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An economical and high throughput alternative for endoglucanase activity determination

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ABSTRACT

The search for new strains with good productivity of cellulolytic enzymes is always necessary to improve the processes are usually employed. Screening protocols used for these purposes require a low cost and high throughput assay for determination of endoglucanase activity. However, the test proposed by the IUPAC and other microadaptations not meet this requirement. The aim of our work is to obtain an economical and high-performance proposed by IUPAC for determination of endoglucanase activity assay adaptation. For this we reduced the assay volume and investigate the effect of this reduction on endoglucanase activity determination. As a result a miniaturized assay statistically correlated with the assay proposed by IUPAC reduced 10-fold lower scale was obtained. As a result a new economic and high performance adaptation for endoglucanase activity assay based on the IUPAC protocol was obtained.
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KEYWORDS

INTRODUCTION

The development of efficient enzyme mixtures for the treatment of lignocellulosic material is one important goal of modern industrial biotechnology. The screening of different enzyme activities is of fundamental importance to pursue a rational design for specific applications. Three major groups of enzyme activities have been described in *Trichoderma spp.* cellulose degrading machinery: endo-β-glucanase (EC 3.2.1.4; endoglucanase; EG), exo-β-glucanase (EC 3.2.1.91; exoglucanases; CBH) and β-glucosidase (EC 3.2.1.21)[1]. The filter paper assay (FPA), generally applied for cellulose activity assessment, accounts for the depolymerization of cellulose considering the three activities as a whole[1,2]. Other methods allow evaluating particular enzyme activities within complex enzyme mixtures broadening the information of the enzymatic profile within the mixture. In particular, endoglucanase activity is often represented by carboxymethylcellulase activity (CMCase) due to the inability of cellobiohydrolases to attack substituted cellulose substrates[3,4]. This activity is relevant in applications pursuing viscosity reduction of cellulosic sub-

strates[5].

The screening for particular enzyme profiles involves hundreds of strains and several rounds of selection, and then efficient assay methods are required[1]. Dealing with large amounts of samples requires methodological procedures able to produce good quality results with minimal turnaround time, cost and waste.

The miniaturization of already established techniques is a viable approach to develop adequate assay procedures. The scaling down of good performance known methods to around hundred microliter volume and adaptation to a microplate format would result in numerous advantages: reduced sample volume, less reagent use, reduction of reagent waste. Furthermore, the laboratory space involved in the assay procedure would be reduced, time for pipetting shortened and then cost per assay[6,9,10]. In this context, Xiao and co-workers[9] establish a microassay protocol for endoglucanase activity determination based on 96-well plate format, but this microassay requires the use of a thermocycler equipment per plate thereby limiting the processing of multiple plates in parallel.

The aim of this work is to find a new microsasay protocol for endoglucanase activity determination that allows multiple plates in parallel and studying the correlation to the IUPAC assay widely used.

MATERIALS AND METHODS

Culture conditions and enzyme: The enzyme preparations were obtained from culture supernatants from the hypercellulolytic *Trichoderma reesei* mutant RUT C30. Cultures were performed in a 5l lab scale bioreactor using modified Mandel's media with microcrystalline cellulose as carbon source[2]. The reactor was inoculated with $1x10^6$ espores/mL obtained from 14 days old potato dextrose agar (PDA) cultures. Fermentation was performed at pH 4.8, 28 °C and 500 rpm agitation for 7 days. The supernatant was recovered by vacuum assisted filtration using Whatman N° 1 filterpaper, then concentrated by tangential flow filtration (10 KDa nominal cutoff polyethersulphone hollow fiber cartridge), and it was finally formulated in 0.1 % potassium sorbate; 10 % sodium chloride and 10% glycerol.

IUPAC CMCase assay

CMCase measurements were performed following the IUPAC method compiled by Ghose[4]. Briefly, 0.5 mL of the enzyme dilution was mixed with 0.5 mL 2% carboxymethylcellulose in 0.05 M citrate buffer, pH 4.8 and kept at 50°C for 30 min. Then, 1 mL of the mixture was assayed for glucose equivalent of reducing sugar by DNS method[10].

