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Review of lignocellulolytic enzyme activity analyses and scale-down to microplate-based assays

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ABSTRACT

With the increasing use of enzymes in environmental applications, there is a need for analytical methods adapted to large factorial experiments. Existing reference methods are chemical and labor intensive and unsuitable to analyze in parallel a large number of samples. Based on an extensive literature review and on experimental results, this work compares reference and microplate adapted methods to define the most adequate filter paper, carboxymethylcellulase, β-glucosidase and xylanase activity tests. In the adapted methods, the total reaction volume was reduced from 2.2–24.5 mL to 0.21–0.24 mL. Statistical analysis of the activities measured on enzyme mixtures by applying the 96-well plate reduced methods showed that they were not significantly different to the activities obtained with reference tests.

1. Inroduction

Emerging applications in industrial biotechnology are multiplying mainly those using enzymes in biofuel production and waste treatment of lignocellulosic matrices. But the three structural polymers of lignocellulose (cellulose, hemicellulose and lignin) present a complex configuration as shown in Fig. 1. Cellulose is a linear homopolymer of glucose units (Fig. 2); the chains of cellulose tend to forms microfibrils with alternating crystalline and amorphous regions. It is hydrolyzed by cellulase, a complex of at least 3 groups of enzymes [3,4]: endoglucanase (endo-1,4-β-D-glucanase) which acts randomly on soluble and insoluble cellulose chains, exoglucanase (Exo-1,4-β-D-glucanase, cellobiohydrolase) which liberates cellobiose from the reducing and non-reducing ends of cellulose chains and β-glucosidase (cellobiase) which liberates glucose from cellobiose. Hemicelluloses are polymers composed of monomeric components mainly xylose, mannose, galactose, arabinose and methylglucuronic acid (Fig. 2). Xylanases are involved in the degradation of hemicellulose. Similar to cellulases, they can act synergistically to achieve hydrolysis. Predominant enzymes within this system are endoxylanases which attack the polysaccharide backbone and β-xylosidases which hydrolyze short xylo-oligosaccharides to xylose [5,6]. Finally, lignin is a complex aromatic polymer, made of different types of phenylpropane units, namely syringyl, guaicayl and also p-hydroxymethyl units (in herbaceous plants), linked by different ether (mainly β-O-4) and C–C bonds. This polymer is usually degraded by a family of ligninolytic peroxidases which include lignin peroxidase (LiP), manganese peroxidase (MnP) [7,8] and more recently versatile peroxidases (VP) [9].

Besides the diversity of enzymes that can be involved, both operating and capital costs of using enzymes in environmental applications for bioethanol or biogas production are very high. It becomes then essential to follow-up the fate of enzymes in the process in terms of their corresponding enzymatic activity. For that purpose, reference methods exist but they are labor-intensive, time consuming, chemical intensive and most importantly not adapted to large factorial experiments. Although some reduced protocols have been reported in the literature, they were most often not checked against the reference method. Therefore research results across the literature cannot be compared.

The main objective of this work is thus to review reference and other existing protocols for four enzymes: total cellulase or filter paper activity (FPase), carboxymemthylcellulase (CMCase), β-glucosidase and xylanase. Using enzyme solutions, chosen methods are experimentally compared. New microplate-based methods are proposed when existing reduced methods do not compare well with the reference.

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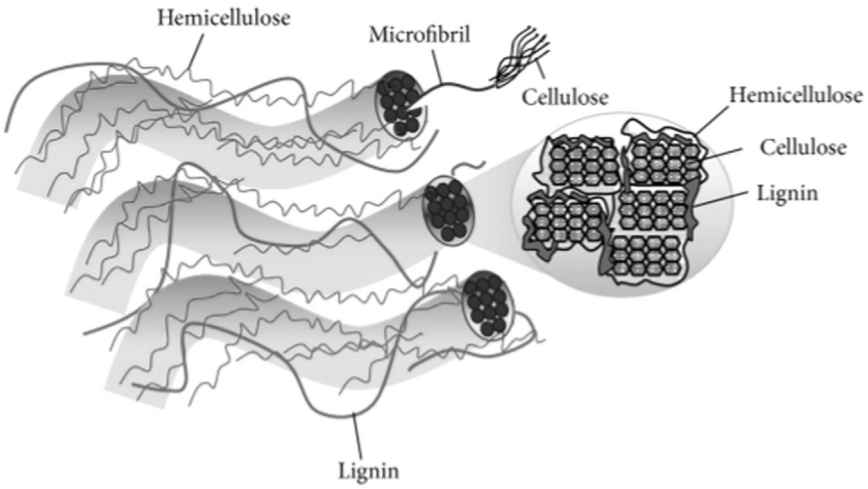

Fig. 1. Schematic diagram of the three components of lignocellulose: cellulose, lignin and hemicellulose [1].

2. Materials and methods

2.1. *Analytical protocols for measuring enzyme activity.*

2.1.1. Filter paper activity (FPase, total cellulase, filter paper cellulase, FP cellulase, exo-1,4-β-D-glucanase, exoglucanase)

One Filter Paper Unit (FPU) is defined as the amount of enzyme that releases one μmol of glucose per minute in the assay reaction. FPase estimates the total cellulase activity in a medium. It is generally assayed by measuring the release of reducing sugars in a reaction mixture containing Whatman No.1 filter paper as substrate in 50 mM sodium citrate buffer (pH 4.8) at 50 °C for up to 60 min. Using three enzyme mixtures (FP1, FP2 and FP3), three methods were compared. The reference method is indicated by the International Union of Pure and Applied Chemistry (IUPAC) and described in Ghose [10]. It is based on using 50 mg of substrate with a final total reaction volume of 24.5 ml. A 96-well plate adapted method using 3.4 mg substrate presented in Xiao et al. [11] was also tested. It followed the same steps described in the reference method but at liquid/solid ratio of 28.24; compared to 30 in the reference. Finally, a new method (FPase.mod) was proposed based on the IUPAC reference with a 1/20 reduction in volume.

