

ARTICLE

Easy Estimation of Endoglucanase Activity Using a Free Software App for Mobile Devices

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This study describes the estimation of the enzymatic activity of endoglucanase based on the 3,5-dinitrosalicylic acid (DNS) method and carboxymethylcellulose (CMC) substrate using glucose as the analyte. As an alternative to measuring the colored product obtained by the reaction between glucose and DNS, a free software app for mobile devices was used for estimating the color intensity of solutions, replacing the need of a UV-Vis spectrophotometer (reference method). The app is able

to convert images in color histograms to red (R), green (G) and blue (B) scales and to provide univariate and multivariate calculations of the image data. The chemometric technique partial least squares (PLS) was employed with the app and presented accurate results that were comparable to those of the reference method. A correlation coefficient of 0.983 was obtained for the linear range between 0.60 and 2.60 mg mL⁻¹ of glucose, and the standard error of cross-validation (SECV) was 0.219 mg mL⁻¹. The proposed method (imaging – PLS) was applied for samples of cellulase enzymes from *Aspergillus niger* and *Trichoderma reesei*, revealing an interesting alternative to estimate the enzymatic activity of endoglucanase.

Keywords: Endoglucanase activity, digital imaging, PLS, free software app.

INTRODUCTION

Lignocellulosic biomass has been shown to be an important natural raw material due its abundance and versatility in terms of applications in areas such as energy, materials, pharmaceuticals and food science [1,2]. Therefore, lignocellulosic biomass has grown as an important alternative to raw fossil-based materials, not only from an industrial perspective but also from an economic and environmental perspective. Currently,

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the biomass conversion process involves three main steps: delignification pretreatment, saccharification and fermentation. The challenge in the use of lignocellulosic biomass as a renewable raw material has been the obtention of reducing sugars, which can be converted into several value-added chemicals by several chemical and biologic routes, in the saccharification step [3,4].

Cellulase enzyme complexes hydrolyze β -1,4 linkages in cellulose chains and play an important role in cellulose saccharification. Endoglucanase hydrolyzes glycosidic bonds at amorphous regions of the cellulose, generating long chain oligomers, which are hydrolyzed into short chains, especially cellobiose, by the action of the exoglucanase. Finally, cellobiose is broken down to glucose by β -glucosidase enzymes [5]. In addition to being the first to act in cellulose hydrolysis, endoglucanase enzymes are outstanding for their importance in different industrial applications, such as in textiles, detergent and paper [6].

Several methods have been employed to determine endoglucanase activity in cellulase complexes from fungi, such as *Trichoderma reesei* and *Aspergillus niger*, which have been extensively studied due to their high cellulolytic activity during biomass conversion processes [7]. Basically, these methods have been carried out by colorimetric techniques, such as 3,5-dinitrosalicylic acid (DNS) and mainly spectrophotometric measurements [8,9], which requires a large consumption of energy and expensive instruments, hampering, for example, field analyses.

On the other hand, for this application, it is possible to use methods that minimize energy consumption and the use of expensive instruments and decrease waste generation but that provide results comparable to the reference method with high analytical frequency, simplicity and low cost [10,11], as an example, analyses using digital images from software apps for mobile devices.

Software apps for mobile devices that use digital images enable qualitative and even quantitative analyses [12], as shown in the study of Masawat et al. [13] in which a software app for mobile devices (iPhone) named "ColorConc" was developed that utilizes an image matching algorithm to determine the concentration of tetracycline in bovine milk. The proposed app was able to determine concentrations in a range from 0.5 to 10 $\mu\text{g mL}^{-1}$ and provided a limit of detection (LOD) of 0.5 $\mu\text{g mL}^{-1}$. Moonrungsee et al. [14] developed a software app for mobile devices named "Phosphorus Analysis" that recorded digital images and used information from the Red-Green-Blue (RGB) color system to determine the content of phosphorus in soil. The calibration was performed by measuring the blue color intensity of standard phosphorus solutions (0.0 to 1.0 mg L $^{-1}$). The results obtained agreed well with the spectrophotometric reference method, with a detection limit of 0.01 mg L $^{-1}$, analytical frequency of 40 samples per hour, accuracy in terms of relative error smaller than 5% and precision in terms of relative standard deviation smaller than 2%, in addition to providing fast and low-cost method, which are convenient for in-loco analysis.

