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Reliable simultaneous zymographic method of characterization of cellulolytic enzymes from fungal cellulase complex

Short Communication

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List of abbreviations: carboxymethyl cellulose (CMC), 4-methylumbelliferyl-β-D (MUC)

Total number of words: 2307

Received: 14-Nov-2014; Revised: 01-Apr-2015; Accepted: 02-Apr-2015

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/elps.201400541.

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Abstract

A method for zymographic detection of specific cellulases in a complex (endocellulase, exocellulase and cellobiase) from crude fermentation extracts, after a single electrophoretic separation, is described in this paper. Cellulases were printed onto a membrane and, subsequently, substrate gel. Cellobiase isoforms were detected on the membrane using esculine as substrate, endocellulase isoforms on substrate gel with copolymerized carboxymethyl cellulose (CMC), while exocellulase isoforms were detected in electrophoresis gel with MUC. This can be a useful additional tool for monitoring and control of fungal cellulase production in industrial processes and fundamental research, screening for particular cellulase producers or testing of new lignocellulose substrates.

Key words: cellulase, endocellulase, exocellulase, β-glucosidase, *Trichoderma*, zymogram, SSF

Cellulases are currently the third most produced category of industrial enzymes worldwide, owing to their application in food, paper and detergents industries, and may become the first if ethanol from lignocellulosic biomass becomes a substantial fuel [1]. Cellulase complex is a multiple enzyme complex consisting of endocellulase (endo-1,4-beta-D-glucanase EC 3.2.1.4), exocellulase (cellobiohydrolase EC 3.2.1.91) and β-glucosidase (cellobiase EC 3.2.1.21).

Cellulases exploited in industrial applications, derive from filamentous fungi [1, 2]. Much research is directed towards the study of metabolic pathways and improvement of cellulase production by gene manipulation techniques [3]. *Trichoderma* sp. fungi are widely used and most studied among fungal cellulase producers [2, 4]. *Trichoderma reesei* is the

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most potent cellulase producer, producing two cellobiohydrolases, eight endo-glucanases and seven β-glucosidases (identified on gene level), and has been fully studied in detail [5, 6].

An enzymatic assay often does not provide enough information on the type or properties of a given cellulase and which is the reason why cellulase zymograms were first mentioned already in 1973[7]. A zymographic technique for endocellulase was developed first, using agar gel with copolymerized CMC [8, 9]. Agar gels are now considered obsolete and have been replaced by polyacrylamide (PAA) gels due to easier handling and attainment of better resolution. The commonly used zymographic technique for endocellulase is based on detection after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) with incorporated CMC [2, 10, 11]. The major disadvantage of this zymographic technique is that only enzymes capable of renaturing are detectable. In addition, electrophoretic separation of native proteins (enzymes) based on their pI values – isoelectric focusing (IEF) can be used to separate the different enzymes prior to zymographic detection. The enzymes are maintained in their original conformation enabling enzymatic activity to be detected directly after electrophoretic separation. Endocellulases of a wide pI range are detected by replication on agar CMC gels after IEF [12, 13].

Multiple-layer zymograms have been developing as a separate category among other zymographic techniques in the last few years, responding to the need for a fast and reliable method of identifying two or more different, industrially important enzymes from a single sample[14, 15]. A zymogram for simultaneous detection of all cellulase subtypes has not been developed up till now. The differentiation of specific cellulase types is achieved by performance of standard enzymatic assays using different 4-methylumbelliferyl glycoside substrates, (but) in the presence of specific inhibitors [16].

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Detection of a cellulase and expressed isoforms by the zymographic technique developed here can assist in answering to the question as to which type is expressed and at what ratio. Utilization of this zymographic technique could be useful additional tool in direction of research aimed at production of only one specific cellulase type or all three cellulase subtypes in specific ratios. Samples of crude fermentation extracts obtained from *Trichoderma* sp. isolates were selected in order to demonstrate broad applicability of the zymographic technique presented here.

Fermentation: *Trichoderma* sp. isolates from soil (spore concentration 1 x 10^5) were used in submersed fermentation (SmF), performed in duplicate in 250 mL Erlenmeyer flasks with microbiological cotton wool caps. Fermentation was performed at 28°C and 150 rpm for a period of 7 days. Medium was composed of nutrient salt (g/L): NH_4NO_3 (2.0), K_2HPO_4 (1.0), NaH_2PO_4 x H_2O (0.4), $MgSO_4$ x $7H_2O$ (0.5), yeast extract (2.0) was used in all fermentations and different lignocellulolytic substrates (described below). SmF is the technique of choice when optimizing fermentation, or testing the effects of gene manipulation techniques or induction effects on the production of cellulase in fungi[1].

