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Determination of the degree of deacetylation of chitosan by potentiometric titration preceded by enzymatic pretreatment

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ABSTRACT

A novel method for determining the degree of deacetylation of chitosan is described. This involves two-abrupt-change potentiometric titration preceded by enzymatic pretreatment (EP-TPT) with chitosanolytic enzyme (cellulase) to reduce the viscosity of chitosan solution. Acidic titration is preferred for the rapid response within the two abrupt changes of pH. Half-deacetylated chitosan (almost soluble in all pH range) was used to prove that the enzyme did not interfere with the measurement. Compared with other methods such as ^{1}H NMR, XRD, FT-IR and linear potentiometric titration, the proposed method is simple, low-cost, accurate, reliable and easy to operate for industrial application.

1. Introduction

Chitin, a β-(1→4)-linked polymer of 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine), exists in the exoskeletons of insects, crustaceans and the cell walls of fungi and algae. Its N-deacetylated derivative, chitosan, can be obtained using concentrated alkali and high temperature. Recently, chitosan is receiving more and more attention since it can be widely used in many areas such as biomedical engineering, fiber industry, environmental protection, functional food and cosmetics. The quality and chemical properties of both chitin and chitosan are closely related with the degree of deacetylation (namely the *DD* value), which is defined as the molar fraction of deacetylated units in the polymer chain. For example, products with different *DD* values have different solubility, viscosity, ion-exchange capacity, ability of flocculation and reaction with amino group (Liu, Chen, Sun, Fan, & Hua, 2003). Thus an accurate, quick and low-cost method for the determination of *DD* value is crucial to meet the industry requirements. Unfortunately, no existing method can satisfy all above-mentioned criteria. The following briefly describes the existing methods and their limitations.

^{1}H NMR method is relatively accurate, and unlike other methods, ^{1}H NMR avoids precise weighting of samples (Lavertu et al., 2003). However, expensive equipments, complicated procedures and high-qualified staff are necessary in this method, which makes ^{1}H NMR unsuitable for industrial on-line monitoring. Compared with ^{1}H NMR method, UV-spectrophotometric determination has lower requirement on equipment. The first derivative UV-spectrophotometric (^{1}DUV) method (Muzzarelli & Rocchetti, 1985; Tan, Khor, Tan, & Wong, 1998) is widely known. However, the use of D-glucosamine would interfere with the determination of N-acetyl-D-glucosamine since the two share the same maximum absorption wavelength (Liu et al., 2003). A new protocol for the UV method using phosphoric acid as a solvent for highly acetylated chitin has been proposed recently (Wu & Zivanovic, 2008). Unfortunately, the improved method has its own shortcoming: the *DD* value would decrease sensitively once the temperature and heating time become slightly higher or longer since the acetyl is combined with phosphoric acid. Infrared spectroscopy method seems feasible since it avoids solubility problem by using dry samples. However, this method needs an extremely well dried sample (this is hard to achieve) since residual water would negatively influence the *DD* measurement, i.e. absorption peak of NH_2– and OH– is difficult to be distinguished in IR spectrum (Kasaai, 2008; Prashanth, Kittur, & Tharanathan, 2002; Yang, Li, Song, & Fan, 2003). Elemental analysis is used to determine the elemental composition of chitosan, and the *DD* was calculated based on the carbon/nitrogen ratio. Therefore, elimination of protein residues present in a chitosan sample is necessary, which may increase nitrogen content and thus overestimate the *DD* value (Jiang, Chen, & Zhong, 2003; Santos, Caroni, Pereira, Silva, & Fonseca, 2009).

Compared with other methods, potentiometric titration methods seem more convenient and cost-saving. These methods include pH-titration, linear titration, and two-abrupt-change titration. The

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first method (pH-metry) is found to be limited due to the residual acid or alkali impurities which can severely influence the experimental result. In particular, chitosan is so easy to absorb moisture that even a short-time exposure to the air would increase its moisture content dramatically (Balázs & Sipos, 2007). The second method, linear potentiometric titration, is good at avoiding the first abrupt change of pH value where the measurement of high-deacetylated chitosan is not accurate due to the flocculation. However, residual acid or alkali in the dry samples can still cause negative interference on the measurement of the first abrupt pH change. The third method, two-abrupt-change potentiometric titration, is superior since it eliminates the potential effect of extra acid or alkali in the sample, and the only flaw is that the unavoidable flocculation of chitosan at alkaline pH makes the solution viscous and may cause measurement error (Jia & Li, 2001; Zhang & Xia, 2004). Even the highly deacetylated chitosan (*DD* > 90%) cannot be determined with this method. These flaws are similar to those of conductometric titration methods, although the latter methods are more accurate than the former because of the easy distinguishable transition points (Jia & Li, 2002; Rusu-Balaita, Desbrieres, & Rinaudo, 2003; Santos et al., 2009).