Enzyme mixtures were diluted at different concentrations. Table 1 summarizes the different steps of the three analytical

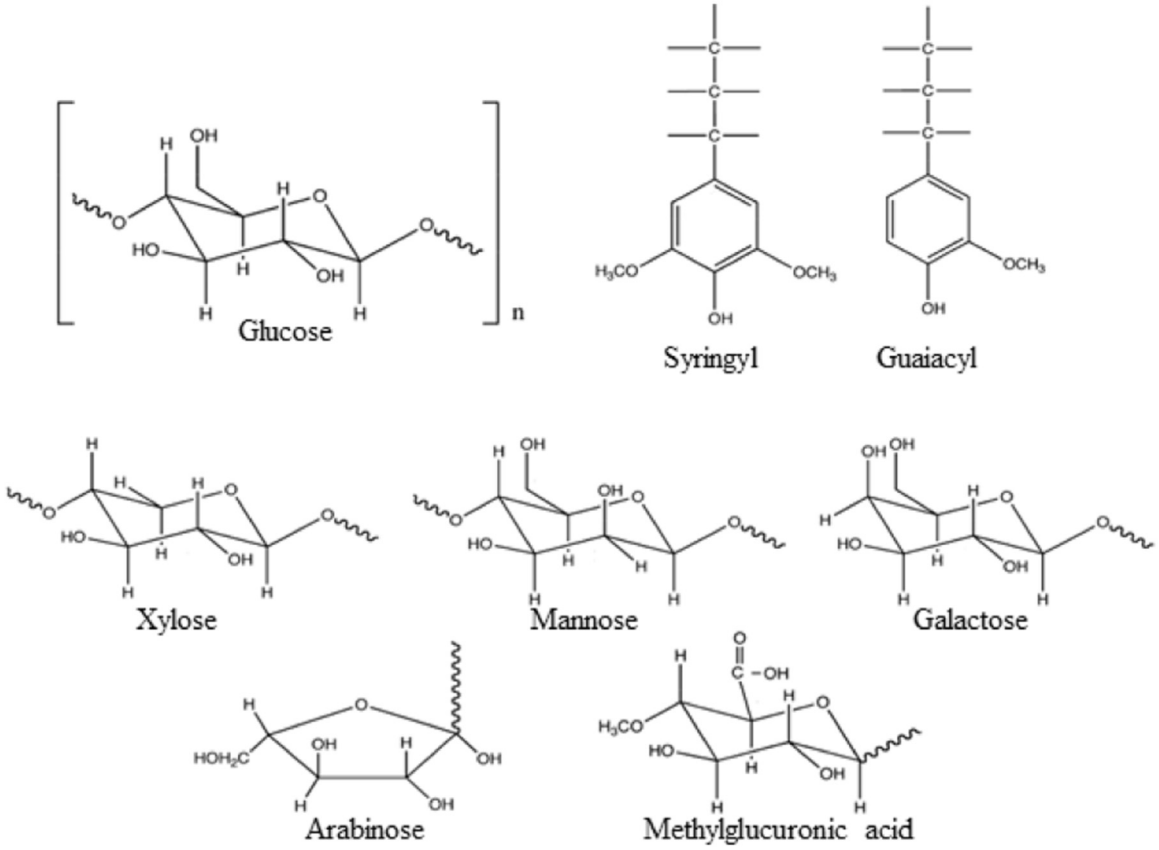

Fig. 2. Chemical structures of the different monomeric components: glucose of cellulose, syringyl and guaiacyl of lignin, xylose, mannose, galactose, arabinose and methylglucuronic acid of hemicellulose [2].

Table 1
Summary table of the three compared methods for FPase analysis with Ghose [10] as standard IUPAC, Xiao et al. [11] and FPase mod.

	Ghose [7] – reference method	Xiao et al. [8]	FPase mod.
Substrate	Whatman no 1 paper		
Amount of substrate	50 mg	3.4 mg	2.4 mg
Buffer type	50 mM sodium citrate buffer (pH 4.8)		
Buffer volume	1 ml	0.064 ml	0.048 ml
Sample volume	0.5 ml	0.032 ml	0.024 ml
Liquid/solid ratio	30	28.24	30
Total reaction volume	1.5 ml	0.096 ml	0.072 ml
Incubation at 50 °C for 60 min	Test tubes in water bath	96-well microplate in dry bath	96-well microplate in dry bath
Volume of DNS	3 ml	0.1 ml	0.144 ml
Color development	100 °C for 5 min	95 °C for 5 min	100 °C for 5 min
Dilution	Add 20 ml H_2O	Mix 0.16 ml H_2O to 0.036 ml color developed mixture	Mix 0.196 ml H_2O to 0.044 ml color developed mixture
Reading wavelength	545 nm	540 nm	545 nm

methods for FPase analysis.

2.1.2. Carboxymethylcellulase (CMCase, Endo-1,4-β-D-glucanase, EC 3.2.1.4)

One unit (U) of CMCase activity is defined as the amount of enzyme that liberates 1 μmol of reducing sugar as glucose per minute under the analysis conditions.

The IUPAC reference method for CMCase was also described by Ghose [10]. Xiao et al. [12] presented a protocol adapted to 96-well plates, in which 0.03 ml sample and 0.03 ml of 2% CMC were mixed and incubated for 30 min at 50 °C. After incubation, 0.06 ml DNS were added and the mixture heated at 95 °C for 5 min. Later, 0.01 ml of color developed mixture was transferred for absorbance reading at 540 nm. In addition to the two above mentioned methods; eight different combinations of a modified method (CMCase.mod) were tested with varying substrate concentrations and substrate to sample ratios. All methods were compared using two enzyme mixtures CMC1 and CMC2. Table 2 summarizes the different analytical methods used to measure CMCase.

2.1.3. β-glucosidase (β-D-gluside glucohydrolase, EC 3.2.1.21)

Unlike all other three enzymes studied in this work, β-glucosidase test does not require the use of a dinitrosalicylic acid (DNS) solution. One unit (U) of β-glucosidase activity is defined as the amount of enzyme which produces 1 μmol of p-nitrophenol (pNP) per minute from 4-nitrophenyl α-D-glucopyranoside (pNPG).

The reference method for the enzyme activity was described by Kubicek and Pitt [13]. A reaction mixture containing 1 ml of pNPG (1 mM), 1.8 ml of acetate buffer (pH 4.8) and 0.2 ml of sample was incubated at 50 °C for 30 min. The absorbance was read at 412 nm. A reduced volume protocol was presented by López Abelairas [14] although not adapted for 96-well plates. A reaction mixture 0.1 ml of 1 mg/ml pNPG and 0.1 ml sample was incubated at 50 °C for 30 min and then the reaction was stopped by adding 0.5 ml of 2% Na_2CO_3. Absorbance was measured at 410 nm. Therefore for the purpose of the present work, three modified methods based on the reference (BGase.mod1) and another modified (BGase.mod2) based on López Abelairas [14] were tested. Results were compared using two enzyme mixtures BG1 and BG2. Table 3 summarizes the different analytical methods used to measure β-glucosidase.

2.1.4. Xylanase (1,4-β-Dxylan xylanohydrolase EC 3.2.1.8)

One unit (U) of xylanase is defined as the amount of enzyme, which produces 1 μmol reducing sugar as xylose per min in the reaction mixture under the specified conditions.

The reference method was reported by Ghose and Bisaria [15]. For the assay, 0.5 ml sample and 0.5 ml of 1% beechwood xylan prepared in 50 mM sodium citrate buffer (pH 4.8) were mixed and incubated at 50 °C for 30 min. After incubation, 3 ml DNS were added and the mixture was put in boiling water bath for 5 min for color development. The reaction was then diluted with 20 ml H_2O and the absorbance was determined at 540 nm. The method presented in Bailey et al. [16] was also tested since it was the result of an interlaboratory study. A mixture of 1.8 ml of 1% xylan and 0.2 ml sample were incubated at 50 °C for 5 min. Three ml DNS were then added and the mixture boiled for 15 min before a final addition of 10 ml of water. Absorbance was measured at 540 nm. The protocol of Cianchetta et al. [17] was added to the comparison study since it was the only 96-well adapted protocol found in the literature. The authors applied a 25-fold reduction of the reference method. In addition to the methods presented above, 12 different combinations of a modified method (Xase.mod1) based on the reference method and 2 combinations of another modified method (Xase.mod2) based on that of Bailey et al. [16] are proposed.

