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A simple and rapid colorimetric method for the estimation of chitosan produced by microbial degradation of chitin waste

Abhik Mojumdar[a], Anjani Kumar Upadhyay[a], Vishakha Raina[a], Lopamudra Ray[a,b,*]

[a] School of Biotechnology, KIIT Deemed to be University, Bhubaneswar, Odisha 751024, India
[b] School of Law, KIIT Deemed to be University, Bhubaneswar, Odisha 751024, India

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ABSTRACT

Chitin is one of the most abundant biopolymers present in the environment. Chitosan being its major derivative can be obtained by hydrolysis of chitin, especially by microbial degradation. Estimation of resulting chitosan produced by chitin degradation is crucial to the process. Usefulness of the method of Badawy (Badawy, 2012) for estimation of chitosan is limited by interference resulting from susceptibility to variation in the pH of the sample and thiobarbituric acid. This work presents an improvement of the method proposed by Badawy for colorimetric determination of chitosan by using 3, 5-Dinitrosalicylic acid (DNSA) reagent instead of thiobarbituric acid, after one step depolymerization and deamination of chitosan with sodium nitrite ($NaNO_2$). Eventually colorimetric estimation was carried out at 540 nm. With the use of DNSA reagent, the limitation of thiobarbituric acid are overcome. This method is easy, cost effective, and sensitive for quantitative determination of chitosan. This new improved method was applied for evaluation and quantification of chitosan produced by microbial degradation of chitin waste by different novel *Streptomyces* strains.

1. Introduction

Chitosan is a linear binary hetero-polysaccharide composed of β-(1, 4) linked *N*-acetyl glucosamine units (GlcNAc) and glucosamine units (GlcN). In recent years, chitosan has attracted much interest because of its physical and chemical properties. It is excellent in biocompatibility, high bioactivity, and biodegradability (Shahidi et al., 1999), selective permeability, ability to form gel and film, chelation ability and absorptive capacity (Shigemasa and Minami, 1996). Chitosan has many reported applications such as antimicrobial (Rabea et al., 2003) and antioxidant (Ngo and Kim, 2014), anti-tumor (Gibot et al., 2015) properties, drug carrier for controlled release (Felt et al., 1998), decalcification of dental enamel, promotes osteogenesis, fat absorbent action and obesity treatment (Han et al., 1999), promotes the healing of ulcers and lesions (Ito et al., 2000; Dai et al., 2011).

Keeping in mind the huge application of chitosan in various sectors, an easy recovery and rapid estimation method for chitosan is required. However, a simple, rapid and cost effective method for quantitative analysis of chitosan is lacking. Chitosan has glycosidic linkages that are resistant to acid hydrolysis due to the presence of a positively charged amino group (Varum and Smidsrod, 1997). Deacetylation of chitin under high alkaline condition does not necessarily lead to significant depolymerization (Ottoy et al., 1995). As a result methods for total carbohydrate analysis by anthrone or phenol-sulphuric acid methods (Daniel et al., 1994) are difficult to apply. Moreover availability and costs limits the use of chitinase for depolymerization of chitosan (Izume and Ohtakara, 1987). There are several methods reported for determination of chitosan by chromatographic and colorimetric techniques. The determination of aminoglucose by indole (Dische and Borenfreund, 1950) and with ninhydrin (Prochazkova et al., 1999) are commonly used methods which do not involve hydrolysis of chitosan. The bound chitosan may also be quantified by adsorption of anionic dyes such as C.I. Acid Orange 7, C.I. Acid Red 13, C.I. Acid Red 27, Orange II, Alizarin S, Alizarin GG, Congo Red, Cibacron Brilliant Red 3B-A and determined colorimetrically (Roberts and Taylor, 1989; Muzzarelli, 1998), but the reactions between free amino groups of chitosan and the reacting agents cannot determine chitosan in the presence of substances bearing primary amino groups, including peptides (Sekmokiene et al., 2006). Spectrophotometric determination of chitosan based on one step depolymerization and reaction with thiobarbituric acid was reported (Badawy, 2012) but susceptibility to variation in the pH of the sample and thiobarbituric acid results in interference.