Unit calculation

All determinations were carried out by triplicate and all activities were calculated according to IUPAC criteria using the formulas proposed in Ghose[4]. Briefly, for enzyme formulations releasing 0.5 mg of glucose equivalents (concentrated enzyme formulations), the CMC units were calculated using the formula: CMC (units/mL) = 0.185 (units/mL)/ enzyme concentration to release 0.5 mg glucose, where concentration = (vol. enzyme in dilution)/(total volume of dilution). For preparations displaying glucose release lower than 0.5 mg (diluted enzyme formulations), units were calculated as CMC (units/ mL) = mg glucose released x 0.37 (units/mL).

Miniaturized CMCase assay

CMCase measurements were performed with a reduced volume version of the IUPAC protocol. Briefly, 50 µL of the enzyme dilution and 50 µL 2 % carboxymethylcellulose both prepared in 0.05 M citrate buffer pH 4.8 was transferred to 96 wells plated in separated wells and preheated for five minutes; then the well containing the enzyme dilution was mixed with the substrate and mixed by pipetting up and down. The enzymatic reaction was carried out according IUPAC protocol at 50°C for 30 min.

Colorimetric assay in microplate

The colorimetric assay was performed according to Miller´s protocol for IUPAC CMCase assay and the reduced version of this protocol was assayed in deep well plates. In the reduced protocol, 300 µL of DNS was added to each sample (mixed by pipetting up and down) and then the plate was held for 5 min at 100°C in water bath, finally 200 µL of

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each sample were diluted with 1 mL of deionized water and absorbance measured at 540 nm.

Experimental design and statistical treatments

Twenty enzymatic dilutions were tested for glucose equivalent release (n=3) by using the miniaturized CMCase assay and the IUPAC assay. The results from the two methods were compared by linear regression and Pearson's correlation test. The enzymatic activity of samples was measure by using both methods in parallel. Four samples of concentrated and diluted enzymes formulations were assayed. The results of all the determinations were analyzed in the first place by a Fisher test to evaluate homoscedasticity, and then the significant difference between the two methods was analyzed by Student's test using the Statgraphics Software (Statpoint Technologies, Inc.).

RESULTS

Evaluation of miniaturized CMCase assay

The assessment of the scaling down of the IUPAC test consisted in a stepwise validation of the assay procedures. Firstly, we studied the glucose equiva-

lents released in the enzymatic reactions performed by using low and high enzyme concentrations in order to obtain glucose equivalents values around the 0.5 mg recommended by the IUPAC methodology[4] (Figure 1). Twenty enzymatic dilutions were tested for glucose equivalent release (n=3) by using the miniaturized CMCase assay and the IUPAC assay. The descriptive statistical analysis accomplished to compare two sets of data showed a normal distribution of data (results not shown). When results from the two methods were compared by linear regression and Pearson's correlation test, we found a R^2= 99.06 %, a coefficient of correlation 0.995 and a standard error of estimation of 0.027, all values were estimated with a p value < 0.05. These results confirmed that there is a strong linear relationship between the two methods along the data set tested, with a confidence level greater than 95%.