The different analytical protocols were compared using two enzyme mixtures X1 and X2. Table 4 summarizes the 17 different analytical methods tested to measure xylanase activities.

2.2. Protocol for measuring reducing sugars

The 2-hydroxy-3,5-dinitrobenzoic acid most commonly known as DNS is very often used for the analysis of reducing sugars released during the enzymatic activity tests. The solution was prepared by dissolving 10 g of DNS in 500 ml of distilled water at 50 °C. The mixture was then cooled down to room temperature

Table 2
Summary table of the ten compared methods for CMCase analysis with Ghose [10] as reference, Xiao et al. [12] and FPase mod. (eight different combinations).

	Ghose [7] – reference method	Xiao et al. [9]	CMCase.mod (8 different combinations)
Substrate	Carboxymethylcellulose salt (CMC salt)		
Buffer	50 mM sodium citrate buffer (pH 4.8)		
Substrate concentration	2% CMC in buffer	2% CMC in buffer	2% *or* 4% CMC in buffer
Substrate volume	0.5 ml	0.03 ml	0.03 ml
Sample volume	0.5 ml	0.03 ml	0.03 ml *or* 0.06 ml
Total reaction volume	1 ml	0.06 ml	0.06 ml *or* 0.09 ml
Incubation at 50 °C for 30 min	Test tubes in water bath	96-well microplate in dry bath	96-well microplate in dry bath
Volume of DNS	3 ml	0.06 ml	0.15 ml and 0.18 ml
Color development	100 °C for 5 min	95 °C for 5 min	95 °C for 5 min
Dilution	Add 20 ml H_2O	N/A	Wihout dilution *or* Mix 0.2 ml H_2O with 0.04 ml color developed mixture
Reading wavelength	540 nm	540 nm	540 nm

Table 3
Summary table of the five compared methods for β-glucosidase with Kubicek and Pitt [13] as reference, BGase.mod1 (three different combinations) and BGase.mod2.

	Kubicek and Pitt [10] – reference method	BGase.mod1 (3 combinations)	BGase.mod2
Substrate	pNPG		
Buffer	50 mM sodium citrate buffer (pH 4.8)		
Substrate concentration	10 mM pNPG	10 mM pNPG	1 mg/ml pNPG
Substrate volume	1 ml	0.1 ml	0.1 ml
Sample volume	0.2 ml	0.02 ml	0.1 ml
Total reaction volume	1.2 ml	0.12 ml	0.2 ml
Incubation	40 °C for 10 min	40 °C for 10 min *or* 50 °C for 10 min	50 °C for 30 min
Color development/ Reaction stopping	1 ml of 1 M Na_2CO_3 (followed by centrifugation)	0.1 ml of 1 M Na_2CO_3 *or* Mix 0.06 ml sample with 0.15 ml of 2% Na_2CO_3	86 μL of color developed reaction mixture mixed with 214 μL of 2% Na_2CO_3
Absorbance reading	412 nm	412 nm	410 nm

(RT) before adding 20 ml of 2 N sodium hydroxide solution and 300 g of Rochelle salts (potassium sodium tartrate tetrahydrate). The volume was finally brought to 1 L with distilled water. The solution was kept at RT in the dark.

2.3. ***Measuring absorbance***

In non-reduced analytical protocols, an Uvi Light XTD 2 spectrophotometer (Secomam, ALES cedex, France) was used to measure absorbance. For 96-well plate adapted tests, a PowerWave XS2 microplate spectrophotometer (BioTek Instruments, Inc., Vermont, USA) with Gen5™ software was used.

It is important to note that in reduced protocols, given the small volumes used in the analysis, the plates were always covered with adhesive seals to reduce evaporative losses.

2.4. *Calculating enzyme activities*

Defining the critical value is the first step before carrying out any unit calculation of the enzymatic activity. This term was first introduced in Ghose [10] as the middle point in the calibration range. The origin of the middle point goes back to the works of Working and Hotelling [18], in which this point was defined as that with the highest probability in a trend. Therefore, for the different enzymatic activity calculations, the critical value should be first defined based on a specific calibration range. Then, the corresponding enzyme activity unit equation was derived. Note that enzyme samples were diluted at different concentrations and at least two dilutions were taken into account to derive the enzyme activity; the corresponding concentration to the critical value should fall between the two chosen enzyme concentrations.

2.4.1. *FPase*

A linear glucose standard curve was drawn with absolute glucose amounts in mg plotted against absorbance. Absorbance values of the different dilutions of the enzyme mixtures (after subtraction of the enzyme blanks) were thus translated into glucose. The critical value was defined as F_c (mg glucose). By plotting glucose against enzyme concentration (=1/dilution), the critical concentration releasing F_c was determined.

One FPU is 1 μmol of glucose/min which corresponds to 0.18 mg glucose/min. Therefore, F_c is produced by a given sample volume (ml) in a given time (min). FPU units are calculated as follows:

$$FPU = \frac{\frac{Fc}{0.18 * samplevolume * incubationtime}}{criticalconcentration} \frac{\mu mol}{(min.\ ml)} = \frac{units}{ml}$$

2.4.2. *CMCase*

The calculations are identical to the FPase units considering a critical value C_c. The enzymatic activity is thus expressed as:

$$CMC = \frac{\frac{Cc}{0.18 * samplevolume * incubationtime}}{criticalconcentration} \frac{\mu mol}{(min.\ ml)} = \frac{units}{m}l$$

2.4.3. *β-glucosidase*

Absolute pNP amounts in μmol were plotted against absorbance. Absorbance values of the different dilutions of the enzyme mixtures (after subtraction of the enzyme blanks) were thus

Table 4
Summary table of the 17 compared methods for xylanasee with Ghose and Bisaria [15] as reference, Bailey et al. [16], Cianchetta et al. [17], Xase.mod1 (12 different combinations) and Xase.mod2 (two different combinations).

