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An alternative method for the determination of polysaccharide cleavage enzymes activities

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ABSTRACT

Most of usual methods used to detect and quantify polysaccharide cleavage enzymes (PCE) are not adapted to high throughput. In this work, enzymatic activities of three enzymes, namely an endocellulase (EC 3.2.1.4), an exocellulase (EC 3.2.1.91) and an alginate lyase (EC 4.2.2.3) were characterized by classical Michaelian model (K_m, V_{max}). Enzymatic activities were then determined by the use of the Enzyme Ring Test® (ERT®) method which allowed following the depolymerization of polysaccharides by PCE. Kinetic parameters were determined and compared to the ones obtained by classical methods. Besides, the ERT® technology clearly showed the possibility to discriminate endolytic and exolytic enzymatic mechanisms.

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1. Introduction

Polysaccharides are abundant in all organisms in which they have several biological functions essential for life (energy, mechanical structure, water retention, regulation of cell function, and others). Owing to their specific and various structures, poly- and oligosaccharides exhibit a structural diversity higher than the diversity of proteins and nucleic acids [1,2]. This structural diversity of PS and their abundance leads to numerous applications in food, pharmaceutical or nutraceutical industries [3,4]. Nowadays, manufactured oligosaccharides obtained from depolymerization of polysaccharides, using physical, chemical and enzymatic methods are widely studied [5–12]. Among them specific depolymerization of polysaccharides by PCE, *i.e.* glycoside hydrolases (EC 3.2.1.-) and polysaccharide lyases (EC 4.2.2.-) is of interest [13–17]. PCE are widely used in many other processes dedicated to food, textile, paper or cosmetic industries [18–21]. Moreover, several studies aim to modify enzymes, *i.e.* by random mutagenesis, site directed mutagenesis or directed evolution, to modify, improve or create new PCE activities [22,23]. In this way, the identification of original PCE activities, native or recombinant, and the development of new PCE cocktails are of primary interests. Yet, the screening of PCE activities requires rapid and reliable tools to detect and measure depolymerization of polysaccharides. Numerous methods are currently used to detect and quantify the activity of glycoside hydrolases and polysaccharide lyases. Some plate screening methods are based on the interaction of dyes with functional groups of the polysaccharides and on their rheological properties [24]. Other ones are premised on the precipitation of polysaccharides in presence of ethanol or cetylammonium bromide (CTAB) [25]. These methods are specific of a couple PCE/polysaccharide but time consuming and not quantitative. Colorimetric assays, *e.g.* dinitrosalicylic (DNS) assay [26] or 2,2′-bicinchoninate (BCA) assay [27], are common, accurate and reproducible ways to measure PCE activities by following quantity of reducing sugars. However, these biochemical methods do not allow the screening and quantification of PCE activities at high throughput as they are only qualitative in the majority and/or involved several steps of heating at high temperatures [28]. Some chromatographic, electrophoretic and spectroscopic (FTIR) methods based on the following of the polymerization degree during the enzymatic reactions can be also used [29,30]. Finally, rheological measurements, dedicated to hydrosoluble polysaccharides, also allow to detect and quantify PCE activities measuring a drop of viscosity during the depolymerization of polysaccharide [31].

The Ring Test® technology is a microplate method based on the immobilization of magnetic beads in a polysaccharide solution. Initially, the method was dedicated to the evaluation of biofilm

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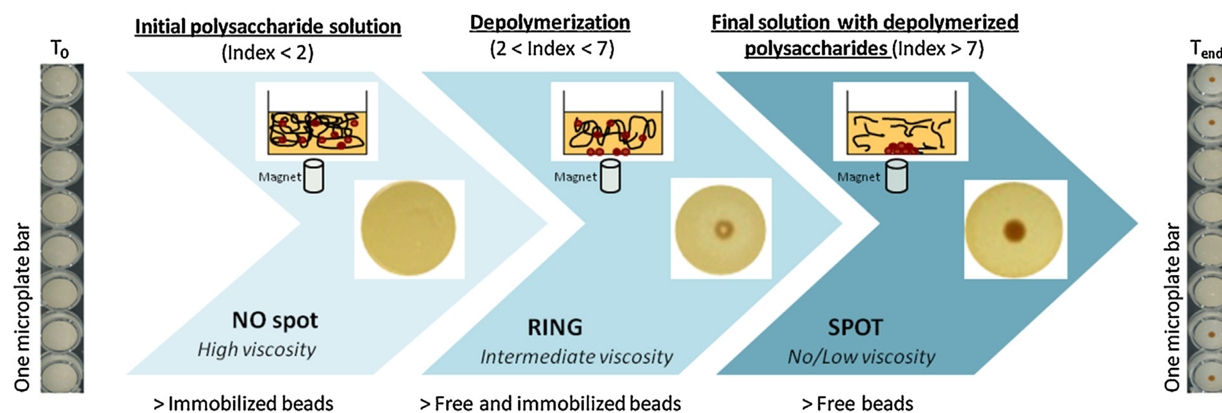

Fig. 1. Monitoring of enzymatic depolymerization of polysaccharide solutions by using Enzyme Ring Test®.

formation by microorganisms [32]. In practice, application of a magnetic field allows the aggregation of free beads, which is visible through the formation of a "spot". In contrary, magnetic particles immobilized in the biofilm do not focus upon a magnetic field. Badel et al. investigated the possibility to adapt this method for the detection of PCE. In this case, magnetic beads allow microviscosity measurements in polysaccharidic solutions [33]. It was then possible to detect the activity of specific PCE by the decrease of viscosity of the polysaccharidic solution due to enzymatic cleavages of polymers. For that, blocking concentrations, corresponding to the minimal concentration of polysaccharides required to immobilize beads have to be determined [33].