In this context, we used a free software app called PhotoMetrix [15] to estimate the enzymatic activity of endoglucanase enzymes. PhotoMetrix has been used for efficiently helping to determine iron in vitamin supplement tablets [16], iodine in biodiesel [17], ethanol in sugarcane spirit samples [18], and for monitoring of acid-base titrations on paper platforms [19], estimation of pH in milk samples [20] and chromium speciation in leather samples [21].

Thus, the goal of this study was to estimate the enzymatic activity of endoglucanase enzymes using color information data from digital images and reference values from the standard 3,5-dinitrosalicylic acid (DNS) method, by performing analyses with a free software app for mobile devices.

MATERIALS AND METHODS

Chemicals

Cellulase enzymes from *Aspergillus niger* (AN) and *Trichoderma reesei* (TR), and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma-Aldrich (St Louis, MO, USA). Carboxymethylcellulose (CMC) sodium salt substrate was obtained from Neon (Suzano, SP, Brazil). No additional purification was required for the chemicals.

Instrumentation

A smartphone ASUS ZE550KL (Beitou, TPE, Taiwan) equipped with a camera with 13-megapixel resolution and an Android 6.0 operating system Marshmallow were used. The software app for mobile devices PhotoMetrix Pro that includes the chemometric technique PLS computed with the NIPALS algorithm was downloaded for free from a website [15]. For spectrophotometric measurements, a Perkin Elmer Lambda 465 spectrophotometer (Waltham, MA, USA) equipped with a UV-Vis photodiode array detector was used.

Endoglucanase activity assay

The enzymatic activity assay was determined by measuring reducing sugar production after the reaction of the endoglucanase with the CMC, based on the DNS method and using glucose as a standard [8], as shown in Figure 1. The cellulase enzymes AN and TR were solubilized in citrate buffer, pH 5, with an initial concentration of 5.0 mg mL⁻¹ and previously diluted before the reaction began. The reaction mixture contained 0.9 mL of 0.44% CMC solution in citrate buffer, pH 5, and 0.1 mL of enzymatic solution. The CMC hydrolysis reaction was carried out for 1 h at 50 °C, after which 1.5 mL of DNS reagent was added to stop the reaction.

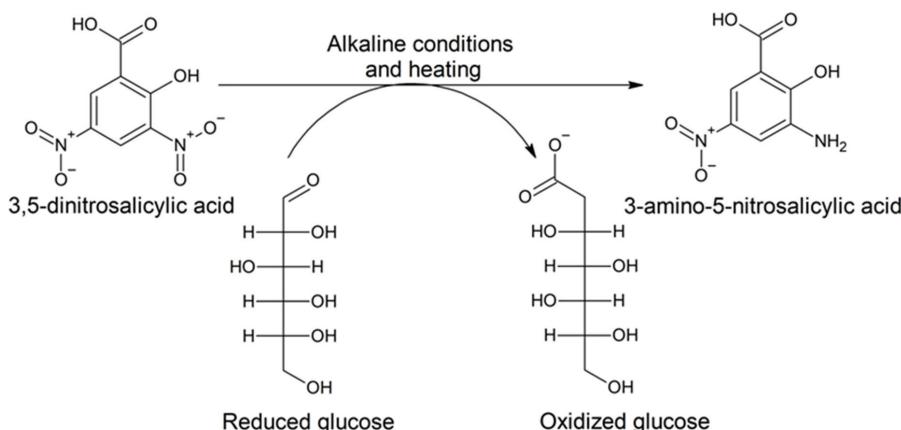


Figure 1. Chemical reaction of the 3,5-dinitrosalicylic acid (DNS) method for glucose determination.