	Ghose and Bisaria [12] – reference method	Bailey et al. [13]	Cianchetta et al. [14]	Xase.mod1 (12 combinations)	Xase.mod2 (2 combinations)
Substrate	1% beechwood xylan in buffer				
Buffer	50 mM sodium citrate buffer (pH 4.8)				
Substrate volume	0.5 ml	1.8 ml	0.02 ml	0.02 ml to 0.09 ml	0.09 ml
Sample volume	0.5 ml	0.2 ml	0.02 ml	0.01 ml to 0.03 ml	0.01 ml
Total reaction volume	1 ml	2 ml	0.04 ml	0.06 ml to 0.1 ml	0.1 ml
Incubation	50 °C for 30 min	50 °C for 30 min	50 °C for 20 min	50 °C for 30 min	50 °C for 30 min
Volume of DNS	3 ml	3 ml	0.12 ml	0.18 ml DNS to 0.06 ml mixture	0.15 ml
Color development	Boil in water bath for 5 min	Boil for 15 min	Boil for 15 min	Boil for 5 to 15 min	Boil for 15 min
Dilution	20 ml H_2O	Add 10 ml H_2O	0.04 ml of color developed reaction mixture mixed with 0.2 ml of H_2O	0.04 ml of color developed reaction mixture mixed with 0.2 ml of H_2O *or* without dilution	0.04 ml of color developed reaction mixture mixed with 0.2 ml of H_2O *or* without dilution
Absorbance reading	540 nm	540 nm	540 nm	540 nm	540 nm

translated into pNP. The critical value was defined as B_c (μmol pNP) and its corresponding critical concentration determined.

One unit (U) of β-glucosidase activity is defined as 1 μmol pNP/min. Therefore, B_c is produced by a given sample volume (ml) in a given time (min). This leads to the following equation:

$$\beta - glucosidase = \frac{\frac{Bc}{samplevolume * incubationtime}}{criticalconcentration} \frac{\mu mol}{(min.\ ml)} = \frac{units}{ml}$$

2.4.4. Xylanase

Absolute xylose amounts in μmol were plotted against absorbance. Absorbance values were then translated into xylose. The critical value is defined as X_c (μmol xylose); by plotting xylose against enzyme concentration, the critical concentration releasing X_c was determined.

One unit (U) of xylanase activity is defined as 1 μmol xylose/min. Therefore, X_c is produced by a given sample volume (ml) in a given time (min). This leads to the following equation:

$$Xylanase = \frac{\frac{Xc}{samplevolume * incubationtime}}{criticalconcentration} \frac{\mu mol}{(min.\ ml)} = \frac{units}{ml}$$

2.5. Statistical analysis

For each enzyme activity method, enzyme mixtures were diluted at least at 5 different levels and three replicates were carried out for every dilution. All data was then analyzed using the statistical package XLSTAT (version 2012.6.06). An analysis of variance (ANOVA) followed by a Tukey HSD (Honest Significant Difference) at 95% confidence level was run to determine which test was least significantly different from the corresponding reference protocol.

For each statistical test per enzyme activity type, all data were compared taking into account all the enzyme activity values of all enzyme mixtures. These latter were compared among the different tested methods. An arithmetic average value will be shown in the corresponding ANOVA tables.

3. Results and discussion

In each of the following sections, an overview of the existing analytical methods is first presented to justify the choice of the tested protocols. Calibration curves and statistical results are shown followed by a detailed description of the best 96-well adapted enzyme activity test.

3.1. FPase

Although the IUPAC method was described in Ghose [10], the oldest referenced method for FPase is that of Mandels et al. [19]. This latter was used by many researchers such as Chahal [20] and Krishna [21]. The IUPAC method is actually based on that of Mandels et al. [19] but differs with the amount of water added (20 ml instead of 16 ml) and the wavelength at which the absorbance is read (540 nm instead of 550 nm). It was widely used by many researchers such as Camassola and Dillon [22], Deswal et al. [3] and Hideno et al. [23]. Krishna et al. [24] and Guowei et al. [25] used it as well although in the former Na-citrate buffer was at 100 mM (pH=5) and in the latter incubation took place for only 30 min. More recently, Adney and Baker [26] proposed a modified version of the same method, in which the dilution before reading the absorbance was increased from a water to color developed mixture ratio of 4.44:1 to 12.5:1.

For the reduced protocols, Decker et al. [27] proposed a method with a total reaction volume of 0.08 ml using 2.67 mg of cellulose powder in 50 mM citrate buffer. Plates were incubated at 50 °C in custom modified microtiter plate incubators for 60 min. Later 150 μL DNS reagent were added before incubation at 98 °C for 10 min for color development. After this stage, 200 μL deionized water were mixed with 10 μL color developed mixture before reading the absorbance at 540 nm. This protocol was not tested since it used powdered cellulose as the substrate as compared to Whatman no 1 paper referenced in all other reviewed work. Two additional reduced methods were reported by Xiao et al. [11]. In the first, the enzymatic reaction volume was reduced to 60 μL (40 μL buffer and 20 μL sample) and in the second to 96 μL (64 μL buffer and 32 μL sample). According to the authors, statistical analyses of the cellulase activities showed no significant difference between the different types of tests. For the comparison study, the protocol based on the larger sample volume was considered. Finally, more recently Camassola and Dillon [28] described another protocol based on a 1/10 volume reduction of that used by Mandels [19]. The total reaction volume was thus reduced to 150 μL with 0.3 ml DNS addition and then a 2:1 dilution ratio of water to color developed mixture. The authors reported that according to their protocol the results were statistically similar to those of Mandels et al. [19]. They added that when using the method of Decker et al. [27] the data variability was important and that the 60-μL protocol of Xiao et al. [11] gave significantly higher values. This method was not included in the comparison because it is not directly applicable to a 96-well plate format; nevertheless the FPase.mod method was based on the same approach of volume reduction.

Statistical analysis showed that the proposed method FPase.mod provides results that are not significantly different from the reference (Table 5). Results based on the Xiao et al. [11] method were lower than those obtained by the reference. This might be explained by the fact that the ratio of enzyme to substrate was lower when compared to the other methods; the amount of enzyme was thus limiting. These interpretations applied to the three tested enzyme mixtures. Fig. 3 shows the calibration lines for the reference method and that of FPase.mod. Table 5 summarizes the average results of the different tested protocols with R^2 value of 0.81.

3.2. CMCase

The IUPAC reference [10] has been used by many authors such as Gokhale et al. [29], Krishna et al. [24], Krishna [21], Camassola and Dillon [22] and Acharya et al. [30]. Many other authors have also modified it. For example, Kalogeris et al. [31] used 4% CMC while Membrillo et al. [32] and Guowei et al. [25] used 1% CMC. Deswal et al. [3] used the buffer at pH 5.5, Hideno et al. [23] ran the incubation at 45 °C and Kiranmayi et al. [33] used 1.5 ml of buffer at pH 5.0 and ran the incubation at 30 °C. Finally, Kim and Kim [5] performed the reactions at 30 °C for 60 min in 50 mM Tris–HCl buffer (pH 7.2). A reduced method was described by Konig et al. [34]. A mixture of 0.03 ml sample and 0.03 ml of 4%

Table 5
ANOVA table for FPase (R^2=0.81). Average values indicated in the table correspond to arithmetic average of enzyme activities for FP1, FP2 and FP3 per tested method.