The work presented here, describes a simple colorimetric method

* Corresponding author at: School of Law, Campus -16, Adjunct Faculty, School of Biotech, Campus -11, Kalinga Institute of Industrial Technology (KIIT), Bhubaneswar 751024, Odisha, India.
E-mail addresses: lopamudra.ray@kls.ac.in, lray@kiitbiotech.ac.in (L. Ray).

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which has advantages such as accuracy and specificity over the method proposed by Badawy (Badawy, 2012). The proposed method is based on determination of chitosan after depolymerization by $NaNO_2$ (Bosworth and Scott, 1994) which is a weak base. The depolymerization mechanism involves deamination of (1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose unit forming 2, 5-anhydro-D-mannose at the new reducing end. The end product contains 2, 5-Anhydromannose molecules which are determined colorimetrically using DNSA reagent (Miller, 1959) instead of thiobarbituric acid as proposed by Badawy.

$$\text{Chitosan} + NaNO_2 \rightarrow 2,5 - \text{Anhydromannose}$$

The proposed method was optimized for many parameters such as hydrolysis conditions, reaction time, $NaNO_2$ concentration and pH, which influence the accuracy of the method.

2. Materials and methods

2.1. Media, chemicals and cultures

All microbiological media, ISP media and chemicals such as chitin, chitosan etc. used in the current study were purchased from Hi Media, Pvt. Ltd. Mumbai, India; Merck, Germany; SD Fine, India. The pure cultures of *Streptomyces* strains used in the study were obtained under the framework of a project previously carried out in the laboratory (Ray et al., 2013 & Ray et al., 2014).

2.2. Culture conditions and media

The pure isolates were grown and maintained routinely on inorganic salts starch agar medium (International *Streptomyces* Project-ISP medium 4; Shirling and Gottlieb, 1966) fortified with seawater (50%, *v*/v) as the basal medium at 30 °C. Broth cultures were obtained by growing the isolates in starch casein medium (pH 7.0) followed by incubation in a shaker incubator (Innova New Brunswick, Germany) at 30 °C for 72 h.

2.3. Assay reagents

2.3.1. Chitosan solution

A stock solution of chitosan (5 mg/ml (w/v) was prepared in 1% (v/v) acetic acid. Suitable dilutions were prepared from the stock using distilled water (1 mg/ml, 2 mg/ml, 3 mg/ml, and 4 mg/ml).

2.3.2. Sodium nitrite reagent (HiMedia)

An aqueous solution of 0.5 M $NaNO_2$ was prepared and stored at 4 °C.

2.3.3. Dinitrosalicylic (DNSA) acid reagent (HiMedia)

DNSA reagent (50 ml) was prepared by dissolving 15 g of sodium potassium tartrate, 10 ml of 2 N NaOH and 0.5 g of DNSA and the volume was made up to 50 ml using distilled water.

2.4. Preparation of a standard curve for mannose

A standard curve of commercially available mannose (HiMedia, India) was prepared (shown in Fig. 3) for a concentration range of 100 μg/ml to 1000 μg/ml by following standard assay protocol using DNSA reagent (Miller, 1959).

2.5. Preparation of a standard curve for 2, 5-Anhydromannose (Deamination product of chitosan after treatment)

A standard curve for 2, 5-Anhydromannose was prepared by using commercially available chitosan (HiMedia, India) for a concentration range of 100 μg/ml to 1000 μg/ml followed by treatment of the same with $NaNO_2$. The end product was assayed with standard assay protocol using DNSA reagent (Miller, 1959).

2.6. Assay procedure

0.1 ml of $NaNO_2$ (0.5 M) was added to the tubes containing sample, blank and standard (1 ml each). The reaction mixtures were then heated at 80 °C for 45 min in a water bath to complete the depolymerization – deamination reaction and cooled under tap water. 1 ml of DNSA was then added to the reaction mix after adjusting the pH to 4 by adding 1 N HCl. The tubes were then placed in water bath at 75 °C for 15 min. The tubes were cooled under tap water and absorbance was measured at 540 nm.