The cellulase enzymes were heated in boiling water for 5 minutes to allow color formation. After cooling to room temperature, the concentration of reducing sugar was measured at 540 nm on a spectrophotometer. One unit of enzyme activity is defined as the amount of reducing sugar produced per mL of enzyme used over time and was stated as U mL⁻¹ (μmol mL⁻¹ min⁻¹). The units were calculated according to Equation (1):

$$\text{Endoglucanase activity } \left(\text{U mL}^{-1} \right) = \frac{\text{Df} \times \text{reducing sugar } \left(\mu\text{mol mL}^{-1} \right) \times V_T(\text{mL})}{\text{Time (min)} \times V_E(\text{mL})} \quad (1)$$

where Df is the dilution factor of cellulase enzymes: 200 for TR and 25 for AN; VT is the total volume of the reaction mixture (1 mL) and VE is the enzyme volume (0.1 mL).

Reference method using UV-Vis data

The glucose standard curve for the DNS method was prepared by dissolving glucose in citrate buffer, pH 5, for concentrations in the range between 0.60 and 2.60 mg mL⁻¹. The reaction solutions of the standard curve were prepared according to the endoglucanase activity assay. Briefly, 0.1 mL of glucose solution was mixed with 0.9 mL of citrate buffer and incubated for 1 h at 50 °C. Subsequently, 1.5 mL of DNS reagent was added, and the samples were heated in boiling water for 5 minutes to allow color formation. After

cooling to room temperature, the solutions of glucose were measured at 540 nm on a spectrophotometer and submitted to image acquisition analysis. In the blank samples, glucose was substituted for citrate buffer, and the procedure was carried out in the same manner.

Proposed method using image - PLS

The acquisition of images of the solutions of various glucose concentrations was performed under controlled lighting provided by a light emitting diode (LED) lamp (6 W, 12 V), with a white paper underneath the Petri dishes to minimize the background reflectance and the influence of external light [22]. The LED lamp was used to illuminate the environment without interference in image quality [17].

The mobile device was placed on top of the laboratory-made imaging system adapted from Helfer et al. [20], which included a box (20 width x 31 length x 15 height cm) with a hole for the mobile camera device (15 cm above the samples) and another hole for the LED lamp, as shown in Figure 2. Approximately 5 mL of solutions was placed in a Petri dish (5.5 cm diameter x 1.0 cm height), and images were acquired using parallel alignment of mobile device on sample surface in a region of interest (ROI) of 64×64 pixels.

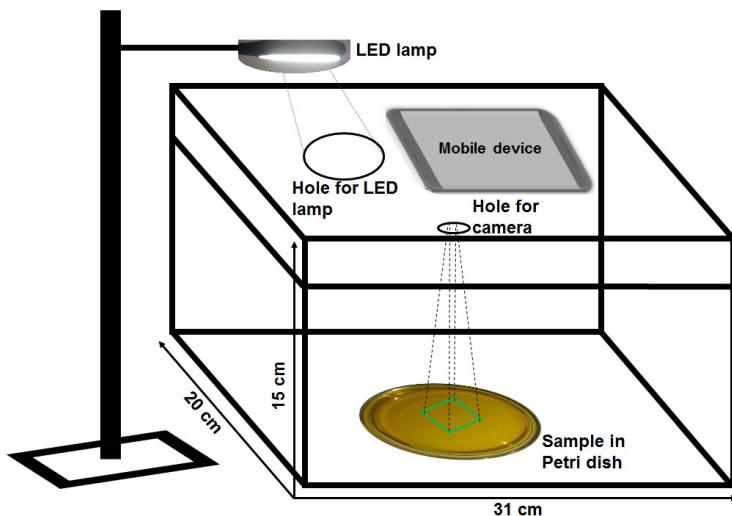


Figure 2. Schematic diagram for acquisition of the digital images.