Tested method	Average (FPU/mL)	Groups
Ghose [7]	59	*A*
FPase.mod	54	A
Xiao et al. [8]	43	B

*Groups followed by the same letter are not significantly different ($P \leq 0.05$).

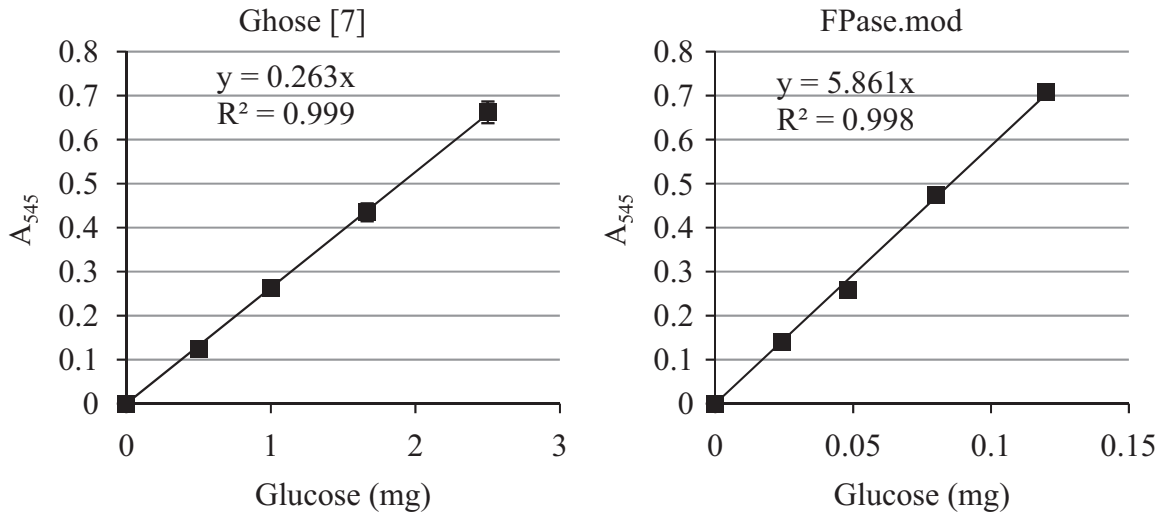

Fig. 3. Calibration curves of glucose in the analysis of FPase activity for the reference method [10] and best 96-well plate adapted method (FPase.mod).

CMC were incubated at 40 °C for 20 min. Later 0.15 ml DNS was added followed by incubation in boiling water bath for 10 min and addition of 1.5 ml water. Absorbance was measured at 530 nm. However, the only 96-well adapted method was that of Xiao et al. [12]. For comparison purposes, the reference method and that of Xiao et al. [12] were tested. For the latter, the authors indicated that their results were highly reproducible and accurately measured the endoglucanase activity compared with the IUPAC standard method. The eight different combinations proposed in Table 2 took into account the variations proposed by the other authors.

Given the results of the statistical analysis shown in Table 6, the protocol of Xiao et al. [12] underestimated the enzymatic activities. Several scenarios of the CMCase.mod protocol did not significantly differ from the reference method. However, the best method was CMCase.mod (#6), which was the least significantly different from the reference. Fig. 4 shows the calibration curves of both the reference and chosen methods. The chosen protocol corresponds to a total reaction mixture of 0.06 ml in which 0.03 ml of 4% CMC in citrate buffer and 0.03 ml of sample were mixed. After incubation at 50 °C for 30 min, 0.18 ml DNS was added and placed at 100 °C for 15 min. Before reading the absorbance at 540 nm, 40 μL color developed mixture were mixed with 200 μL distilled water.

3.3. β-glucosidase

For β-glucosidase, the IUPAC method was described by Wood and Bhat [35] using cellobiose as substrate. At the exception of Chahal [20] who used a 1% salicine solution, all other reviewed works referred to pNPG as substrate. For this reason the most widely used method, Kubicek and Pitt [13] was considered as the reference method; it was cited in Wood and Bhat [35], Christakopoulos et al. [36], Acharya et al. [30] and Deswal et al. [3]. Some reduced methods were reported but not adapted to 96-well plates. Gokhale et al. [29] reduced the reaction mixture to 1 ml with 0.9 ml of pNPG (1 mg/ml) and 0.1 ml sample. The mixture was incubated at 65 °C for 30 min. Later 2 ml of 2% sodium carbonate were added for color development and absorbance was measured at 410 nm. With also 1 ml reaction mixture, Hideno et al. [23] mixed 0.1 ml of 10 mM pNPG, 0.05 ml sample, 0.2 ml 50 mM acetic acid buffer (pH 4.8) and 0.65 ml distilled water. The mixture was incubated at 45 °C for 10 min and the reaction was terminated by adding 0.5 ml of 1 M Na_2CO_3 Absorbance was measured at 420 nm. Finally in Qian et al. [37], the reaction mixture consisted of 0.4 ml of pNPG (8 mM), 0.4 ml sample and 1.2 ml citrate phosphate buffer. The mixture was incubated at 45 °C for 30 min and the reaction was stopped by adding 2 ml of cold 0.5 M sodium carbonate buffer. The absorbance of the liberated p-NP was read at 400 nm.

More recently, López-Abelairas [14] used in the reaction mixture 0.1 ml of 1 mg/ml pNPG and 0.1 ml sample. This mixture was incubated at 50 °C for 30 min and then the reaction was stopped by adding 0.5 ml of 2% Na_2CO_3. Absorbance was measured at 410 nm. Until the end of the incubation, the reaction mixture could fit in a well of a microplate. Therefore for the present work, the dilution step was modified to adapt to the volume limitation. It is important to note that Kim and Kim [5] developed a 96-well adapted method, in which the enzyme activity was measured by incubating enzyme samples with 10 mM pNPG in 50 mM Tris–HCl buffer at a final volume of 0.2 ml for 60 min at 30 °C. The amount of pNP liberated was determined spectrophotometrically at 420 nm. Unfortunately no specific details of the method were published and therefore it was not possible to include it in the comparison study.

As presented in Table 3, the modified methods BGase.mod1 and BGase.mod2 took into account the different variations tested in the literature. Fig. 5 shows the calibration curves of both reference and chosen methods. As shown in Table 7, BGase.mod2 overestimated the enzymatic activities. Only one modified method based on Kubicek and Pitt [13] compared best to the reference method (BGase.mod1 (#3). In this latter, a reaction mixture of 0.1 mL 10 mM pNPG and 0.02 mL sample was incubated at 50 °C

Table 6
ANOVA table for CMCase (R^2=0.897). Average values indicated in the table correspond to arithmetic average of enzyme activities for CMC1 and CMC2 per tested method.

Tested method	Average (U/mL)	Groups			
Ghose [7]	3494	A			
CMCase.mod (#6)	3249	A			
CMCase.mod (#8)	2885	A	B		
CMCase.mod (#5)	2532	A	B	C	
CMCase.mod (#7)	2447	A	B	C	
CMCase.mod (#1)	2098		B	C	
Xiao et al. [9]	2051		B	C	
CMCase.mod (#2)	1659			C	
CMCase.mod (#3)	334				D
CMCase.mod (#4)	289				D

*Groups followed by the same letter are not significantly different ($P \leq 0.05$).