As for this problem, we propose a novel method in which chitosan solution becomes less viscous and gets a better mass transfer by using a trace amount of chitosanolytic enzyme, a low-cost nonspecific cellulase (Zhang, 2005). Thus the determination of the pH becomes more sensitive, accurate and easier to operate. In addition the cost of this method is low and can be widely applied to the industry.

2. Experimental

2.1. Materials

Cellulase (ROCKSOFT$^{\mathrm{TM}}$ ACE P150 from Dyadic International, Inc.), 80,000 U/g, was measured according to the standard procedures of International Union of Pure & Appl. Chem. (Ghose, 1987). Finely powdered (particle size <80 mesh) and purified chitin and its highly deacetylated chitosan (Sample 7), both from shrimp, were supplied by Laizhou Haili Marine Biotechnology Co. Ltd., China. The lower *DD* chitosan samples were prepared as described (Zhang, Xue, Li, Zhang, & Fu, 2006), and the deacetylation time was 15 and 180 min, respectively, for Samples 1, 2, 3, 4, 5 and 6. All other chemicals were of reagent grade.

2.2. Degree of deacetylation measurement

2.2.1. Two-abrupt-change potentiometric titration preceded by enzymatic pretreatment (EP-TPT)

Accurately disperse 0.1000 g chitosan into 25 mL NaCl solution (0.1 mol/L) and then add 10 mL HCl (0.1062 mol/L) of the standard solution to get protonation and thus increase its solubility. Adjust the pH value to 6 by adding NaOH (0.0992 mol/L) of the standard solution and calculate the total volume of NaOH solution consumed for the other replicates, and then add 0.3 mL cellulase solution (160 U/mL). After 40 °C water-bath incubation for 6 h or more (no flocculation occurred when dropped in 0.1 M NaOH solution), chitosan solution is taken out and replenished with HCl solution and the pH is adjusted back to about 2.5. By adding NaCl solution, the total volume maintains at about 50 mL. With the aid of the pH meter (FE20, Mettler Toledo equipped with a 420 electrode), chitosan is titrated with NaOH (alkaline titration) and then, after the two abrupt pH changes (pH > 10.5), re-titrated with HCl solution (acidic titration), making the pH back to <3. In the process of two abrupt pH changes, both the pH value and the consumption of NaOH (or HCl) are recorded. The degree of deacetylation is calculated using the following formula (Wang & Huang, 2001):

$$DD = \frac{203.195 \times w(\mathrm{NH_2})}{16.02262 + 0.42037 \times w(\mathrm{NH_2})},$$

$$w(\mathrm{NH_2}) = \frac{V \times c \times 100 \times 0.016}{W_{\mathrm{dry}}}$$

where V, c, and W_{dry} stand for volume of NaOH (or HCl) consumption between two abrupt changes of pH, concentration of NaOH (or HCl), and the dry weight of chitosan sample (derived from the moisture content) respectively. *DD* and $w(\mathrm{NH_2})$ are based on the percentage.

To prove that the enzyme added does not influence the accuracy of the final result, two groups of control experiments are conducted using half-deacetylated chitosan (Sample 2), which is soluble in aqueous solution at almost all pH range (Zhang et al., 2006). One group is with cellulase while the other is with distilled water instead of cellulase, and six replicates were performed for each group. *F*-test and two-sample *t*-test with unequal variance for equal mean, performed using Microsoft Excel (Microsoft Corp.), were used to determine whether the results from the two groups were statistically significantly different.

Moisture content of chitosan samples was measured by a Rapid Moisture Meter (MX-50, A&D Company Ltd., Japan).