The method was also conducted at semi-micro scale with a final volume of 0.5 ml. Reaction Volumes of the reagents were reduced proportionally. In brief, 250 μl of the sample was mixed with 25 μl of 0.5 M $NaNO_2$ followed by incubation at 80 °C for 45 min in a water bath 250 μl of DNSA was then added to the reaction mix after adjusting the pH to 4 by adding 1 N HCl. The tubes were then placed in water bath at 75 °C and the colour development was observed.

2.6.1. Effect of reaction time on colour development

The effect of different incubation time on the reaction mix for the development of the colour was studied. The reaction containing 0.1 ml $NaNO_2$ (0.5 M) and 1 ml chitosan (3 mg/ml) was incubated for time range from 20 to 80 min. (20, 30, 40, 50, 60, 70, 80 min). The resulting product was analyzed by DNSA reagent (Miller, 1959).

2.6.2. Effect of volume of $NaNO_2$

Effect of different volumes of $NaNO_2$ solution for end product development was studied. Since $NaNO_2$ was used for deamination of chitosan, thus effect of different volumes in the eventual deamination was required to be analyzed. The reaction containing 0.1 ml of 0.5 M $NaNO_2$ and 1 ml chitosan (3 mg/ml) was incubated for optimized time. The resulting product was analyzed by DNSA reagent (Miller, 1959).

2.6.3. Effect of pH

Effect of different pH on the reaction was studied. The chitosan solutions (3 mg/ml) were prepared in different pH (pH 4, 7 and 10). The reaction containing 0.1 ml 0.5 M $NaNO_2$ and 1 ml chitosan (3 mg/ml) was incubated for optimized time. The resulting product was analyzed by DNSA reagent (Miller, 1959).

2.6.4. Comparison of mannose and chitosan

A standard curve for 2, 5-Anhydromannose prepared by the method described in Section 2.5 was compared with the standard curve of mannose (shown in Fig. 3) for a concentration range of 100 μg/ml to 1000 μg/ml.

2.6.5. Quantification of chitosan resulted from chitin degradation by Streptomyces strains

The proposed method was used to quantify chitosan produced by degradation of chitin by novel *Streptomyces* strains. The strains were grown in Mineral salt medium (Hsu and Lockwood, 1975) supplemented with 1% colloidal chitin (*w/v*). Samples (2 ml culture aliquots) were taken out at an interval of 48 h and 72 h and the supernatant was collected by centrifugation. 0.5 ml of the collected supernatant was then mixed with 0.05 ml $NaNO_2$ (0.5 M) in a micro centrifuge tube. The tubes containing the reactions were then placed in a water bath at 80 °C. After 45 min the reaction tubes were taken out and cooled to room temperature and pH was adjusted to 4 by adding 1 N HCl. DNSA (0.5 ml) reagent was then added to the tubes containing the reaction mix and incubated at 75 °C for 15 min. The tubes were then cooled to room temperature and absorbance was measured at 540 nm.

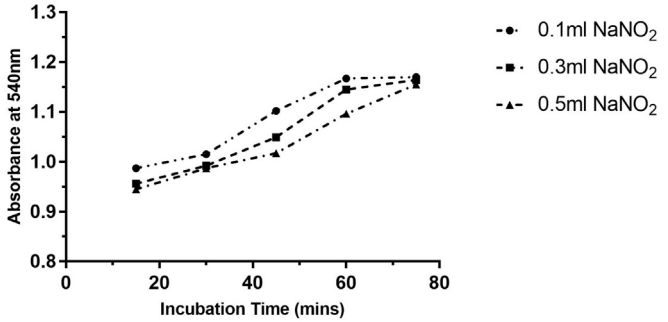

Fig. 1. The effect of different volumes of $NaNO_2$ (0.1 ml–0.5 ml) on reaction mixture with different incubation time (20–80 min) is shown. Concentration of chitosan was 3 mg/ml. Absorbance was measured at 540 nm.