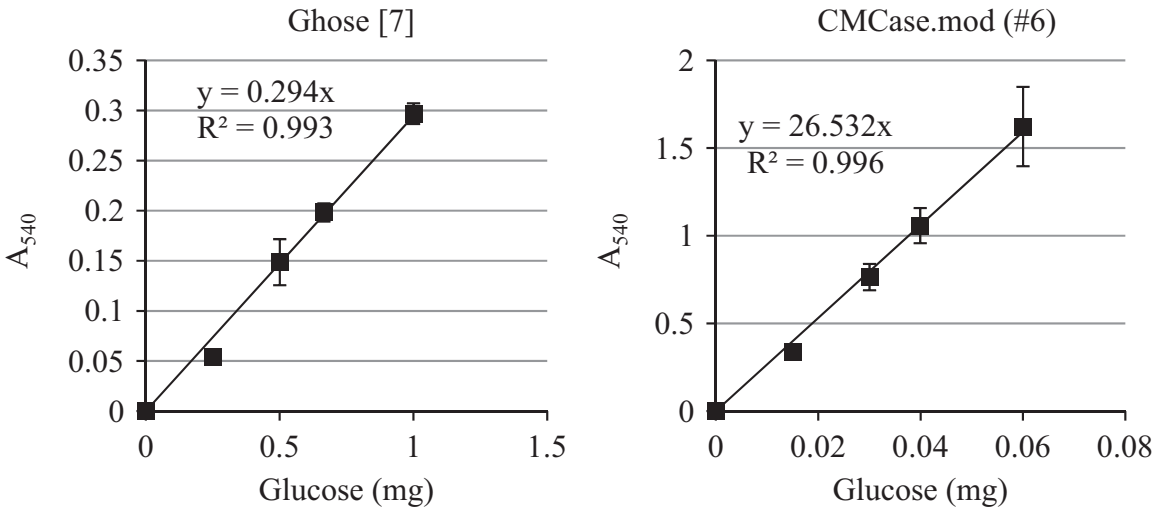

Fig. 4. Calibration curves of glucose in the analysis of CMCase activity for the reference method [10] and best 96-well plate adapted method (CMCase.mod #6).

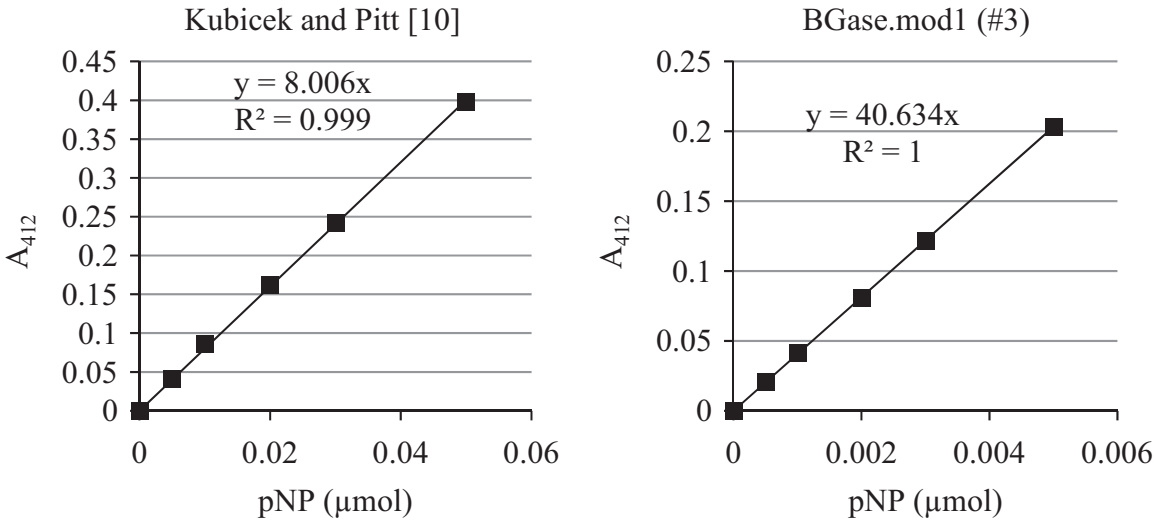

Fig. 5. Calibration curves of pNP in the analysis of β-glucosidase activity for the reference method [13] and best 96-well plate adapted method (BGase.mod #3).

Table 7
ANOVA table for β-glucosidase (R^2=0.990). Average values indicated in the table correspond to arithmetic average of enzyme activities for BG1 and BG2 per tested method.

Tested method	Average (U/mL)	Groups
BGase.mod1 (#2)	766	A
BGase.mod2	432	B
Kubicek and Pitt [10]	*237*	C
BGase.mod1 (#3)	214	C
BGase.mod1 (#1)	149	D

*Groups followed by the same letter are not significantly different ($P \leq 0.05$).

for 10 min. Later 0.06 ml sample was mixed with 0.15 ml of 2% Na_2CO_3 before reading absorbance at 412 nm.

3.4. Xylanase

For xylanase, the reference method was described in Ghose and Bisaria [15] and used also in Gokhale et al. [29]. The protocol proposed by Bailey et al. [16] was also referenced by Loera and Cordova [38], Membrillo et al. [32] and Camassola and Dillon [22].

Besides the two above methods; some reduced protocols have been also referenced. In Konig et al. [34], 0.03 ml sample was pre-

Table 8
ANOVA table for xylanase (R^2=0.970). Average values indicated in the table correspond to arithmetic average of enzyme activities for X1 and X2 per tested method.

Tested method	Average (U/mL)	Groups
Bailey et al. [13]	15447	A
Xase.mod2 (#2)	9954	B
Xase.mod1 (#6)	9647	B
Xase.mod2 (#1)	9543	B C
Xase.mod1 (#5)	9329	B C
Xase.mod1 (#11)	9081	B C
Xase.mod1 (#12)	8638	C D
Ghose and Bisaria [12]	*8094*	*D E*
Xase.mod1 (#8)	7143	E F
Xase.mod1 (#7)	6662	F
Xase.mod1 (#10)	6361	F G
Xase.mod1 (#9)	6198	F G
Cianchetta et al. [14]	5590	G
Xase.mod1 (#2)	4366	H
Xase.mod1 (#1)	4067	H
Xase.mod1 (#4)	2865	I
Xase.mod1 (#3)	2828	I

*Groups followed by the same letter are not significantly different ($P \leq 0.05$).

