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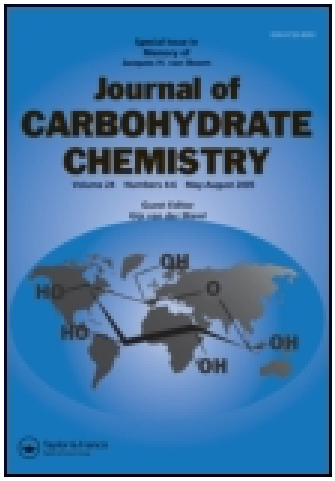

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A UV-Vis Spectrophotometric Method for the Estimation of Aldehyde Groups in Periodate-Oxidized Polysaccharides Using 2,4-Dinitrophenyl Hydrazine

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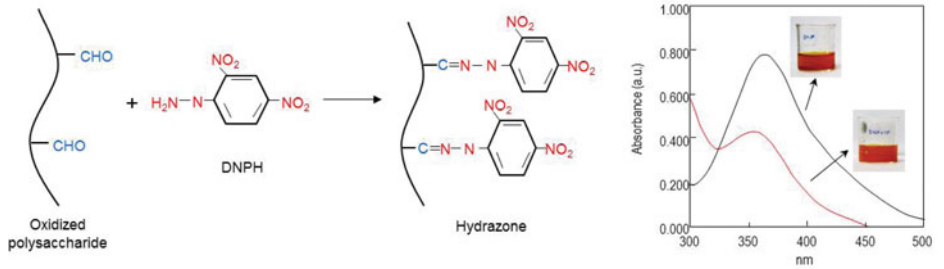
GRAPHICAL ABSTRACT



In this study, efforts have been made to develop a simple approach to determine aldehyde content in periodate-oxidized polysaccharides. A UV-Vis analytical technique using *2,4*-dinitrophenylhydrazine (DNPH) was employed to calculate the aldehyde content. When tested against compounds like acetaldehyde and formaldehyde with known aldehyde content, the DNPH method yielded accurate results. The DNPH assay was

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further utilized to analyze the aldehyde generation during periodate oxidation of pectin, a naturally occurring plant polysaccharide. It was found that the DNPH assay was sensitive even at low concentrations of aldehyde and yielded accurate results. Therefore, this method could be used to control and optimize reaction parameters during the periodate oxidation of polysaccharides.

Keywords Aldehyde analysis; Pectin; Dinitrophenyl hydrazine; UV-Vis; Periodate

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INTRODUCTION

Functionalization of polymers offers an alternative and economic route for product development, as compared to polymer synthesis. Today, a lot of research is devoted to product development using naturally available polymers, such as polysaccharides and proteins.[1–4] Polysaccharides contain a large number of functional groups on their backbones, such as hydroxyl, carboxyl, amine, carboxymethyl groups, and so forth, which render them easily susceptible to functionalization.

In the current article, the emphasis is placed on reaction mechanisms that lead to the inclusion of aldehyde units in the backbone structure of polysaccharides. Aldehyde functional groups are highly reactive and may further be used to form strong covalent linkages between the modified polysaccharide and other biopolymers, such as proteins. Aldehydes are generated primarily through oxidation of hydroxyl functional groups present on the backbone of glucopyranose rings in polysaccharides. Two of the most popular methods used for the oxidation of polysaccharides are the *2,2,6,6*-tetramethyl-*1*-piperidinyloxy (TEMPO)-mediated oxidation using sodium bromide or sodium hypochlorite, and periodate oxidation. While both of these methods are highly selective, the TEMPO-mediated reaction oxidizes primary hydroxyl functional groups to aldehydes or metal salts, depending on the polysaccharide.[29,30] This methodology cannot be used universally to incorporate aldehyde functional groups in the polysaccharide structure, since only those materials containing $-CH_2OH$ groups can be converted to aldehydes. Contrastingly, periodate oxidation, irrespective of the presence or absence of $-CH_2OH$ units, selectively cleaves the C—C bond between the vicinal diols or amino alcohols present on the C1-C2 or C2-C3 carbons of the polysaccharide chain to yield a dialdehyde structure.[31,32] The reaction mechanism is highly selective, and it is necessary that the vicinal diols or amino alcohols are oriented in an equatorial-equatorial or axial-equatorial position. An alternative method for the generation of aldehyde units in polysaccharides was put forth by Kulkarni and Mehta.[33] In a manner similar to periodate oxidation, the vicinal diols present on the C2 and C3 carbons are converted to aldehyde moieties. However, this methodology has not been pursued much due to the difficulty in separating leftover Ce^{4+} from the reaction medium. On the other hand, periodic acid and its salts are highly biocompatible and do not pose toxicity issues even when used in close contact