RESULTS AND DISCUSSION

Figure 3 shows the univariate analytical curve obtained with the UV-Vis spectrophotometer from six glucose concentration values, obtained in triplicate and ranging between 0.60 and 2.60 mg mL^{-1} . As shown in Figure 1, the reducing sugars, such as glucose, have the capability to reduce DNS to its analogue 3-amino-5-nitrosalicylic acid. This aromatic compound absorbs strongly visible light at 540 nm and, therefore, can be used to establish a direct relationship between the amount of the reducing sugar and the colorimetric measurement from a UV-Vis spectrophotometer (reference method) [23]. The mean absorbances \pm standard deviation (SD), ($n = 3$) were (1) 0.116 ± 0.008 ; (2) 0.238 ± 0.01 ; (3) 0.357 ± 0.001 ; (4) 0.484 ± 0.004 ; (5) 0.613 ± 0.01 and (6) 0.730 ± 0.02 . These data are used according to the reference method based on the color formation during the reaction between DNS and the reducing sugars from the CMC hydrolysis to estimate the enzymatic activity of endoglucanase enzymes.

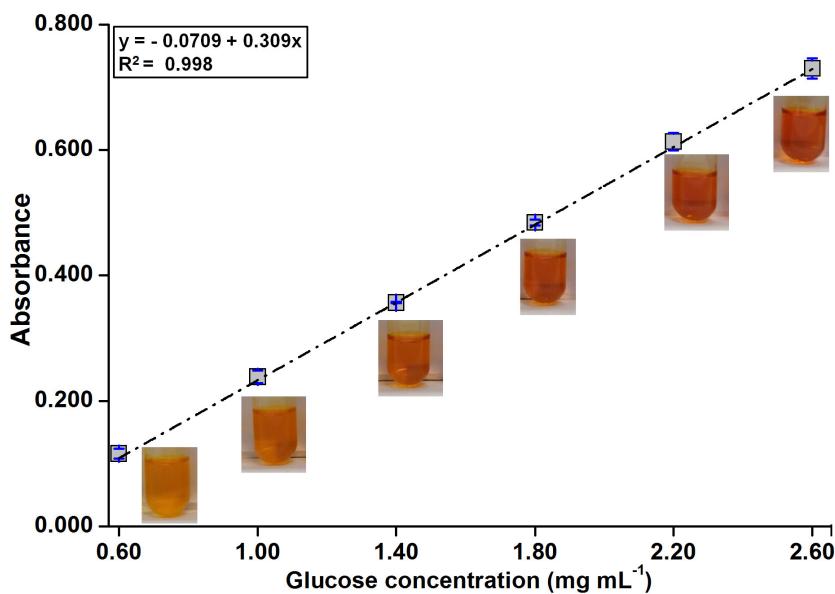


Figure 3. Analytical curve for glucose obtained using the reference method (UV-Vis spectrophotometry) for reducing sugar determination.

The robustness of the analytical curve of the UV-Vis was evaluated using analysis of variance (ANOVA), as shown in Table I. ANOVA revealed that the curve was well adjusted. The calculated F-value for the regression with 1 and 16 degrees of freedom (D.F.) was equal to 8954 at the 95% confidence level, being 1993 times higher than the tabulated F-value of 4.49. The calculated F-value for the lack of fit with 4 and 12 D.F. presented a value 9.42-fold smaller than the tabulated F-value at the 95% confidence level, confirming that the regression did not show a lack of fit. Moreover, neither of the regression coefficients assumed the value of zero, and these coefficients were significant at same level. The residues between the empirical and predicted values showed no heteroscedastic tendency in the data, implying a normal distribution.

Table I. Description of sum of squares from analysis of variance (ANOVA) for the analytical curve for UV-Vis spectrophotometry data

Source	Sum of squares	Degrees of freedom (D.F.)	Mean of squares	F-test	F-tabulated (95%)
Regression	0.801	1	0.801	8954	4.49 (1 and 16 D.F.)
Residual	0.00143	16	0.0000894		
Total	0.802	17	0.0472		
Pure error	0.00128	12	0.000107	0.346	3.26 (4 and 12 D.F.)
Lack of fit	0.000148	4	0.0000370		
R ²	0.998	R	0.999		
R ² maximum	0.998	R maximum	0.999		

After this test, the UV-Vis data were considered appropriate as a reference for estimating enzymatic activities. The glucose concentration (mean \pm SD, n=3) values were equal to 1.90 ± 0.002 mg mL⁻¹ and 2.02 ± 0.03 mg mL⁻¹ for cellulase enzymes from AN and TR, respectively. The relative standard deviations (RSD%) were 0.0853% and 1.27% for AN and TR, respectively.