2.2.2. Linear potentiometric titration

Reagents and procedures are the same as described in Section 2.2.1 except the following steps: when the pH reached around 2, 0.5 mL NaOH solution is added each time stepwise. Both the pH value and the addition of NaOH are recorded. The titration was stopped once the pH reached around 3.2, and the equivalence volume (V_{e}) was obtained from the linear titration curve. Three replicates were performed for each sample (Jiang et al., 2003; Ke & Chen, 1990).

2.2.3. ^{1}H NMR spectra

^{1}H NMR spectra were recorded on a JNM-ECP 600 MHz spectrometer (Japan Electronic Optic Laboratory). Each sample was air-dried at 80 °C for 10 h, followed by 105 °C for 1 h, sealed, cooled with an ice bag and then immediately dissolved in D_2O containing 1% DCl, at a concentration of 10 mg/mL. The experiments were based on Lavertu's method (Lavertu et al., 2003) and run at 70 °C. The chemical shifts are given on the *d* scale relative to sodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS). *DD* was calculated according to the following equation:

$$DD\,(\%) = \frac{100 \times \mathrm{H\text{-}1D}}{(\mathrm{H\text{-}1D} + \mathrm{H\text{-}Ac}/3)}$$

where H-1D and H-Ac are the integrals of the peak of the H-1 anomeric proton of deacetylated monomer (H-1D) and of the peak of the three protons of N-acetyl group (H-Ac), respectively.

2.2.4. FT-IR spectra

The samples were dehydrated as described in Section 2.2.3 and then cooled in a desiccator with CaO. FT-IR spectra were obtained by Fourier transform infrared spectrometer (FT-IR; Bruker Vertex 70) under dry air at room temperature. *DD* of chitosan material was calculated according to the following equation:

$$DD = 100 \times \left(\frac{1 - (\mathrm{A1655}/\mathrm{A3450})}{1.33}\right)$$

which was derived from this absorbance (Brugnerotto et al., 2001; Domard & Rinaudo, 1983; Duarte, Ferreira, Marvao, & Rocha, 2002).

2.2.5. X-ray powder diffraction (XRD) spectra

X-ray diffractograms on powder samples were obtained using a Rigaku D-MAX 2500/PC X-ray diffractometer under the following operating conditions: 40 kV and 150 mA with Cu Ka1 radiation at λ1.5405981 Å and acceptance slot at 0.15 mm. About 20 mg of the sample was spread on a sample stage, and the relative intensity was recorded in the scattering range (2θ) of 5–40° in steps of 0.02°. The crystalline index (CrI; %) was determined using $\mathrm{CrI}_{020} = (I_{020} - I_{\mathrm{am}}) \times 100/I_{020}$, where I_{020} is the maximum intensity below 13° and I_{am} is the intensity of amorphous diffraction at 16° (Focher, Beltranme, Naggi, & Torri, 1990; Kumar, Varadaraj, Lalithac, & Tharanathan, 2004; Zhang, Xue, Xue, Gao, & Zhang, 2005). The linear relationship between CrI_{020} values and their corresponding data of *DD* from ^{1}H NMR was established using Zhang's method (2005):

$$y = -1.636x + 154.7$$

where y and x stand for the crystalline index and the *DD* value, respectively.

3. Results and discussion

3.1. Two-abrupt-change potentiometric titration preceded by enzymatic pretreatment (EP-TPT)

Fig. 1 shows the two abrupt changes of pH in enzymatic hydrolysis–potentiometric titration of chitosans with different *DD* values. From this figure we know that as *DD* value increase, the abrupt changes of pH suffer almost no change except for the volume of NaOH solution. It means our method is suitable for chitosans with both low and high degree of deacetylation. This may be explained by the fact that the cellulase we added makes chitosan degraded and its solution less viscous, and thus better in mass transfer, making the titration more sensitive.

In Table 1, the results of *DD* value measured by EP-TPT method are also compared with the data from other methods such as linear potential titration, XRD, FT-IR and ^{1}H NMR method. Data from ^{1}H NMR method are used as standard value due to its accurate data and low variation of experimental results, as shown in Fig. 2. The results show that the proposed method is not only simple and inexpensive, but also accurate and reliable in the determination of chitosan's *DD*.