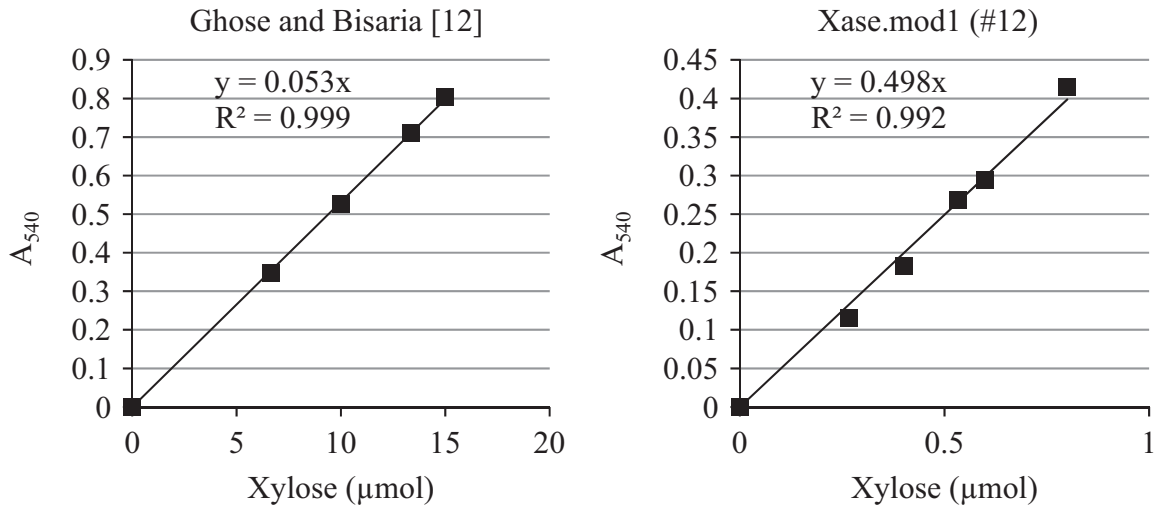

Fig. 6. Calibration curves of xylose in the analysis of xylanase activity for the reference method [15] and best 96-well plate adapted method (Xase.mod #12).

incubated for 5 min at 40 °C and then added to 0.3 ml of 1.5% (w/v) xylan equilibrated at 40 °C. The mixture was incubated at 40 °C for 20 min. Later, 0.15 ml DNS were added and the new mixture was boiled in a water bath for 5 min. Before measuring the absorbance at 530 nm, 1.5 ml of water were added. In Saha [39], xylanase activity was assayed in a reaction mixture of 0.5 ml containing boiled oat spelt xylan (1% w/v), 50 mM acetate buffer and the sample. After 30 min incubation at 50 °C, reducing sugars were measured according to Miller [40]. In Lu et al. [41], the same approach was followed by on a reaction mixture of 2 ml of 0.8% (w/v) oat xylan solution and 2 ml sample incubated at 40 °C for 30 min. It is also the case in Rodriguez-Fernandez et al. [42] where 0.5 ml of 1.0% (w/v) birchwood xylan and 0.5 ml sample were incubated at 50 °C for 10 min before adding 1 ml of DNS. After heating for 5 min in boiling water bath, 5 ml of water were added and absorbance was read at 540 nm. None of the above mentioned protocols were adapted for the 96-well plate, except that reported by Cianchetta et al. [17]. For this reason this latter was considered for comparison protocols. The other proposed protocols in the comparison study took into account the variations found in other reviewed methods.

Out of all tested methods, statistical analysis (Table 8) showed that both Xase.mod1 (#12) and Xase.mod1 (#8) were not significantly different from the reference. The former was chosen because the percent difference was less important (7% vs. 12%). The method proposed by Bailey et al. (1992) largely overestimated the enzymatic activities and that of Cianchetta et al. [17] underestimated them.

In the chosen protocol, 0.08 ml 1% beechwood xylan in citrate buffer and 0.02 ml sample were incubated at 50 °C for 30 min. Later 0.18 ml of DNS was added before boiling for 5 min. Dilution of 0.04 ml color developed mixture in 0.2 ml water was performed before measuring the absorbance at 540 nm. Fig. 6 shows the calibration curves of both the reference and chosen methods.

4. Conclusions

Four enzymes, FPase, CMCase, β-glucosidase and xylanase, were identified of interest to degrade the lignocellulosic complex under anaerobic conditions. In this context, large factorial experiments need to be conducted but most existing measurement protocols of those enzymes are not adapted. This work identifies through extensive and systematic analytical work microplate-based protocols for these enzymes of interest: 0.072 mL reaction volume with Whatman no1 paper as substrate for FPase, 0.06 mL reaction volume with 4% CMC as substrate for CMCase, 0.12 ml reaction mixture with 10 mM pNPG as substrate for β-glucosidase and 0.1 mL mixture with 1% beechwood xylan as substrate for xylanase. If those protocols are used by other researchers, results in the literature could be easily compared and more effective transfer of information could be achieved.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.12.073.

References

[1] H.V. Lee, S.B.A. Hamid, S.K. Zain, Conversion of lignocellulosic biomass to nanocellulose:Structure and chemical process, Sci. World J. (2014) 1–20.
[2] E. Santoni, A. Callone, J. Sandak, S. Sandak, Dirè, Solid state NMR and IR chacaterization of wood polymer structure in relation to tree provenance, Carbohydr. Polym. 117 (2015) 710–721.
[3] D. Deswal, Y.P. Khasa, R.C. Kuhad, Optimization of cellulose production by a brown rot fungus *Fomitopsis sp.* RCK under solid state fermentation, Bioresour. Technol. 102 (2011) 6065–6072.
[4] S.A. Jabasingh, C.V. Nachiyar, Response surface approach for the biodegradation of pretreated coir pith using *Aspergillus nidulans* SU04 for cellulase production, Second international conference on sustainable energy and intelligent system (SEISCON 2011).
[5] S. Kim, C.H. Kim, Production of cellulase enzymes during the solid-state fermentationo f empty palm fruit bunch fiber, Bioprocess Biosyst. Eng. 35 (2012) 61–67.
[6] S. Montoya, C.E. Orrego, L. Levin, Growth, fruiting and lignocellulolytic enzyme production by the edible mushroom *Grifola frondosa* (maitake), World J. Microbiol. Biotechnol. 28 (2012) 1533–1541.
[7] E. Fernandez-Fueyo, F.J. Ruiz-Dueñas, P. Ferrerira, et al., Comparative genomics of *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective lignolysis, PNAS (2012) 5458–5463.
[8] F.J. Ruiz-Dueñas, T. Lundell, D. Floudas, J.M. Barrasa, D.S. Hibbett, A.T. Martinez, Lignin-degrading peroxidases in Polyporales: an evolutionary survey based on 10 sequenced genomes, Mycologia 105 (2013) 1428–1444.