The endoglucanase enzymatic activity calculated from the reference method according to Equation 1 was 44.0 ± 0.04 and 375 ± 5 U mL⁻¹ for cellulase enzymes from AN and TR, respectively. Generally, *Trichoderma* spp. show almost four times more endoglucanase activity than *Aspergillus* spp. [24]. The higher endoglucanase activity found for TR can be attributed to its high endoglucanase production. Almost 20-36% of the cellulase production of TR was attributed to endoglucanase enzymes, which makes TR an excellent fungus for cellulose hydrolysis [25].

Afterwards, pictures were taken from the same solutions used in the reference method (UV-Vis spectrophotometry). The Photometrix Pro app automatically converted the images into color information data by representing the frequency of the pixels (histograms) of the RGB color system, generating 768 variables per image, with 256 variables for each individual color. According to ANOVA shown in Table II, there is no significant difference at the 95% confidence level, since that the F-value for the regression with 1 and 16 D.F. was equal to 467, being 104 times higher than the tabulated F-value of 4.49.

Table II. Description of sum of squares from analysis of variance (ANOVA) for imaging-PLS method

Source	Sum of squares	Degrees of freedom (D.F.)	Mean of squares	F-test	F-tabulated (95%)
Regression	7.85	1	7.85	467	4.49 (1 and 16 D.F.)
Residual	0.269	16	0.0168		
Total	8.11	17	0.477		
Pure error	0.0283	12	0.00236	25.5	3.26 (4 and 12 D.F.)
Lack of fit	0.241	4	0.0602		
R ²	0.967	R	0.983		
R ² maximum	0.997	R maximum	0.998		

At the same app, it is possible to predict the glucose concentration using the chemometric technique PLS as an in-app tool. The histograms from the RGB colors, represented by color counts, were the independent variables and the glucose concentrations obtained by the reference method UV-Vis spectrophotometry were the dependent variables. The preprocessing applied to the independent variables included mean-centering for the counts of each color. The PLS model was built with only 3 latent variables (LVs) with leave-one-out cross-validation that explained 97% of the data variance. The glucose concentrations (mean \pm SD, n=3) in mg mL⁻¹ predicted by the PLS model were (1) 0.760 ± 0.03 ; (2) 0.927 ± 0.01 ; (3) 1.35 ± 0.1 ; (4) 1.83 ± 0.01 ; (5) 2.01 ± 0.006 and (6) 2.72 ± 0.03 . The correlation between predicted (image – PLS) and reference values (UV-Vis) was equal to 0.983 and the value of coefficient of determination was 0.967, as shown in Figure 4. The SECV was used to evaluate the predictive ability of the PLS model and presented a low value equal to 0.219 mg mL⁻¹. The absolute errors ranged between -0.190 and 0.200, with an estimated bias of -0.000556.

