Original Research Article

Determination of Levan from *Bacillus licheniformis* by Ultraviolet Spectrophotometry

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Abstract

Purpose: To establish a first-order derivative ultraviolet spectrophotometric (FODUS) method with good reproducibility for the determination of levan, a D-fructofuranosyl polymer with a β-(2-6) backbone and β-(2-1) branching.

Methods: Levan was isolated from fermentation broth by alcohol precipitation and ultrafiltration. Factors influencing the determination of the ultraviolet (UV) spectrophotometric method was compared with a single-factor analysis. The UV spectra of levan reaction solutions in the absorbance range of 200 - 400 nm were obtained. An orthogonal experimental design was applied to optimize the sulfuric acid hydrolysis conditions in the spectrophotometric determination. FODUS method was validated by analyzing its linearity, reproducibility, stability and recovery.

Results: Factors influencing absorbance for the determination were confirmed and two regression equations were established. UV absorbance at 320 nm of sulfuric acid-hydrolyzed sample was stable for 5 h. The FODUS method developed had a good reproducibility (RSD = 2.1 %, n = 5), linearity (ranging from 1.6 µg/mL to 12.8 µg/mL), R^2 = 0.9996) and recovery (95.90 %, RSD = 1.7 %, n = 3).

Conclusion: The developed FODUS method is convenient, efficient and robust for the determination of microbial levan. The method provides a valuable approach for the determination of polysaccharides.

Keywords: Derivative spectrophotometry, Bacillus licheniformis, Levan, Total carbohydrate determination

INTRODUCTION

It is always required to determine total carbohydrates in aqueous solution for polysaccharide application development (with a good reproducibility) in most cases [1,2]. Diversity and heterogeneity of polysaccharides have led to the development of numerous chromatographic and spectroscopic methods for measuring carbohydrates [3,4]. However, these methods require sophisticated skills and advanced equipment.

Phenol-sulfuric acid method, a most frequently applied colorimetric method for determining total sugar, has several drawbacks due to the application of the volatile, unstable and toxic phenol [5,6]. The sulfuric acid–UV (SAU) method eliminates phenol for visible coloration of furfural derivatives produced by sulfuric acid treatment and capitalizes on the UV absorption potential of

the furfural intermediates [1]. Derivative ultraviolet-visible absorption spectrophotometry (DS) method has been widely used to enhance the signal and decrease the interference of impurities with closely adjacent peaks in complex mixtures for determining drugs, food and other compounds [7]. DS, however, found few applications in determining polysaccharides due to its poor absorbance in the UV region.

Levan, a linear polymer of fructose with a β- (2-6) backbone and β-(2-1) branching usually purified from fermentation broth, has a great potential in food and pharmaceutical industry [8-11]. In this paper, a first-order derivative sulfuric acid-UV (FODUS) method was investigated and validate for the determination of levan (Figure 1).
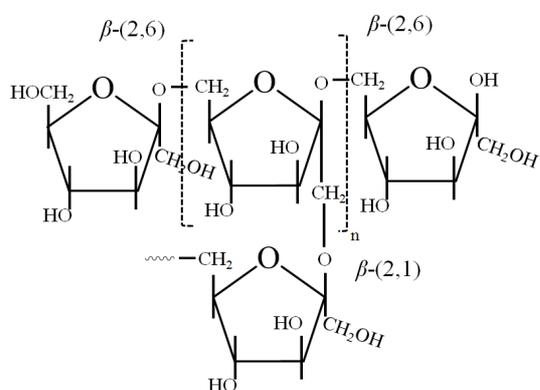
Figure 1: Structure of levan

EXPERIMENTAL

Materials

Levan was extracted from the fermentation broth of *B. licheniformis* tjgws 14278 (a microbial strain producing levan and screened by our group) by centrifugation, alcohol precipitation and membrane fractionation sequentially [12,13]. Two fractions of the extracellular polysaccharide (EPS) from *B. licheniformis* tjgws 14278 were separated: HML (high molecular weight levan, 540 kDa) and LML (low molecular mass fraction, 8.5 kDa) were isolated and characterized by our group. Only HML was determined in this study. Other chemicals used in the work were of analytical reagent grade.