In FT-IR methods (Fig. 3), there is no unique reference band that can be used as a reference band for the entire range of the *DD* because of the spectrum of chitosan changing with the *DD* (Kasaai, 2008). In addition, water content induces interferences and difficulties to obtain accurate results especially half-deacetylated chitosan when drying or ambient measuring condition is not appropriate (e.g. Sample 2).

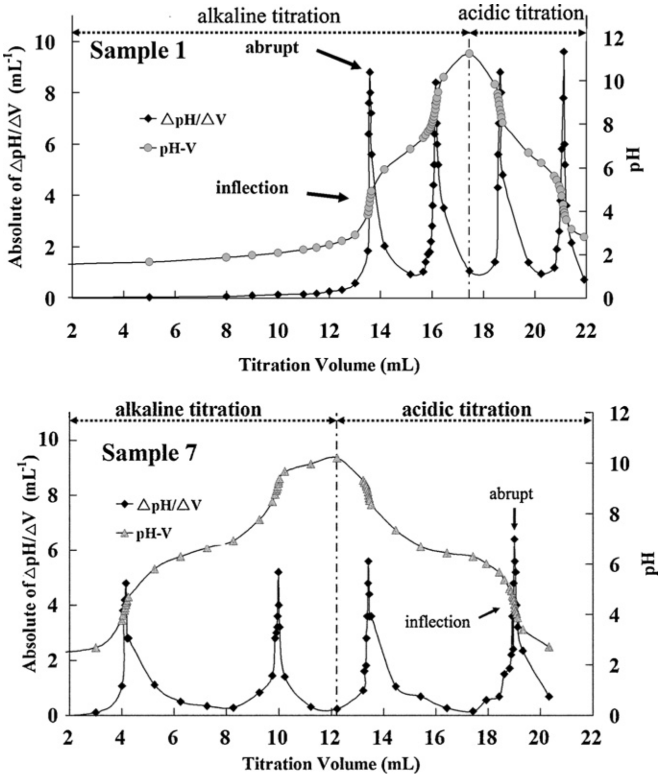

Fig. 1. EP-TPT titration curves of chitosans with different *DD* value. Samples 1 and 7 are described in Table 1.

In XRD method, the regression equation is derived from the *DD* values of ^{1}H NMR and their corresponding data of $\mathrm{CrI}_{020,}$ which vary in different instruments of XRD and measurement condition. Care should be taken in the preparation of the chitosan sample to make the procedure consistent. It is noted that 2θ value of (0 2 0) reflection is an important reference for the evaluation of *DD*, in that (0 2 0) plane moved ordinarily with the increase of *DD* (Fig. 4). However, it is noted that the corresponding spectrum of high *DD* chitosan (*DD* > 90%) indicates that the degree of crystallinity is so poor that a large error would be involved when reading the (0 2 0) intensity (e.g. Sample 7).

Linear potentiometric titration needs refined acid/alkali-free chitosan. These residuals may interfere with the measurement, but may not interfere with our method (EP-TPT) which is based on the deprotonation (by NaOH) or protonation (by HCl) of amino group. Comparing of these two methods, some chitosan samples are believed to have residual alkaline or acid, as shown in Table 1.

Table 1
Degree of deacetylation of chitosan measured by EH-TPT method and by other methods: ^{1}H NMR, XRD and FT-IR method (data from ^{1}H NMR are used as standards).

Sample	EH-TPT (%)		^{1}H NMR	FT-IR (%)	XRD (%)	Linear potentiometric titration (%)
	Titrated by NaOH	Titrated by HCl				
1	41.60 ± 0.34	42.93 ± 0.51[a]	43.3	49.9	47.3	44.23 ± 0.54[a]
2	49.65 ± 0.17[b] 49.76 ± 0.41[c]	50.06 ± 0.44[b] 49.96 ± 0.26[c]	51.5	51.2	51.5	48.34 ± 0.40[c]
3	51.78 ± 0.51[a]	52.91 ± 0.31[a]	52.4	51.7	52.6	55.20 ± 0.64[a]
4	63.66	63.92 ± 0.29[a]	63.3	63.5	62.2	63.20 ± 0.66[a]
5	71.32	72.88 ± 0.25[a]	72.6	70.8	67.0	71.85 ± 0.76[a]
6	79.62	80.45 ± 0.50[a]	79.4	75.7	75.1	75.00 ± 0.56[a]
7	93.18	93.84 ± 0.87[a]	94.0	93.9	96.6	92.47 ± 0.11[d]

[a] Mean ± SD, $n = 3$.
[b] Mean ± SD, $n = 6$.
[c] Mean ± SD, $n = 6$ without cellulase.
[d] Mean ± SD, $n = 8$.