with biological systems. Therefore, periodate oxidation seems the most viable method to incorporate aldehyde units in polysaccharide structures. Periodate-oxidized polysaccharides have been used for a variety of biomedical applications such as wound care,[5,6] tissue engineering,[7–9] and drug delivery.[10–12,15] Apart from biomedical applications, periodate-oxidized polysaccharide products have been used as flocculating agents[34] and for ion-exchange separation.[35] A recent patent outlines the method for production of nonwoven fibrous mats with high wet and dry strength by crosslinking periodate-oxidized cellulose with transition metal containing crosslinking agents.[36] In each case, the aldehyde content determined the suitability of the material for a given application. Additionally, periodate oxidation is concomitantly accompanied by degradation of the glycosidic linkages. Therefore, to control the loss in molecular weight and optimize the reaction parameters, a precise analysis regarding the degree of oxidation is mandatory.

A large number of methods have been reported earlier to determine the aldehyde content. Maekawa et al. calculated the degree of oxidation in partially oxidized *2,3*-dicarboxy cellulose by UV-Vis spectrophotometric analysis of residual periodate ions.[13] At the end of the oxidation reaction, the absorbance of the unreacted periodate ions was measured and a calibration equation was employed to calculate the degree of oxidation. Since oxidation is an instantaneous and continuous process, the time gap between the end of the reaction and measurement plays a crucial role in this process, thus allowing for the possibility of an error. One of the most popular procedures used for the determination of aldehyde content is the oxime conversion method.[14,16–19] The aldehyde groups generated are converted to oximes by Schiff base reaction with hydroxylamine hydrochloride. These were further analyzed by titrimetry, NMR analysis, elemental analysis, and thermogravimetric analysis. A major drawback of titrimetry is the ambiguity involved in determining the end point by the operator. While elemental, NMR, and thermogravimetric analysis yield accurate aldehyde content values, they are expensive and tedious methods.

Alternatively, a UV-Vis analytical technique using *t*-butyl carbazate and trinitrobenzenesulfonic acid was utilized to decipher the degree of oxidation.[20,21] Aldehyde moieties on the oxidized polysaccharide backbone reacted with *t*-butyl carbazate to form Schiff bases. Unreacted carbazate units were then allowed to react with trinitrobenzenesulfonic acid and the colored complex thus formed was quantified spectrophotometrically. In order to further simplify the aldehyde assay, we designed a one-step procedure using *2,4*-dinitrophenyl hydrazine (DNPH). It has already been established that hydrazines react with carbonyl compounds to form hydrazones.[22–24] DNPH has previously been used to quantify protein carbonyl markers[25] and stereoisomers of low-molecular-weight aldehyde compounds[26] by HPLC analysis. In the current study, we developed a new and easier method to analyze periodate oxidation using DNPH assay spectrophometrically. The DNPH assay

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was initially employed against low molecular weight compounds with known aldehyde content as the control with a view to check the accuracy of the obtained results. Pectin, a naturally occurring polysaccharide, was subsequently oxidized by periodic acid and the aldehyde content generated was analyzed using the DNPH assay.

RESULTS AND DISCUSSION

Pectin is a naturally occurring polysaccharide, poly(*1,4*-galacturonic acid), obtained from the cell walls of terrestrial plants. Under suitable conditions, the vicinal diols present on the C2 and C3 carbons of the anhydro-D-glucopyranose ring undergo oxidation by periodic acid and are converted to aldehyde units. The periodate oxidation is instantaneous, selective, and highly dependent on the reaction parameters. Due to the formation of hemiacetals between aldehyde units and neighboring hydroxyl groups, complete oxidation is not possible. In addition, acid-catalyzed cleavage of the *β-1,4*-glycosidic bonds takes place, leading to degradation.[17] That being the case, a precise control over aldehyde generation is important so as to retain the applicability of the polymer. In the current study, pectin was oxidized by 0.5 M periodic acid at 60°C for different periods of time. The reaction mechanism is shown in Figure 1(a). While various methods are available in the literature for aldehyde analysis, most of them are subject to tedious sample preparation and inaccuracy. Therefore, we have attempted to develop a simple method for detecting and quantifying aldehyde generation in periodate-oxidized polysaccharides by using DNPH.