Main equipment used

UV-Vis spectrometer (TU-1810SPC, Beijing Purkinje General Instrument Co Ltd, China) and vortex mixer (DG-800, Beijing Dingguo Cangsheng Biotech Co Ltd, China) were used. Monosaccharide analysis experiments were performed on Agilent HP 1100 pump with RI detector and CO-201 column oven (Agilent Technologies Inc, Santa Clara, CA, US). Shodex SUGAR KS801-801 coupled column (Showa Denko Inc, JP) was chosen.

Method development

Methods of adding concentrated sulfuric acid influenced the reproducibility and the slope of calibration curve in the phenol sulfuric acid method [5,14]. Pre-cooled levan solution (1 mL) was rapidly mixed with 4 mL concentrated sulfuric acid in a test tube with screw cap and vortexed for 30 s in ice bath. The temperature of the mixture rises rapidly within 10 - 15 s after addition of 4 mL sulfuric acid directly on the liquid surface. Then, vortex mixer was applied to mix the liquid for 30 s. Next, the reactant was heated at 70 °C in water bath for 2 min and then the mixture was cooled in ice bath for 2 min. Later, the test tube was brought to 25 °C in water bath for 5 - 10 min. Finally, derivative UV spectra at 306 and 337 nm were read using a UV spectrophotometer. Reference solution was prepared following the same procedure as the above in which the carbohydrate aliquot was replaced with distilled water. Absorbance at 320 nm can also be recorded for a fast determination. To create a calibration curve, a series of pre-cooled levan solution aliquots were prepared and treated with the above procedure for scanning UV spectra from 200 to 400 nm. Their derivative spectra were processed by the UVWin software. The standard curve was created by taking the weight of polysaccharide (μg) as the abscissa and the absorbance as the ordinate.

Recovery determination

Different volumes (0.10, 0.15 and 0.20 mL) of fructose stock solutions (20 μg/mL) were precisely pipetted into 10 mL graduated test tubes with screw caps respectively, then 0.5 mL levan stock solution (20 μg/mL) was added to each test tube, and then the final volume was adjusted to 1.0 mL for each test tube by adding water. Then, levan concentration in each test tube was tested and calculated according to the regression equation.

Monosaccharide analysis

Firstly, polysaccharide sample was tentatively analyzed using thin layer chromatography to confirm its composition. Thereafter, 10 mg sample was hydrolyzed with 2 M CF₃COOH at 110 °C for 2 h in an oven. The hydrolysate was evaporated on a rotary evaporator until dry. Methanol (5 mL) was added for facilitating further

removal of CF_3COOH with the rotary evaporator. And then, the sample was dissolved into double distilled water and analyzed with HPLC. The conditions for HPLC analysis were: temperature 50 °C (with jacket), mobile phase double distilled water; flow rate 0.8 mL/min.

Software

Data were processed with Excel 2007 (Microsoft Inc, USA). Orthogonal design results were analyzed with Orthogonal Assistant 3.1 (Sharetop Software Studio, China). UV spectra and derivative spectra were acquired and processed with UVWin 5.1.0 (Beijing Purkinje General Instrument Co Ltd, China).

Statistical analysis

All experiments were performed at least three times and the data expressed as mean ± standard deviation. Statistical analysis was carried out using one-way analysis of variance to compare different groups with the assistance of Excel 2007 (Microsoft Inc, USA) and Orthogonal Assistant 3.1 (Sharetop Software Studio, China). $P < 0.05$ was considered statistically significant.

RESULTS

UV spectra

UV spectra of levan solutions in the range of 200 to 400 nm were scanned and the derivative spectra were transformed by UVWin software (Figures 2 and 3). The maximum absorption wavelength (λ_{max}) of levan solutions was 320 nm. Derived from the first-order derivative spectrum, the peak maximum is 306 nm and the peak minimum is 337 nm. The small peak (221 - 274 nm) may be a response of the furfural intermediates (Figure 2) [6].