Activity determination in both methods

To evaluate the performance of the miniaturized CMCase assay, the enzymatic activity of samples was measured by using both methods in parallel. Samples of concentrated and diluted enzymes formulations were assayed (Figure 2). The p value obtained in Student's test was higher than 0.05 (p> 0.05) confirm-
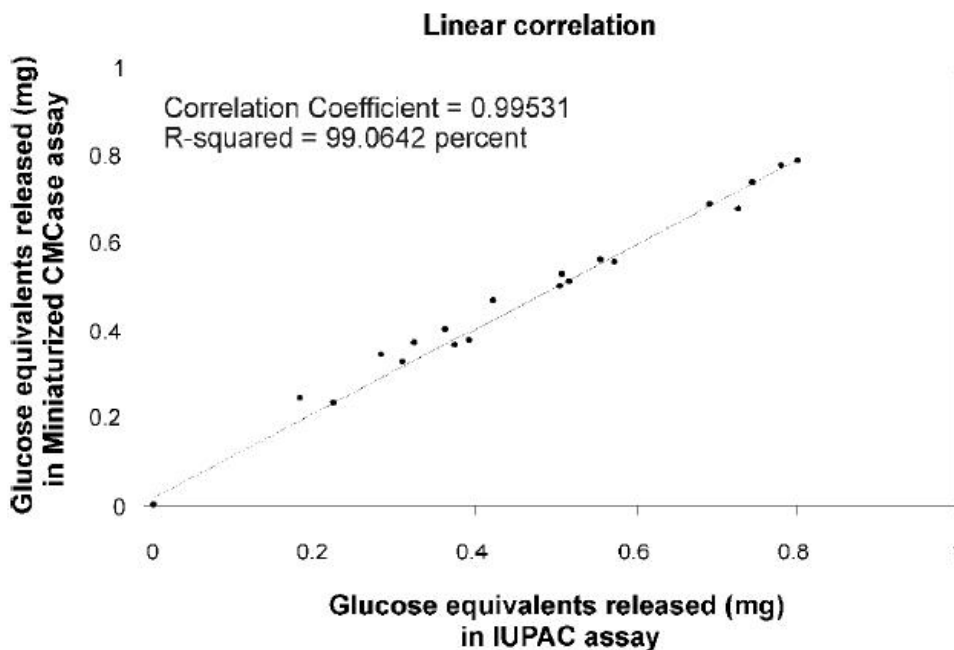

Figure 1 : Correlation model between glucose equivalents (mg) released by IUPAC assay (x-axis) and miniaturized CMCase assay (y-axis). Each point represents the mean of three independent replicates of glucose equivalents released in the enzymatic reactions performed using the miniaturized CMCase assay and the IUPAC assay respectively. The straight line represents the linear regression showing the correlation between the two methods
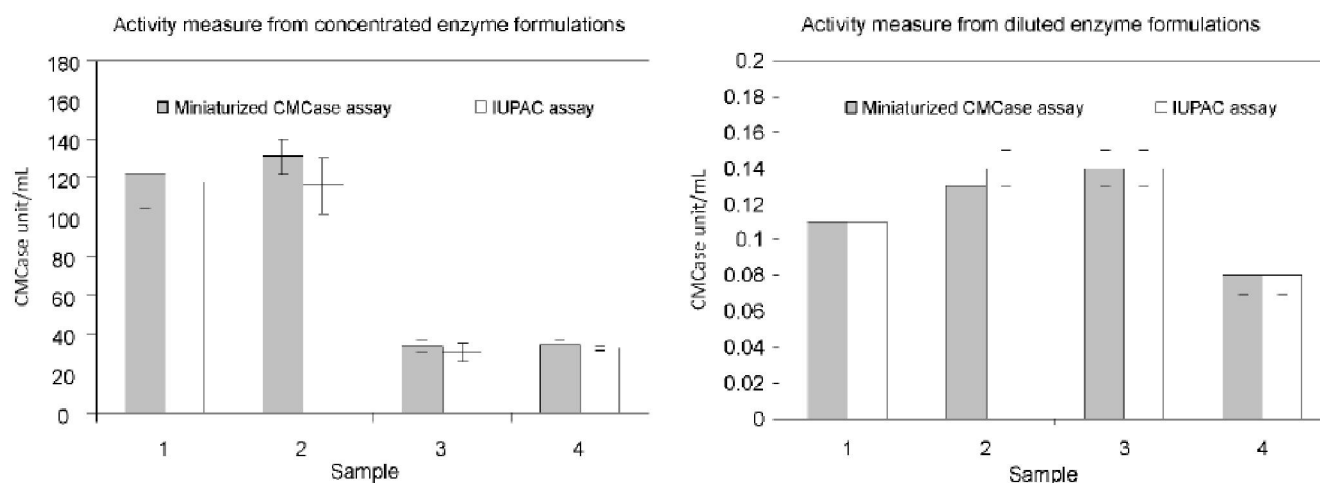
Figure 2 : Comparison of activity determined by both methods. A) Concentrated enzyme formulation. B) Diluted enzyme formulation