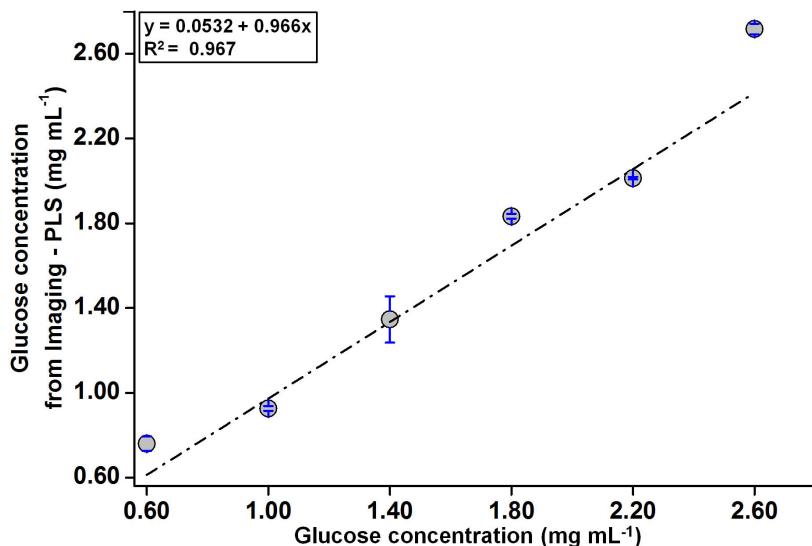


Figure 4. Correlation between values of glucose concentrations (mg mL^{-1}) of the standard samples versus predict by imaging-PLS model using Photometrix®.

Samples of cellulase enzymes from AN and TR were used in triplicate to evaluate the predictability of the PLS model. The glucose concentrations values (mean $\pm \text{SD}$, $n=3$) for AN and TR predicted by the PLS model were $1.74 \pm 0.05 \text{ mg mL}^{-1}$ and $2.08 \pm 0.1 \text{ mg mL}^{-1}$, respectively. The results of the SEP and the correlation coefficient of prediction were 0.164 mg mL^{-1} and 0.867 , respectively. The range of absolute errors was between -0.208 and 0.171 with -0.0547 of bias. Using Equation 1, the cellulase enzymes from TR showed higher endoglucanase activity of $385 \pm 27 \text{ U mL}^{-1}$ than those of AN at $40.2 \pm 1 \text{ U mL}^{-1}$.

The variances between the methods, UV-Vis spectrophotometry and imaging – PLS, were calculated. For the AN enzyme, the variance between the methods was not comparable, with a calculated F-value of 775 and a tabulated F-value of 19.0 with 2 D.F. (3 replicates measured per method equal to 2 D.F.), which suggests a 40.8-fold increase. Comparison of means was performed using a paired Student's *t*-test for same samples with different variances since the tabulated *t*-value was 4.30 and the calculated *t*-value was 6.25 (2 D.F.).

For cellulase enzymes from TR, the endoglucanase activities obtained were $375 \pm 5 \text{ U mL}^{-1}$ for UV-Vis and $385 \pm 27 \text{ U mL}^{-1}$ for the image-PLS model. In this case, the variance was 31.8 for calculated F-value and 19.0 for tabulated F-value with 2 D.F. In this case, the variances between methods were still not comparable. Afterwards, a paired Student's *t*-test was calculated for the same samples with different variances, showing that there was no significant difference at the 95% confidence level between the two mean concentration values because the calculated *t*-value of 0.630 is smaller than the tabulated *t*-value of 4.30, and thus the mean was comparable. The advantages and limitations from both methods, UV-Vis and imaging - PLS were summarized in Table III.

Table III. Advantages and limitations between reference and proposed method

Parameter	Reference method UV – Vis Spectrophotometer	Proposed Method Imaging – PLS Mobile device
Cost	high	low
Robustness	high	high
Analysis <i>in loco</i>	no	yes

Table III. Advantages and limitations between reference and proposed method (Cont.)

Parameter	Reference method UV – Vis Spectrophotometer	Proposed Method Imaging – PLS Mobile device
Maintenance cost	high	low
Portability	no	yes
Analytical frequency	medium	high
User friendly interface	no	yes
Energy consumption	medium	low

CONCLUSIONS

The proposed method developed by using digital images processed by a cellphone app provides potential for evaluating enzymatic activity in cases where the enzymatic activity is higher than 385 U mL⁻¹, taking advantage of the calibration curve that can be quickly assessed using only a cellphone. The imaging – PLS method showed good agreement with the results of the colorimetric method using the DNS reaction at the 95% confidence level.

The traditional method using a spectrophotometer could be replaced by quick screening of enzymatic activity for *in loco* analysis in industries or processes that require this estimation, mainly those dedicated to biomass processing.

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Conflicts of interest

All authors declare that they have no conflict of interest.

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