Regression equations

Regression equations were constructed from Figures 1 and 2 to quantify sugar concentration as follows:

$dA/d\lambda = 0.0087\ C + 0.00004\ (R^2 = 0.9996)$ …..........(1)
$A = 0.1095\ C + 0.0154\ (R^2 = 0.9976)$ ………………(2)

where $dA/d\lambda$ is the difference between the first-order derivative absorbance at 306 nm ($A'_{306\ nm}$) and the first-order derivative absorbance at 337 nm ($A'_{337\ nm}$); A is the absorbance at 320 nm ($A_{320\ nm}$); and C is the concentration of sugar (µg/mL).
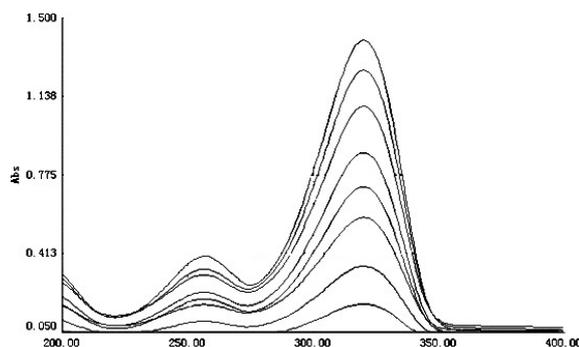
Figure 2: UV spectra of levan solutions (2 - 16 µg/mL) at the absorbance range of 200 - 400 nm; abscissa = wavelength (nm); ordinate = UV absorbance. Absorption maxima ranged from 317 to 320 nm for the various solutions with the maximum at 320 nm
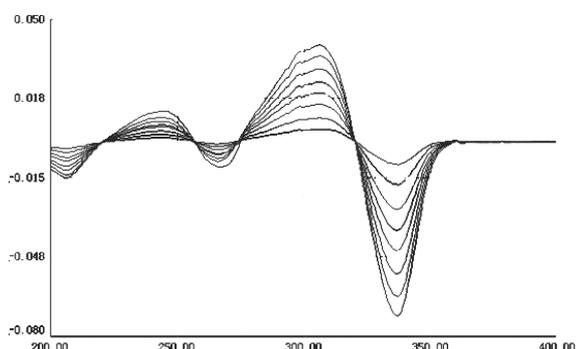
Figure 3: The first-order derivative UV spectra of levan solutions (2 - 16 µg/mL); abscissa = wavelength (nm); ordinate = the first derivative absorbance

Single-factor analysis

(1) Addition of sulfuric acid

We compared several ways of adding sulfuric acid for studying the effect of addition using the SAU procedure [1]: (1) Sulphuric acid addition directly over the surface of the solution (group I); (2) Sulphuric acid addition over the side of the tube (group II); and (3) cool the sample to about 4 °C before adding of sulfuric acid (group III). We prepared a working curve for each group, and then compared the relative standard deviation (RSD) among the three groups at the same sugar concentration (10.05 µg /mL)]. Results for the addition of sulphuric acid demonstrated that the linearity of group I was very poor ($R^2 = 0.8306$; regression equation of working curve "*A = 0.0547 C + 0.0694*"); whereas the other groups' were better ($R^2 = 0.9623$; regression equation "*A = 0.0609 C + 0.0039" (group II)*; $R^2 = 0.9370$, regression equation "*A = 0.0661 C + 0.0560" (group III)* respectively).

We found no statistically significant difference between group I and group II in terms of the slope of the curve, which was inconsistent with the work of Cuesta [5,14]. RSD of each group was 6.6 % (group I), 9.5 % (group II) and 0.3 % (group III) respectively. In addition, concentrated sulfuric acid was added directly over the surface of the solution, although it seemed that "through-the-surface" addition or "over-the-side" addition made no difference in the method not using phenol.

(2) Effect of reaction temperature

Heating between 50 $^{\circ}$C and 70 $^{\circ}$C increased the absorbance (Figure 4). However, further heating at 80 and 90 $^{\circ}$C decreased the absorbance.
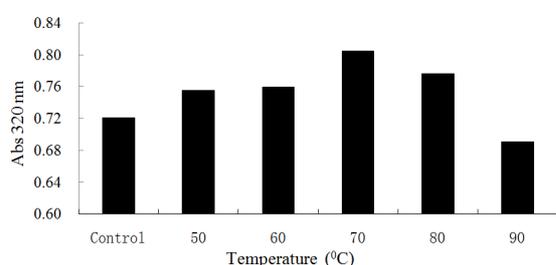
Figure 4: Effect of temperature on the absorbance at 320 nm. Levan solution was 10.00 µg/mL (n = 3). Control group (no heating) was assayed according to the sulfuric acid–UV method. Solutions in other groups were heated for 2 min before determining UV absorbance.