TABLE 1: Statistical summary of activity determinations for both methods, The p value higher than 0.05 (p> 0.05) indicates that the homoscedasticity criteria is met (Fisher's test) and there are not significant differences between the methods (Student's test)

	Concentrated sample			
	Miniaturized CMCase assay (n=3)	**IUPAC Assay (n=3)**	**Statistics**	
Sample	Mean in CMC [units /mL]	Mean in CMC [units /mL]	Fisher test (p value)	Student test (p value)
1	122 ± 17	118 ± 18	0,95	0,75
2	131 ± 9	116 ± 14	0,37	0,49
3	34 ± 3	31 ± 5	0,58	0,22
4	35 ± 2	33 ± 1	0,79	0,14
	Dilutedsimple			
	Miniaturized CMCaseassay (n=3)	**IUPAC Assay (n=3)**	**Statistics**	
Sample	Mean in CMC [units /mL]	Mean in CMC [units /mL]	Fisher test (p value)	Student test (p value)
1	0,11 ± 0,01	0,11 ± 0,01	1,00	0,52
2	0,13 ± 0,01	0,14 ± 0,01	0,40	0,42
3	0,14 ± 0,01	0,14 ± 0,01	1,00	0,23
4	0,08 ± 0,01	0,08 ± 0,01	1,00	1,00

ing that there were not significant differencesin the enzymatic determinationsbetween the methods(TABLE 1).

DISCUSSION

The IUPAC technique presented by Ghose[4] has been used for decades in the determination of endoglucanase activity. The precise assessment of the CMCaseactivity within a sample by using this method required several dilutions of the sample to achieve a glucose equivalent release around 0.5 mg of glucose[1,4].

The method presented here poses an order of magnitude reduction in the reagent volume, space usage, pipetting effort and the use of water bath equipment allows multiples plates processing in parallel (TABLE 2). Thus, allowing high throughput determination of this activity more feasible and economically. On the other hand during the method optimization it was observed that the geometric parameters of the reaction container greatly affected the activity result, in particular when using PCR multiwell plates (results not shown).

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TABLE 2 : Procedure comparison between standard IUPAC CMCase activity assay and miniaturized assay

Assay	IUPAC assay	Miniaturized CMCase assay
	Comparison of methods	
2% CMC in Buffer	0.5mL	50 μL
Buffer volume in blank and standard curve	0.5 mL	50 μL
Enzyme Dilution or glucose standard volume	0.5 mL	50 μL
Enzyme reaction at 50°C for 30 min	Test tubes in water bath	96 deep-well microplate in water bath
Volume of DNS	3.0 mL	300 μL.
Color development	100°C for 5 min	100°C for 5 min
Amount of H2O added prior to measurement	20 mL	1 mL of water to 200 μL of the reaction/DNS mixture
Read at 454 nm	1 mL in spectrophotometer cuvette	200 μL in standard optical 96 well plate

It is noteworthy that the correlation between the miniaturized method and the IUPAC method was observed in the whole dynamic range of the standard assay; as a consequence not only CMC units but also international units (IU) would be determined when the glucose release is lower than the critical value (0.5 mg of glucose equivalents) as stated by Ghose[4].

CONCLUSION

The results obtained in this paper indicated that a 10 fold scale down version of IUPAC CMCase assay linearly correlates with the classical assay even at low concentrations of enzymes. The equipment used and assay reduction in the present work, diminish the amount of DNS used (contaminant reagent), increases the number of samples that can be processed in parallel per assay in the same work area. In conclusion, the assay reduction result in a lower cost per test and then poses it as the preferred option to carry out high-throughput screening.

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