The reaction of carbonyl compounds with DNPH forming the corresponding hydrazones is one of the most important qualitative methods in organic analysis.[26] In addition to detecting the presence of carbonyl compounds, the DNPH assay could also be used to identify the specific aldehyde or ketone. During this addition-elimination reaction, nucleophilic addition of the -NH_2 group to the carbonyl group takes place with the removal of a water molecule. In the current study, we have harnessed this simple reaction mechanism and developed a UV-Vis-based analytical technique to quantify the aldehyde content in periodate-oxidized polysaccharides. Initially, the DNPH UV-Vis methodology was tested against low-molecular-weight aldehydes, such as formaldehyde, glutaraldehyde, and acetaldehyde, to evaluate its accuracy and efficiency. A representative reaction between formaldehyde and DNPH is depicted in Figure 1(b). Immediately upon addition of aldehyde to the DNPH solution, a yellow precipitate typical of an aliphatic compound is formed. A distinct shift and loss in intensity of the absorption maxima was observed when the UV-Vis spectra of the supernatant (unreacted DNPH) fluid were recorded, as shown in Figure 2. A strong UV band in between 355 and 360 nm was observed. This is attributed to transitions due to the excited resonance state of DNPH in

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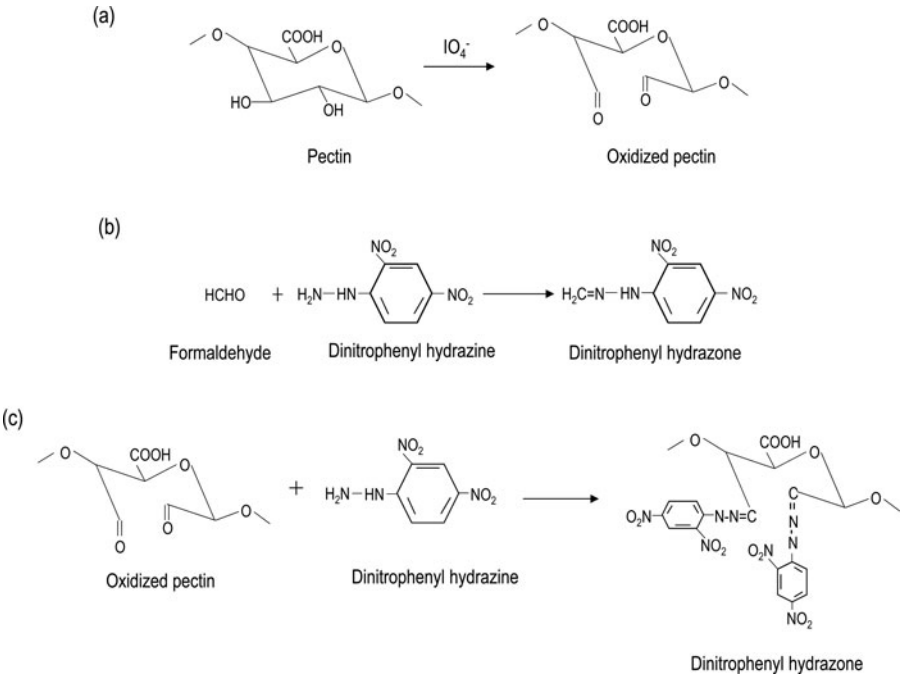

Figure 1: (a) Periodate oxidation of pectin. (b) Reaction of formaldehyde with DNPH. (c) Hydrazone formation upon reaction of DNPH with periodate-oxidized pectin. Reaction temperature 25°C; reaction time 1 h.

solution form.[28] While the absorption maximum for DNPH was recorded at 360 nm, it shifted to ~357 nm after reaction with formaldehyde. Uchiyama et al.[26] theorized that due to stereoisomerism, the absorption maximum experiences a shift. Therefore, the DNPH UV-Vis assay can also be used to identify the type of isomer. In order to calculate the aldehyde content, a calibration equation was formulated at 357 nm from the calibration curve presented in Figure 3. The theoretical and actual values of aldehyde content in formaldehyde, acetaldehyde, and glutaraldehyde at varying concentrations were compared and are found to be in reasonably good agreement, as shown in Figure 4. Therefore, it was surmised that the DNPH UV-Vis assay gave an accurate measure of the carbonyl content.