(3) Effect of reaction time

Maximum absorption of levan was reached after heating for 2 min (Figure 5).
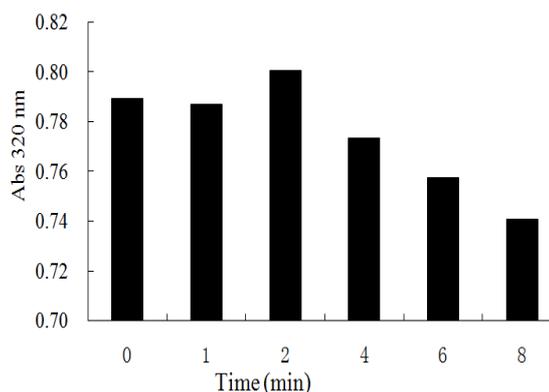
Figure 5: The effect of time on the absorbance at 320 nm. Hydrolysis temperature was 70 $^{\circ}$C (n = 3)

Orthogonal design and variance analysis

Range analysis of the orthogonal test showed that sulfuric acid had a greater effect than temperature and time. However, results demonstrated that no factor significantly influenced the UV absorbance ($p < 0.05$) in the orthogonal test (Table 1). To further validate our modifications by orthogonal design, we selected linearity, recovery, reproducibility and stability to evaluate the proposed method (Table 1).

Method validation

(1)   Linearity

According to the regression equations (1) and (2), concentration of levan ranging from 1.6 to 12.8 µg/mL was proportional to the absorbance (A_{320}) or to the dA/dλ respectively and the coeffi-

Table 1: Results of the orthogonal design

Level	Temp	Time	SA		dA /dλ
E1	1	1	1	1	0.056
E2	1	2	2	2	0.067
E3	1	3	3	3	0.070
E4	2	1	2	3	0.068
E5	2	2	3	1	0.072
E6	2	3	1	2	0.055
E7	3	1	3	2	0.070
E8	3	2	1	3	0.055
E9	3	3	2	1	0.067
Ave 1	0.064	0.065	0.055	0.065	
Ave 2	0.065	0.065	0.067	0.064	
Ave 3	0.064	0.064	0.071	0.064	
Range	0.001	0.001	0.016	0.001	

Note: Ave denotes the average of dA/dλ for each factor and each level. Temp stands for temperature. SA means sulfuric acid. Temperature (65, 70, and 75 $^{\circ}$C for level 1, 2 and 3 respectively); time (1.5, 2.0, and 2.5 min for level 1, 2 and 3 respectively) and sulfuric acid (3.0, 3.5, and 4.0 mL for level 1, 2 and 3 respectively) were chosen as factors for the orthogonal design according to the single-factor analysis. Measurement conditions were modified, followed by the result of the orthogonal test: temperature = 70 $^{\circ}$C, time = 2 min and sulfuric acid = 4 mL, were then adopted except for the experimental level

cient of determination approximated 1 (R² = 0.9996).

(2) Recovery

Levan concentration was calculated according to the regression equation. The average recovery was 95.90 % and the RSD was 1.7 % (n = 3), which indicated that the accuracy of the FODUS method was acceptable. Moreover, the high recovery also suggested that the acid treatment in the FODUS procedure did not decompose the sugar significantly. In addition, further polished levan sample may improve the recovery.

(3) Reproducibility

The absorbance at 320 nm was measured by the FODUS method for the levan solution (6 μg /mL) repeatedly for 5 times (RSD = 2.1 %), which suggested that it was a reproducible method.

(4) Stability

Levan solution (6 μg/mL) was sequentially determined for 5 h by the FODUS method. Each group of data was determined thrice. Results showed that the absorbance was stable within 5 h (RSD = 0.6 %, n = 8). FODUS method outperformed anthrone-sulfuric acid method in terms of stability [15].

DISCUSSION

Total sugar determination in aqueous solution is important for quality control and process development for extracellular polysaccharide fermentation and purification [2,14]. Phenol-sulfuric acid method, a simple and cheap colorimetric method, is generally applied in total sugar determination [16-18]. However, it is required to create a qualification curve according to monosaccharide contents; then, a series of correction factors are established for quantification [19]. Additionally, the complexity of some polysaccharides and differences of chemical reactivity between neutral and acid sugar components bring about inaccuracies. The SAU method is a method that can determine total sugar in aqueous solution using no phenol. Levan is an essentially homogeneous polysaccharide composing roughly pure fructose. Thus, the SAU method circumvents the laborious calibration procedure for calculating correction factors.

