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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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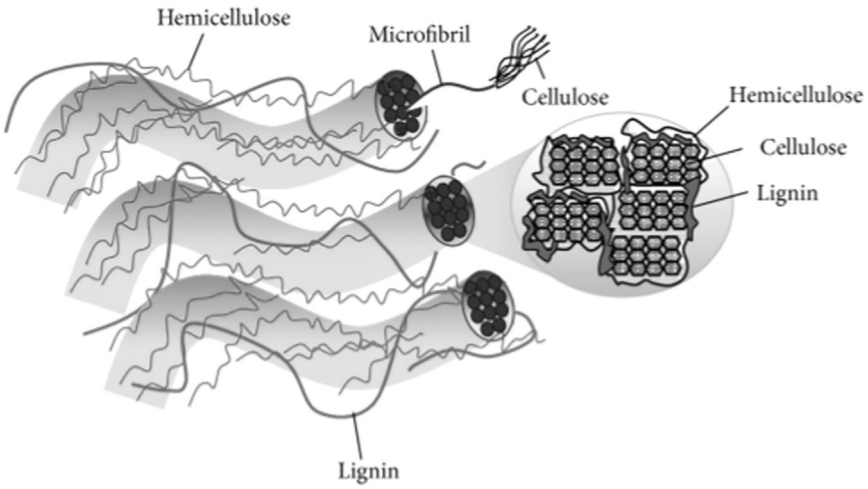


**Fig. 1.** Schematic diagram of the three components of lignocellulose: cellulose, lignin and hemicellulose [1].

## 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

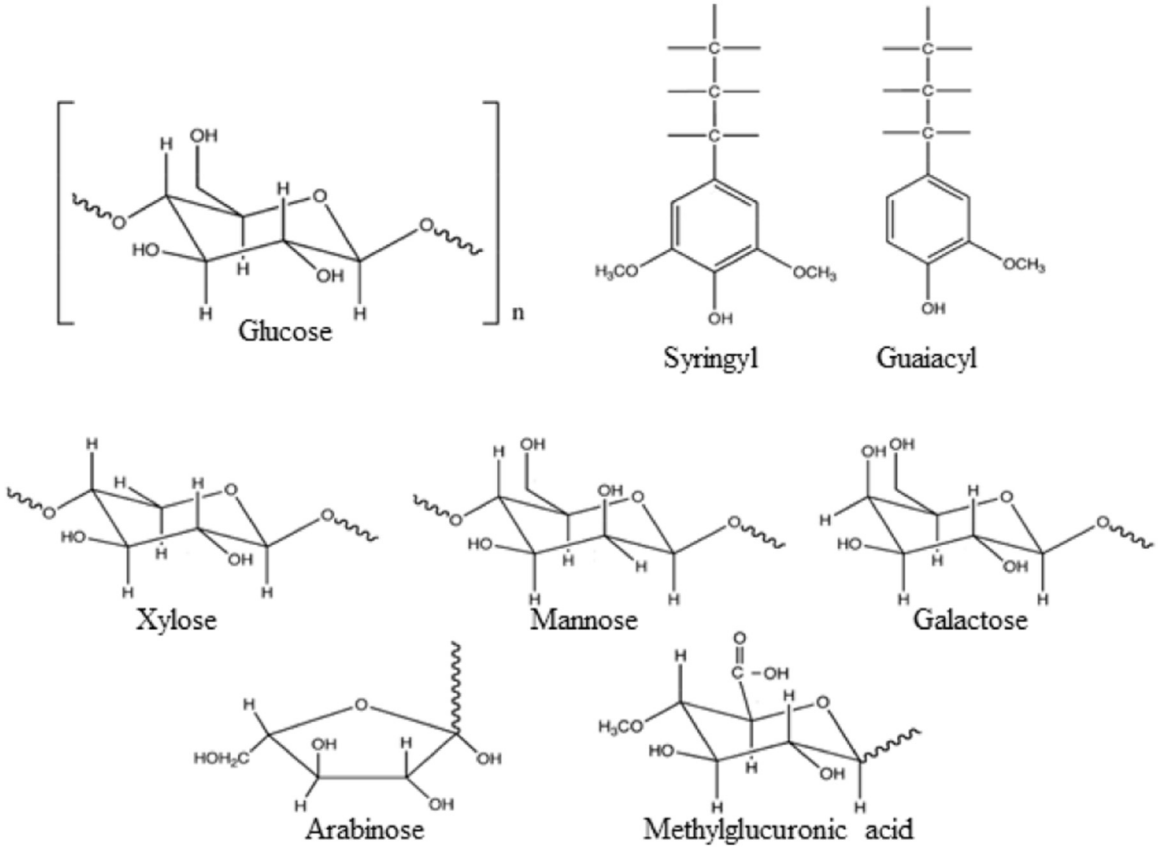


**Fig. 2.** Chemical structures of the different monomeric components: glucose of cellulose, syringyl and guaiacyl of lignin, xylose, mannose, galactose, arabinose and methylglucuronic acid of hemicellulose [2].