[9] M. Pérez-Boada, F.J. Ruiz-Dueñas, R. Pogni, et al., Versatile peroxidase oxidation of high redox potential aromatic compounds: site-directed mutagenesis, spectroscopic and crystallographic investigation of three long-range electron transfer pathways, J. Mol. Biol. 354 (2005) 385–402.
[10] T.K. Ghose, Measurement of cellulase activities, Pure Appl. Chem. 59 (1987) 257–268.
[11] Z. Xiao, R. Storms, A. Tsang, Microplate-based filter paper assay to measure total cellulase activity, Biotechnol. Bioeng. 88 (2004) 832–837.
[12] Z. Xiao, R. Storms, A. Tsang, Microplate-based carboxymethylcellulose assay for endoglucanase activity, Anal. Biochem. 342 (2005) 176–178.
[13] C.P. Kubicek, D.E. Pitt, Effect of dilution rate on β-glucosidase secrection and cell wall metabolism in *Trichoderma aureoviride* cultivated in a continuous air lift fermenter, Eur. J. Appl. Microbiol. Biotechnol. 16 (1982) 189–196.
[14] López Abelairas, Biological pretreatment of lignocellulosic materials for the production of ethanol. MSc Thesis, University of Santiago de Compostela, Dept. Chem. Eng. 2010, 82pp.
[15] T.K. Ghose, V.S. Bisaria, Measurement of hemicellulose activities – part 1: Xylanases, Pure Appl. Chem. 59 (1987) 1739–1752.
[16] M.J. Bailey, P. Biely, K. Poutanen, Interlaboratory testing of methods for assay of xylanase activity, J. Biotechnol. 23 (1992) 257–270.
[17] S. Cianchetta, S. Galletti, P.L. Burzi, C. Cerato, Hydrolytic potential of *Trichoderma sp*. strains evaluated by microplate-based screening followed by switchgrass saccharification, Enzym. Microb. Technol. 50 (2012) 304–310.
[18] H. Working, H. Hotelling, Applications of the theory or error to the interpretation of trends, J. Amer. Stat. Assoc. 24 (1929) 73–85.
[19] M. Mandels, R. Andreotti, C. Roche, Measurement of saccharifying cellulase, Biotechnol. Bioeng. Symp. 6 (1976) 21–33.
[20] D.S. Chahal, Solid state fermentation with *Trichoderma reesei* for cellulase production, Appl. Environ. Biotechnol. 49 (1985) 205–210.
[21] C. Krishna, Production of bacterial cellulases by solid state bioprocessing of banana wastes, Biores. Technol. 69 (1999) 231–239.
[22] M. Camassola, A.J.P. Dillon, Biological pretreatment of sugar cane bagasse for the production of cellulases and xylanases by *Penicillium echinulatum*, Ind. Crop. Prod. 29 (2009) 642–647.
[23] A. Hideno, H. Inoue, K. Tsukahara, S. Yano, X. Fang, T. Endo, S. Sawayama, Production and characterization of cellulases and hemicellulases by *Acremonium cellulolyticus* using rice straw subjected to various pretreatments as the carbon source, Enzym. Microb. Technol. 48 (2011) 162–168.
[24] J. Krishna, S.K. Khare, A.P. Gandhi, Solid state fermentation of soyhull for the production of cellulase, Biores. Technol. 54 (1995) 321–322.
[25] S. Guowei, H. Man, W. Shikai, C. He, Effect of some factors on production of cellulase by *Trichoderma reesei* HY07I, Procedia Environ. Sci. 8 (2011) 357–361.
[26] B. Adney and J. Baker, Measurement of cellulase activities, http://www.nrel.gov/biomass/analytical_procedures.html#lap-013.
[27] S.R. Decker, W.S. Adney, E. Jennings, T.B. Vinzant, M.E. Himmel, Automated filter paper assay for determination of cellulase activity, Appl. Biochem. Biotechnol. 105 (2003) 689–703.
[28] M. Camassola, A.J.P. Dillon, Cellulase determination: Modifications to make the filter paper assay easy, Fast, Pr. Effic. 1 (2012) 125, http://dx.doi.org/10.4172/scientificreports.125.
[29] D.V. Gokhale, S.G. Patil, K.B. Bastawade, Optimization of cellulase production by *Aspergillus niger* NCIM 1207, Appl. Biochem. Biotechnol. 30 (1991) 99–109.
[30] B.K. Acharya, S. Mohana, R. Jog, J. Divecha, D. Madamwar, Utilization of anaerobically treated distillery spent wash for production of cellulases under solid-state fermentation, J. Environ. Manag. 91 (2010) 2019–2027.
[31] E. Kalogeris, P. Christakopoulos, P. Katapodis, A. Alexiou, S. Vlachou, D. Kekos, B.J. Macris, Production and characterization of cellulolytic enzymes from the thermophilic fungus *Thermoascus aurantiacus* under solid state cultivation of agricultural wastes, Process. Biochem. 38 (2003) 1099–1104.
[32] C. Membrillo, M. Sanchez, E. Meneses, O. Favela, Loera, Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by *Pleurotus ostreatus* strains, Bioresour. Technol. 99 (2008) 7842–7847.
[33] M.U. Kiranmayi, S. Poda, P.B.B.N. Charyulu, M. Vijayalakshmi, P.V. Krishna, Studies on influence of natural biowastes on cellulase production by *Aspergillus niger*, J. Environ. Biol. 32 (2011) 695–699.
[34] J. Konig, R. Grasser, H. Pikor, K. Vogel, Determination of xylanase, β-glucanase, and cellulase activity, Anal. Bioanal. Chem. 374 (2002) 80–87.
[35] T.M. Wood, K.M. Bhat, Methods for measuring cellulase activities, Methods Enzymol. 160 (1988) 87–112.
[36] P. Christakopoulos, P.W. Goodenough, D. Kekos, B.J. Macris, M. Claeyssens, M. K. Bhat, Purification and characterisation of an extracellular β-glucosidase with transglycosylation and exo-glucosidase activities from *Fusarium oxysporum*, Eur. J. Biochem. 224 (1994) 279–385.
[37] L.-C. Qian, S.-J. Fu, H.-M. Zhou, J.-Y. Sun, X.-Y. Weng, Optimization of fermentation parameters for β-glucosidase production by *Aspergillus niger*, J. Anim. Vet. Adv. 11 (2012) 583–591.
[38] O. Loera, J. Cordova, Improvement of xylanase production by a parasexual cross between *Aspergillus niger* strains, Braz. Arch. Biol. Technol. 46 (2003) 177–181.
[39] B.C. Saha, Production, purification and properties of xylanase from a newly isolated *Fusarium proliferatum*, Process. Biochem. 37 (2002) 1279–1284.
[40] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (1959) 426–428.
[41] W. Lu, D. Li, Y. Wu, Influence of water activity and temperature on xylanase biosynthesis in pilot-scale solid-state fermentation by *Aspergillus sulphureus*, Enz. Microb. Technol. 32 (2003) 305–311.
[42] D.E. Rodriguez-Fernandez, J.A. Rodriguez-Leon, J.C. de Carvalho, W. Sturm, C. R. Soccol, The behavior of kinetic parameters in production of pectinase and xylanase by solid-state fermentation, Bioresour. Technol. 102 (2011) 10657–10662.