The disadvantage of the SAU method is that it can be interfered by some impurities bearing groups that have UV absorption within the absorption range of the analyte. Derivative spectrophotometry (DS) is expected to enhance the signal and resolve the overlapped peak-signals due to its advantages in differentiating closely adjacent peaks, and identifying weak peaks obscured by sharp peaks [7]. Taking the difference between the peak maximum and the peak minimum gives the best signal-to noise ratio. In addition, a heating process in the FODUS procedure minimized those interferences.

The λ_{max} chosen in the SAU procedure is different from the DuBios method [5]. In the former, the acid and neutral carbohydrates have the same λ_{max}, whereas the latter is 490 nm for most neutral sugars and 480 nm for most acid sugars [5]. The broad range of λ_{max} (480 - 490 nm) for DuBois method perplexed many workers in determining polysaccharides, especially those who want to precisely determine heterogeneous polysaccharides composing neutral and acid carbohydrate residues. It seems that the SAU method simplified the procedure greatly without considering the issue of optimizing λ_{max}. In fact, λ_{max} in this study is slightly different from the reported data [1]. However, it is consistent with the report by Love [16].

The graph of absorbance vs. concentration can be constructed and the amount of analyte derived from the two equations stated earlier. Each regression equation has a good coefficient of determination. The intercept in the regression equation 1 is very small (0.00004), which may suggest that the FODUS method has a very low limit of quantification (LOQ). The concentration of control solution (sulfuric acid solution) influences the intercept of the regression equation, although it seems that there is no detectable UV absorbance from 306 to 337 nm for a sulfuric acid solution. However, considering that concentrated sulfuric acid is caustic and toxic, it is recommended that the control solution select distilled water to substitute for the sulfuric acid solution when the sample concentration is much higher than the LOQ. Equation 2 is also good enough for levan solution determination considering the coefficient of determination approximates to 1 (R² = 0.9976), which can be used for a fast determination when the interference is negligible. The slope of the equation 2 is slightly larger compared with that of neutral polysaccharides (see Table 1 of Albalasmeh's data) [1], which suggests that Eq 2 is more sensitive than the counterpart.

Polysaccharide can be hydrolyzed into monosaccharides, and then dehydrated by concentrated sulfuric acid and finally form furfural derivatives [6]. However, 30 s in the SAU procedure may not be enough for completely transforming levan into furfural derivatives. Macromolecular levan can form aggregates, which may retard the hydrolysis rate. Consequently, we analyzed some factors that possibly influenced the hydrolysis process.

First, we compared the influence of sulfuric acid addition on the absorbance. When concentrated sulfuric acid is added to the sugar solution, heat is obviously produced, sugar is hydrolyzed and furfural derivatives are produced. Temperature influences the yield of furfural derivatives of levan, which may also have an effect on the repeatability of the results. Thus, it is necessary to control the temperature to minimize the absorbance deviation, which is especially important in an extreme climate. Pre-cooling before adding sulfuric acid is useful for decreasing deviation, which can be confirmed by the RSD reduction. In addition, no statistically significant difference was found between groups I and II. Results indicate that the use of ice-bath for carbohydrate sample is significant for decreasing data deviations.

Second, we studied the effect of time and temperature to the sulfuric acid hydrolysis process. With the time and temperature increasing, more furfural derivatives are produces; however, sulfuric acid also degrades monosaccharides or furfural derivates with the hydrolysis time increasing. Optimized heating procedure increases the response, sensitivity and LOQ for determining sugars.

Followed by single-factor analysis, the orthogonal test was designed for further polishing the hydrolysis process to increase UV absorbance response. However, variance analysis results suggest that absorbance is insensitive to the variation of time, temperature and sulfuric acid under the optimized conditions, which may contribute to the improved reproducibility. Hence, the FODUS method is a robust method under the optimized condition.

CONCLUSION

FODUS method for the quantitative determination of microbial levan has been established. The method is convenient, efficient, low-cost and readily available. The required equipment is minimal and highly skilled personnel are not required. Its reproducibility, linearity, stability and recovery meet the requirements for both scientific investigation and quality control in pilot processes. With all these merits, a similar approach should be considered for the analysis of other polysaccharides.

ACKNOWLEDGEMENT

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