3. Results and discussions

3.1. Optimization of the reaction conditions

Reaction conditions were optimized based on factors that influence the reaction and colour production such as reaction time, pH, and volume of $NaNO_2$ etc.

3.2. Effect of reaction time on colour development

The development of colour in this reaction depends on formation of an intermediate, which then reacts with DNSA. The maximum absorbance was observed in the sample containing $NaNO_2$ and chitosan solution incubated for 45 min at 80 °C (Fig. 1). Thus it can be concluded that the optimum time required for breakdown of chitosan with $NaNO_2$ is 45 min.

3.3. Effect of volume of $NaNO_2$

Effect of different volume of $NaNO_2$ on the reaction was studied. The results revealed that colour yield for 1 ml of chitosan solution (3 mg/ml) with 0.1 ml of $NaNO_2$ (0.5 M), was more in comparison to 0.3 ml and 0.5 ml of $NaNO_2$ (Fig. 1).

3.4. Effect of pH

The yield of coloured complex was more in the sample whose pH was adjusted to 4 followed by the sample with pH 7 and pH 10 respectively. The range of absorbance values for a particular set of concentration of chitosan was higher at acidic pH (Fig. 2). Hence, acidic pH (near pH 4) was considered as optimum under standard assay conditions.

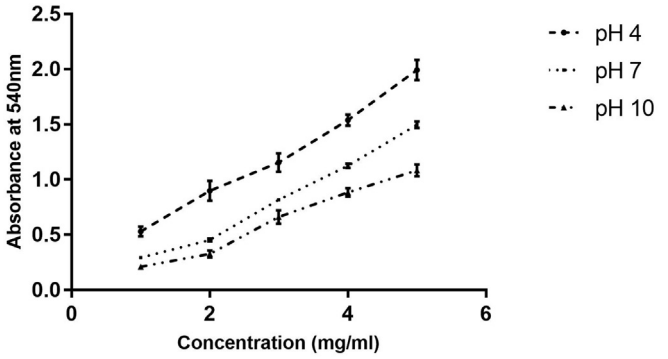

Fig. 2. The effect of pH on the yield of colour. The function of pH i.e., acidic (pH 4), neutral (pH 7) and basic (pH 10), for reaction of the reducing end of chitosan.

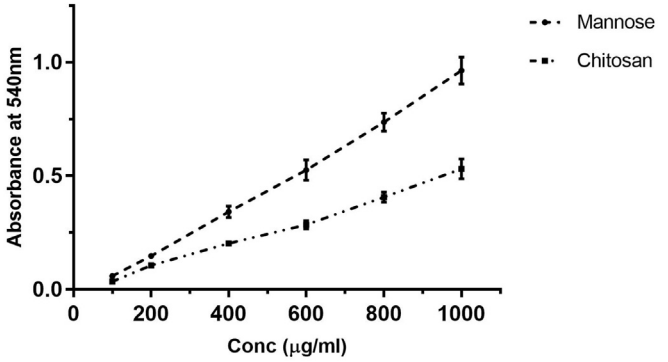

Fig. 3. Standard analytical curve of mannose and chitosan for a concentration range of 100 µg/ml to 1000 µg/ml is compared. Chitosan was pretreated with 0.5 M 0.1 ml $NaNO_2$ for 1 ml chitosan sample. Absorbance is measured at 540 nm. Reliability of regressions: $R^2 > 0.997$.

3.5. Comparison of mannose and chitosan

A standard curve of commercially available mannose (HiMedia, India) was prepared and was compared with the standard 2, 5-Anhydromannose as described in Section 2.5 and 3.4 (Fig. 3) for a concentration range of 100 µg/ml to 1000 µg/ml. It was observed that the slope of the curve for mannose is higher than the slope of chitosan. The probable reason may be the structure of chitosan that is composed of β-(1, 4) linked *N*-acetyl glucosamine units (GlcNAc) and glucosamine units (GlcN), where the carbonyl group is not available for oxidation and depolymerization of chitosan yields monomers and oligomers of GlcNAc units and GlcN units creating free aldehyde group for oxidation by $NaNO_2$.