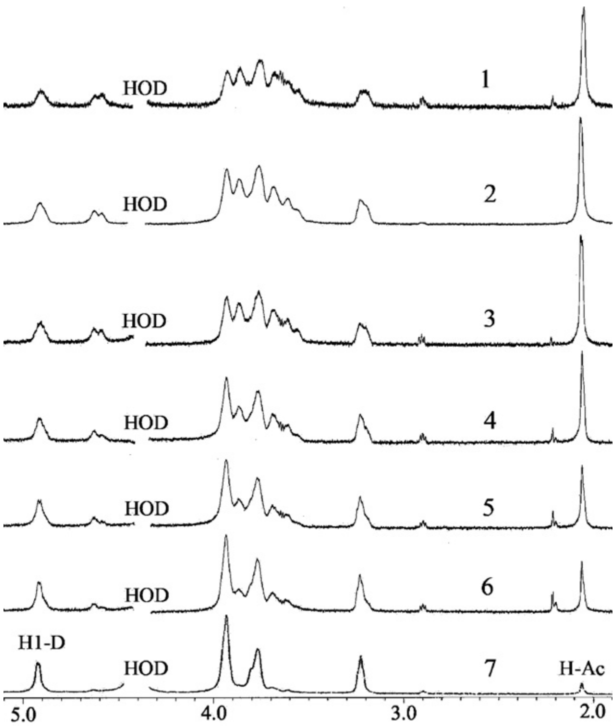

Fig. 2. ^{1}H NMR spectra of chitosan with different degrees of deacetylation (*DDs*), from top to bottom are Samples 1–7 described in Table 1. HOD, H-1D and H-Ac are the peaks of the HOD from H_2O exchange with D_2O, the anomeric proton of the deacetylated monomer and N-acetyl proton, respectively.

3.2. Effect of the enzyme itself on the determination of DD

Does the added enzyme negatively affect the measurement of *DD* value? To eliminate this factor, we conducted two groups of controlled experiment using half-deacetylated chitosan (soluble in aqueous solution at almost all pH range and cellulase is not necessary before titration) as a substrate: one is with cellulase and the other is blank control without any enzyme. The mean values obtained by the former method were statistically indistinguishable ($p = 0.55$ for alkaline titration, $p = 0.65$ for acidic titration) from those obtained by the latter one, on the basis of a Student's *t*-test (two-sample assuming unequal variances). The result shows that the added cellulase not only has little or no effect on the titration but also makes the resulting data stable and precise. Thus the hypothesis, that the enzyme may negatively affect the *DD* determination because of the possibility of containing some certain alkaline groups like amino group and imidazole group, does not hold. And because of its chemical non-interference, this enzyme can be used to measure chitosans with other *DD* values besides half-deacetylated chitosans.

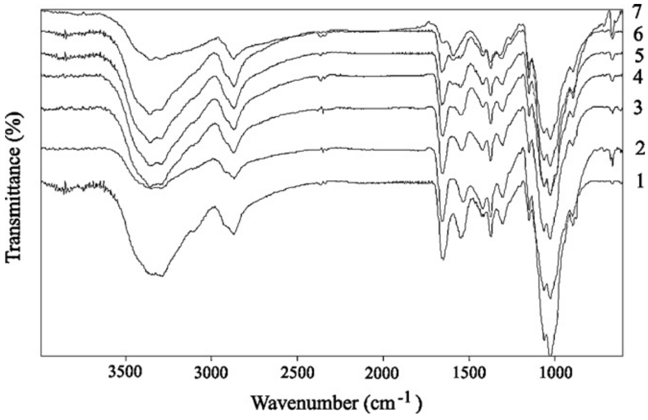

Fig. 3. FT-IR spectra of chitosan samples with different degrees of deacetylation (*DDs*). The *DD* value is rising from Samples 1 to 7.

