Muzzarelli, R. A. A., & Muzzarelli, C. (1998). Native and modified chitins in the biosphere. *ACS Symposium Series*, *707*, 148–162.

Patil, R. S., Ghormade, V., & Deshpande, M. V. (2002). Chitinolytic enzymes: an exploration. *Enzyme and Microbial Technology*, *26*(7), 473–483.

Rudrapatnam, N., Tharanathan, R. N., & Kittur, F. S. (2003). Chitin—the undisputed biomolecule of great potential. *Critical Reviews in Food Science and Nutrition*, *43*(1), 61–87.

Schales, O., & Schales, S. S. (1945). A simple method for the determination of glucose in blood. *Archives of Biochemistry*, *8*(2), 285–292.

Sharrock, K. R. (1988). Cellulase assay method: a review. *Journal of Biochemical and Biophysical Methods*, *17*, 81–106.

Shigemasa, Y., Saito, K., Sashiwa, H., & Saimoto, H. (1994). Enzymatic degradation of chitins and partially deacetylated chitins. *International Journal of Biological Macromolecules*, *16*(1), 43–49.

Somogyi, M. J. (1952). Notes on sugar determination. *Journal of Biological Chemistry*, *195*, 19–23.

Suzuki, K., Sugawara, N., Suzuki, M., Uchiyama, T., Katouno, F., Nikaidou, N., Watanabe, T., & Chitinases, A. (2002). B, and C1 of *Serratia marcescens* 2170 produced by recombinant *Escherichia coli*: enzymatic properties and synergism on chitin degradation. *Bioscience, Biotechnology and Biochemistry*, *66*(5), 1075–1083.

Vlasenko, E. Y., Ryan, A. I., Shoemaker, C. F., & Shoemaker, S. P. (1998). The use of capillary viscometry, reducing end-group analysis, and size exclusion chromatography combined with multi-angle laser light scattering to characterize endo-1,4-β-D-glucanases on carboxymethyl-cellulose: a comparative evaluation of the three methods. *Enzyme and Microbial Technology*, *23*(6), 350–359.