3.6. Calibration curve and final optimized assay

Different concentrations of chitosan ranged from 10 µg/ml to 5000 µg/ml were mixed with 0.5 M 0.1 ml $NaNO_2$. After incubation for 45 min at 80 °C in water bath, pH was adjusted to 4. DNSA reagent was then added and followed by incubation at 75 °C for 15 min in water bath and cooled to room temperature. Absorbance was then measured at 540 nm. The variation of absorbance with respect of concentrations of chitosan are shown in Fig. 4 as a semi log plot and a linear standard analytical curve of chitosan ranged from 10 µg/ml to 5000 µg/ml are shown in Fig. 5.

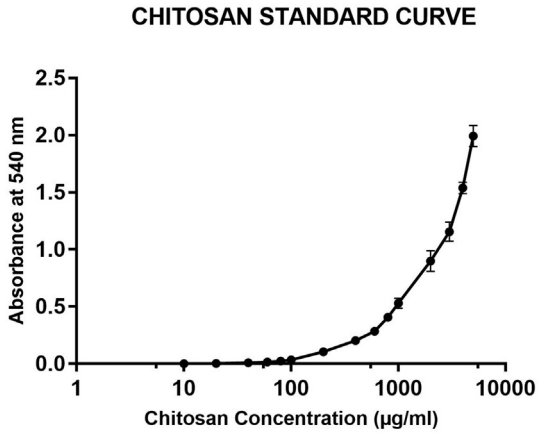

Fig. 4. Semilog plot showing absorbance as a function of chitosan concentration. Different range of chitosan concentration (10 µg/ml to 5000 µg/ml) were examined at optimum conditions in the proposed method at 540 nm. Reliability of regressions: $R^2 > 0.996$.

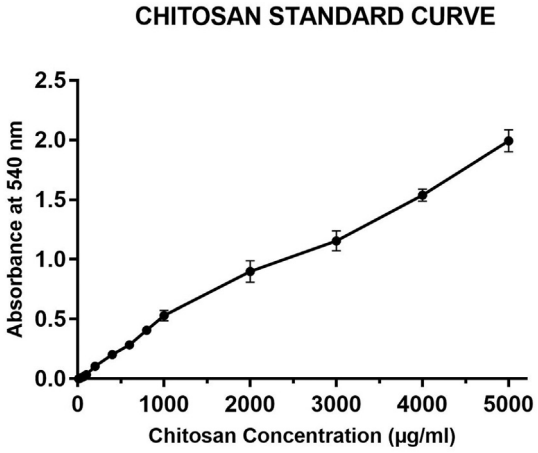

Fig. 5. Absorbance shown as a function of chitosan concentration for the standard analytical curve for chitosan at optimum conditions in the proposed method at 540 nm. Reliability of regression: $R^2 > 0.996$.

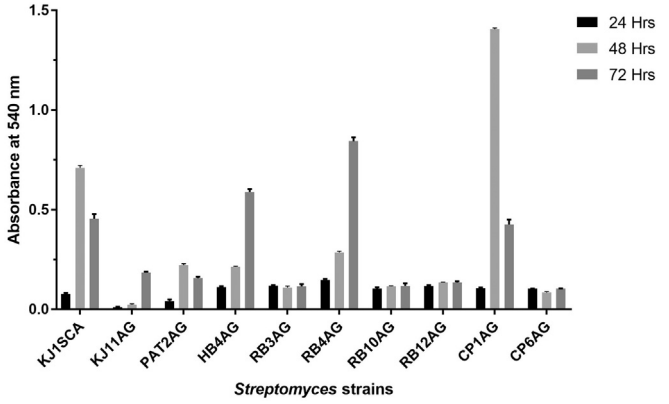

Fig. 6. Screening of novel *Streptomyces* strains by quantifying chitosan produced by degradation of colloidal chitin. In brief 500 µl of the culture supernatant was mixed with 50 µl of $NaNO_2$ followed by heating at 80 °C for 45 min and then pH was adjusted to 4. The mixture was cooled down to room temperature and 500 µl DNSA was added and incubated at 65 °C - 75 °C for 15–20 min. The absorbance was measured at 540 nm.