Subsequently, the DNPH assay was utilized to analyze the aldehyde content generated in periodate-oxidized pectin. The oxidized pectin thus produced was reacted with DNPH; the reaction mechanism and hydrazone formation are depicted in Figure 1(c). The reaction between the aldehyde groups in oxidized pectin and DNPH is instantaneous, resulting in the formation of a yellow precipitate. Though there is a possibility of hydrogen bond formation between the unreacted hydroxyl groups of pectin and the amino groups of DNPH, they are considered to be miniscule. Also, the possible interactions

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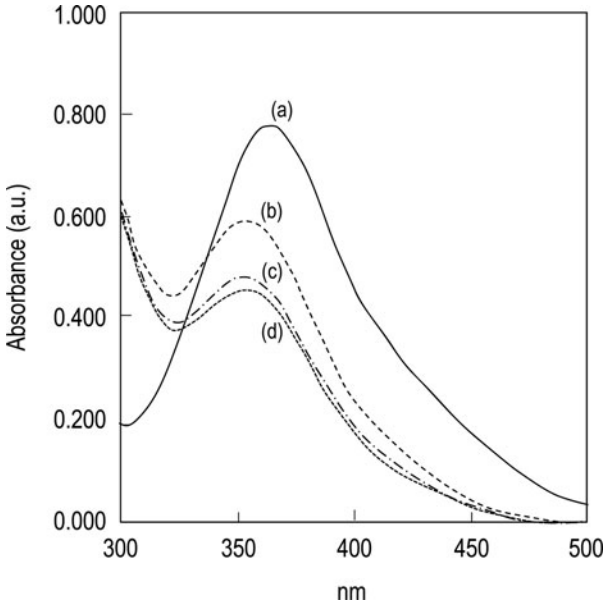

Figure 2: UV-Vis spectra of DNPH solutions upon reaction with formaldehyde at varying concentrations: (a) DNPH alone; (b) with 20% formaldehyde; (c) with 30% formaldehyde; (d) with 37% formaldehyde. Reaction temperature 25°C; reaction time 1 h.

between the carboxylic groups of pectin and the amino groups of DNPH are hindered due to steric factors. The effect of reaction time on the aldehyde content was monitored and is presented in Figure 5. With an increase in reaction time, a progressive increase in the aldehyde content was observed. However,

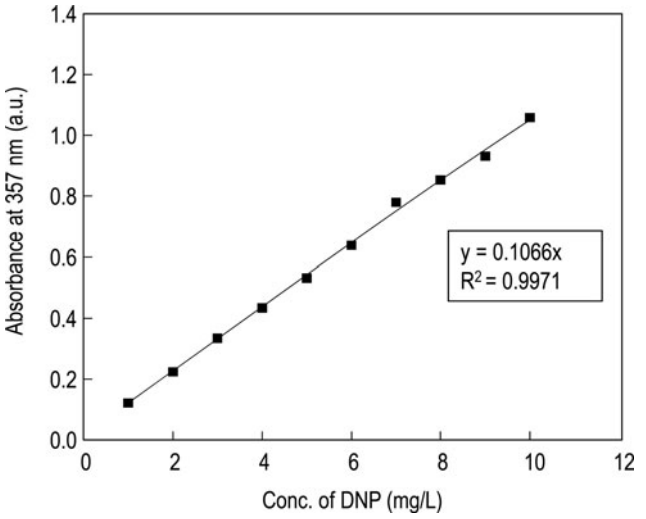

Figure 3: Calibration curve of DNPH in water–ethanol mixture.

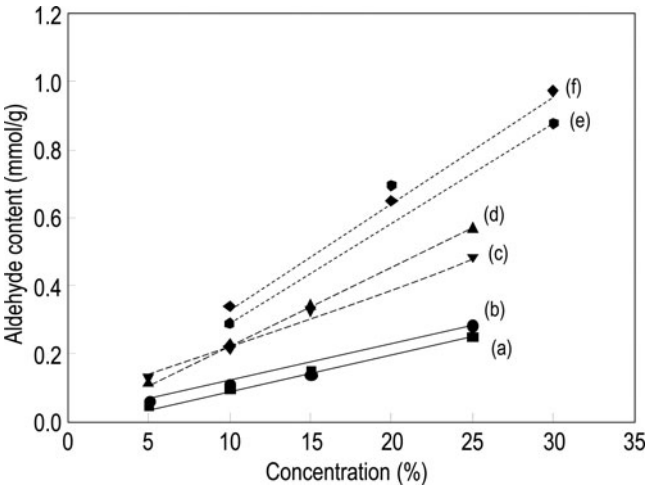

Figure 4: Effect of concentration on the aldehyde content of theoretically calculated (a) glutaraldehyde, (d) acetaldehyde, and (f) formaldehyde, and experimentally observed (b) glutaraldehyde, (c) acetaldehyde, and (e) formaldehyde.

at a reaction time of 3 h, it seems as though the saturation level has been reached, indicating the highest degree of oxidation. Thus, it could be surmised that to achieve maximum aldehyde content with 0.5 M periodic acid at 60°C and solution pH of 3.5, the reaction time should be 3 h.