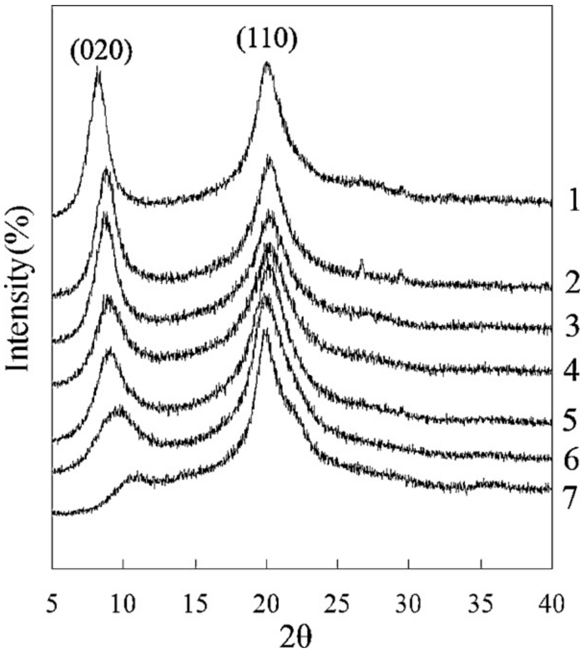

Fig. 4. X-ray diffraction (XRD) spectra of chitosan with different degrees of deacetylation (*DDs*), from top to bottom are Samples 1–7 described in Table 1.

3.3. Effects of enzymatic pretreatment on the determination of DD

In order to decrease the viscosity of chitosan solution, chitosanolytic enzyme is needed especially for the highly deacetylated chitosan samples. Cellulase was proved to be susceptible to aminoglycoside bond and the enzymatic incubation time is increased with the increase of the *DD* (Zhang, 2005). The extent of the enzymatic hydrolysis is determined by the absence of the chitosan hydrogel in alkaline solution. Therefore, in this paper, 6 h is sufficient to degrade high molecular weight of chitosan with the *DD* of 94% (Sample 7).

3.4. Effects of chitosans' hygroscopic nature on the determination of DD

Water absorption is the mean source of measurement error since chitosans own a very high water/moisture affinity. The moisture content of air-dried chitosan can be 15% or more especially for the half-deacetylated chitosan, and a reasonable fraction of this humidity cannot be removed by ordinary drying. Furthermore, hydration will happen when dried chitosan is exposed to the air, even for an extremely short time, which is in agreement with the findings of other authors (Balázs & Sipos, 2007). Therefore, it is important to use a specialized moisture meter to accurately measure a sample's dry weight.

3.5. Alkaline titration and acidic titration

Alkaline titration was used ordinarily in the *DD* measurements. However, in the EP-TPT method, some precipitation (not flocculation, depolymerized chitosan with relatively low-molecular weight and high *DD* is slightly soluble in aqueous solution) occurred in the depolymerized chitosan solutions in a pH region of 6 or more, and in this range the response of the glass-electrode became sluggish and required a long time for equilibrium and measurement. On the contrary, during acidic titration, the precipitates are gradually solubilized due to the protonation of amino groups of chitosan, which makes a much more rapid response than alkaline titration. The acidic titration was modified as follow: NaOH is used instead of HCl to change the pH of the depolymerized chitosan solution from 6 to 10.5 or more, mixed thoroughly with stirrer, ready for the measurement.

Half-deacetylated chitosan (Sample 2) was used for the comparison between the two methods. The mean values obtained by alkaline titration were statistically indistinguishable ($p = 0.08$ with cellulase, $p = 0.35$ without cellulase) from those obtained by acidic titration, on the basis of a Student's t-test (two-sample assuming unequal variances). Therefore, acidic titration with cellulase is preferred for *DD* determination.

4. Conclusion

EP-TPT method is an accurate method in determining the *DD* values, which are in good agreement with the data from the standard ^{1}H NMR method. Considering its accuracy, precision, low-cost and simple operation, this method (EP-TPT) is ideal for *DD* determination especially in industrial application. It is possible for other chitosanolytic (not chitinolytic) enzymes, such as chitosanase, pectinase (Zhang, 2005), to be utilized in the determination of *DD* with this method.

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