This paper deals with the proof of concept of the Enzyme Ring Test® technology for the fine detection and quantification of PCE activities. First, enzymatic activities of endocellulase (EC 3.2.1.4), exocellulase (EC 3.2.1.91) and alginate lyase (EC 4.2.2.3) were determined by classical colorimetric assays and data were treated by the classical Michaelian model (K_m, V_{max}). Then, the Enzyme Ring Test® (ERT®) method was used to follow the depolymerization of polysaccharides by the PCE. Finally, the possibility to distinguish endolytic and exolytic enzymatic mechanisms for the endo- and exocellulases was investigated by using the ERT® technology.

2. Material and methods

2.1. Enzymes and substrates

Endocellulase (EC 3.2.1.4) from *Aspergillus niger* (C1184, Sigma–Aldrich, France) was used in 100 mM sodium acetate buffer at pH 5.6 containing CarboxyMethyl Cellulose (CMC) (21904, Fluka, France) to determine its activity. Exocellulase (EC 3.2.1.91) from *Trichoderma* sp. (E-CBHI, Megazyme, France) was incubated in 500 mM sodium acetate buffer at pH 4.8 supplemented by CMC (21904, Fluka, France) as substrate. Alginate lyase (EC 4.2.2.3) from *Sphingomonas* sp. (E-ALGS, Megazyme, France) was employed in 100 mM Tris–HCl buffer at pH 7.2 containing sodium alginate (RARESEA, Cargill, France) as substrate.

2.2. Enzymatic depolymerization

Endocellulase, exocellulase and alginate lyase were used at various concentrations, *i.e.* 1.2×10^{-1} to 1.2×10^{-3} U/mL, 5×10^{-3} to 5×10^{-4} U/mL and 2×10^{-1} to 1×10^{-3} U/mL respectively, in specific buffers containing their own substrates. In accordance with the commercial descriptions of the enzyme preparations, one unit (U) of (i) cellulase (endo- or exoenzyme) was defined as the amount of enzymes that released 1 μmol of reducing sugar (glucose equivalent) per hour using CMC as substrate and of (ii) alginate lyase was defined as the amount of enzymes required to produce an increase in absorbance of 1.0 per minute at 235 nm (beta-elimination mechanism) using sodium alginate as substrate.

Enzymatic assays were carried out in a Carousel 12 Plus Reaction Station (Radleys, France) at 30 °C temperature for each tested enzyme, under stirring (700 rpm) and were done at least in duplicate. Samples were taken off at different times during the enzymatic depolymerization, then heated at 100 °C during 20 min. After centrifugation (3500 × *g*, 5 min, 4 °C), the supernatant was recovered and finally frozen until colorimetric analyses.

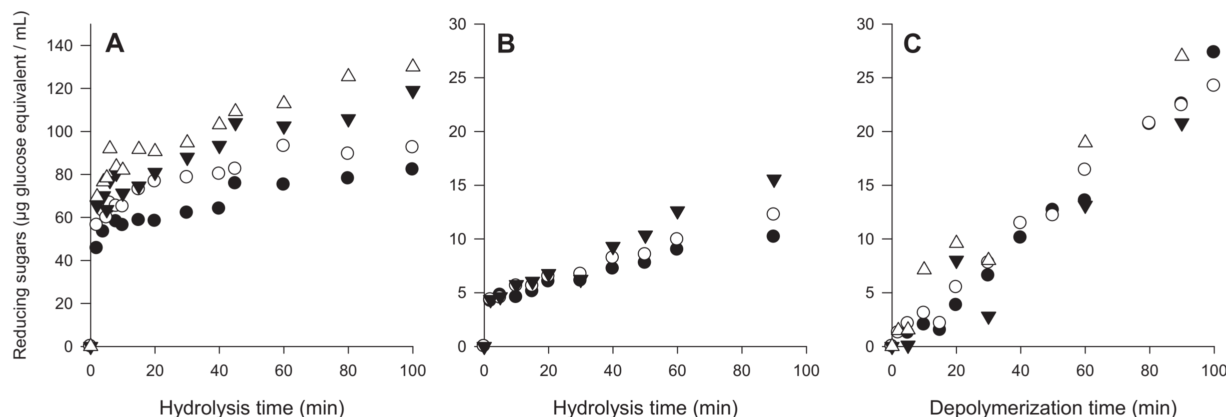

Fig. 2. Effect of variation of substrate concentration on reducing sugars releasing on endocellulase/CMC (A: ● 3, ○ 4, ▼ 5, △ 6 g/L), exocellulase/CMC (B: ● 3, ○ 5, ▼ 6 g/L), and alginate lyase/sodium alginate (C: ● 5, ○ 6, ▼ 7, △ 8 g/L). Reactions occurred respectively at theoretical concentration of enzymes of 1.2×10^{-2}, 5×10^{-4} and 2×10^{-2} U/mL, and in sodium acetate 0.1 M pH = 5.6, sodium acetate 0.5 M pH = 4.8 and Tris–HCl 0.1 M pH = 7.2.

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