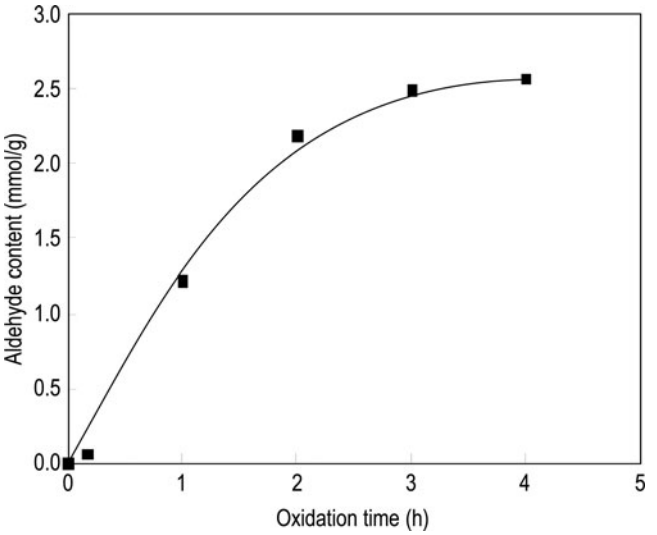

Figure 5: Effect of oxidation time on the aldehyde content. Oxidation temperature 60°C; pH 3.5; periodic acid concentration 0.5 M.

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CONCLUSIONS

A facile and economic analytical approach using DNPH and UV-Vis spectrophotometry has been developed to determine aldehyde content in periodate-oxidized polysaccharides. DNPH reacts with carbonyl groups to form the corresponding hydrazones. A close resemblance was obtained between the theoretical and calculated values of aldehyde content in formaldehyde when it was analyzed using the DNPH UV-Vis assay. The DNPH UV-Vis assay was further employed to analyze aldehyde generation during periodate oxidation of pectin. The results indicate sensitivity and accuracy of the protocol. Therefore, the DNPH UV-Vis assay could be used to optimize and control reaction conditions during the periodate oxidation mechanism.

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EXPERIMENTAL

Materials

Citrus pectin (Mw∼30,000 g/mol, degree of esterification∼72%) and DNPH were purchased from CDH Fine Chemicals (India). They were used as received. Formaldehyde (35%), glutaraldehye (25%), acetaldehyde (25%), periodic acid, sulphuric acid (95%), absolute ethanol, and isopropanol were obtained from Merck Chemicals (India). All other chemicals used were of analytical grade. Millipore water was used for all the experiments.

Oxidation of Pectin by Periodic Acid

Periodate oxidation of pectin was carried out according to the procedure reported in our earlier work.[27] To a 2% solution of pectin in distilled water, 3 mL of 0.5 M periodic acid was added. The pH of the medium was maintained at 3.5 using dilute hydrochloric acid and sodium bicarbonate solution. The reaction was allowed to take place under constant stirring for specific time periods at 60°C. The reaction vessel was wrapped in several layers of aluminium foil and the reaction was carried out in the dark, to prevent autooxidation due to light. At the end of the reaction, oxidized pectin was precipitated out using excess isopropanol, separated by vacuum filtration, and dried at 60°C.

Preparation of *2,4*-dinitrophenylhydrazine Reagent

40 mg of DNPH was dissolved in 0.3 mL of concentrated sulphuric acid. To this slurry, 3 mL of ethanol was added under continuous stirring at 25°C. The mixture was allowed to react for 10 min to achieve homogenization. The volume was then made up to 10 mL with distilled water.

Determination of Aldehyde Content

To 10 mL of freshly prepared DNPH solution, 100 μL of formaldehyde solution (10%–37%), glutaraldehyde (5%–25%), acetaldehyde (5%–25%), or 0.3% oxidized pectin was added. The reaction mixture was allowed to stand for 1 h at 25°C and then centrifuged at 10,000 rpm for 10 min. The absorbance of unreacted DNPH in the supernatant fluid was measured at λ of 357 nm using a Shimadzu UV 2450 UV-Vis spectrophotometer. The amount of aldehyde generated was calculated according to:

$$\text{Aldehyde conc. (mmol/g)} = \left[\frac{\text{Reacted DNP (mmol/g)}/198.14}{\text{Conc.}(\%) \times 10^{-4}} \right],$$

where 198.14 is the molecular weight of DNPH.

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