3.7. Quantification of chitosan resulted from chitin degradation by Streptomyces strains

To evaluate the performance of the method for analysis of real samples, microbial degradation of chitin waste by different novel strains of *Streptomyces* were investigated. The results shown in Fig. 6 revealed that the proposed method is suitable for determination of trace amount of chitosan.

The effects of different time of incubation, pH and volume of $NaNO_2$ are shown in Figs. 4 and 5. The optimal time for deamination by sodium nitrite was 45 min. The optimum volume of $NaNO_2$ required for deamination of 1 ml chitosan sample was 0.1 ml. Increased concentration of $NaNO_2$ did not show any significant deamination. The optimum pH for the reaction is 4.

The rate of colour development is dependent upon the incubation time and also on acidity of the final mixture. At room temperature full colour development required 15–20 min and then it retained the colour till 6–12 h. Sodium nitrite concentration has little effect on the formation of the intermediate compound. It was observed that 0.1 ml of $NaNO_2$ resulted in a better deamination. It was concluded that conditions selected for standard procedure are optimum and slight deviation (+ = 2%) in the volume of sample, $NaNO_2$ volume and DNSA reagent have no significant effect on the assay.

After incubation, the brick red colour developed in the samples where as there was no colour in the blank. Difference in colour was visually distinguishable as the colour varied with the concentration of chitosan. Absorbance was taken at 540 nm. The value of blank was subtracted from the absorbance of chitosan solution of different concentrations and the final absorbance was noted.

The retention of the colour of the final solution after the second incubation was also measured at 0th hour, 6th hour and 12th hour. It was revealed that there was negligible change in the absorbance value hence it can be concluded that the colour is retained for a long period of time.

An analytical method is specific if its signal depends only on the analyte (Vessman et al., 2001; Persson and Vessman, 2001). The matrix may contain both GlcN and GlcNAc units, but no other side reactions were observed since $NaNO_2$ is selective and reacts only with GlcN units. As per the slope of standard (calibration) curve, the analytical sensitivity for determination of chitosan by the proposed method was found to be 0.0011 absorbance/amount for 10 µg/ml chitosan.

Analytical sensitivity varies substantially when different sample matrices are used. Matrix refers to components of the sample other than the analyte i.e. chitosan. It is important to use a medium that has qualities that are similar to the sample matrix. Interference may occur if chitosan is introduced to endogenous substances that originate from within the organism, source etc. or exogenous substances such as powdered gloves, hand cream, etc. Moreover presence of any reducing sugar in the matrix or in the medium will cause interference as DNSA reacts with all reducing sugars and hence it will have an influence on the absorbance.

All the data on the curves in Figs. 1–6 represent a mean value of three replicates. The slopes of the curves for mannose and chitosans were reproduced within + = 2%. The reliability of regression curves in Figs. 4 and 5, expressed as coefficient of determination (R^2) was > 0.996. The probability value or *P* value was found to be < 0.0001.

3.7.1. Applications of this method

The proposed colorimetric method is cost effective, sensitive and rapid. Hence it can be used for routine analysis of multiple samples. Screening of strains that produces chitosan by degrading chitin-based substances can also be carried out. It can be also used in pharmaceutical aspects for determining the chitosan concentrations.

4. Conclusion

The reaction of chitosan with $NaNO_2$ and DNSA is rapid, sensitive, specific and reproducible. The linear standard curves were obtained in the range of 10 µg/ml – 5000 µg/ml of chitosan concentration. This method has been used for quantification of chitosan produced by microbial degradation of chitin waste (from the current study), which is easy and can be used for routine analysis of samples.

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