Preparation of crude fermentation extract: Solid parts (wheat straw with biomass) in fermentations were separated from liquid by a strainer. Liquid was centrifuged at 10956 *g* for 15 minutes. Protein concentrations were detected according to Bradford [17].

List of samples: 1 to 7, crude fermentation extracts of *Trichoderma* sp. and chosen lignocellulolytic, substrate accompanied with protein loadings content: sample 1 *Trichoderma* sp. 1 –mixture of wheat straw and soy bean stalks – 1.28 µg of protein; sample 2 - *Trichoderma* sp. 2 in SmF – raw wheat straw – 14.12 µg of protein; sample 3- *Trichoderma* sp. 3 – raw wheat straw – 16.2 µg of protein; sample 4 - *Trichoderma* sp. 1 – raw wheat straw – 19.24 µg of protein; sample 5 - *Trichoderma* sp. 1 – pretreated wheat straw – 14.16 µg of protein; sample 6 - *Trichoderma* sp. 1 – raw wheat straw in static culture

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conditions (semi SSF) – 16.64 μg of protein; sample 7 - *Trichoderma* sp. 1 – raw wheat straw – 19.24 μg of protein. Wheat straw pretreatment: 0.1% NaOH was added to wheat straw and heated in a microwave to boiling point, 0.25 M HCl was used for neutralization to pH 6.5

Zymograms: Fermentation extracts were separated by IEF according to manufacturer's instructions (Multiphor II, LKB Pharmacia, Uppsala, Sweden), for 2 h and 15 min, at 1054 V, 4.7 mA with constant power of 5 W, using ampholytes in the 3.0–10.0 pH range (Amersham Biosciences, Uppsala, Sweden) on 7.5% acrylamide (AA) gel, at 10ºC. IEF mix 3.6-9.3, Sigma-Aldrich was used as pI standard.

Zymograms for all three cellulolytic enzymes were performed in two steps according to the scheme shown in Figure 1. After electrophoretic separation, proteins were printed onto nitrocellulose membrane (NC) for 15 minutes. NC was placed on IEF gel and two pieces of filter paper were placed above. The sandwich was covered with paper wadding and a weight unit (300 g) was placed on top, enabling transfer of the enzymes from the IEF gel onto NC by capillary force. β-glucosidase isoforms were detected on NC, using 0.1% esculine and 0.03% $FeCl_3$ in 50 mM acetate buffer pH 5.7 as substrate [18]. Visualization of isoforms was accelerated by incubation at 50ºC for 5 minutes, Figure 2a. Cellobiase is most commonly detected by 4-methylumbelliferyl-β-D-glucoside (MUG) as substrate [13, 19]. The method with esculin as substrate is more suitable for cellobiase detection because it requires minimum time and costs.

The IEF gel was later printed on an acrylamide (AA) (7.5%) gel with copolymerized 0.1% CMC (TCI, viscosity of 1% in water of 25ºC is 900-1400 mPa-s) for 30 minutes, Figure 1, right. After dismounting of the printed sandwich, CMC gel was incubated at 50ºC for 5 minutes. Endocellulase isoforms appeared as clear zones on red background, after coloring of the CMC gel with 0.1% Congo-red for 10 minutes. 1M NaCl was used to rinse the gel. Acetic

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acid (7%) was used to change the color of the gel from red to blue in order to obtain better resolution, Figure 2b.

The remaining IEF gel was used for detection of exocellulase, with 4-methylumbelliferyl-β-D-cellobioside (MUC cellobioside) as substrate and a UV transluminator for visualization of the bands [12]. β-glucosidase also has the capacity to hydrolyze MUC but is inhibited completely by D-glucono-δ-lactone [16, 20]. IEF gel was immersed in 0.5 mM D-glucono-δ-lactone for 5 minutes with the aim to inhibit cellobiase, and afterwards in a mixture of 0.5 mM MUC and 0.5 mM D-glucono-δ-lactone in 50 mM acetate buffer pH 5.0 for 5 minutes at 50ºC. Exocellulase isoforms appeared as blue fluorescent bands after exposure of the gel to UV light. Pictures were taken by camera through yellow filter to obtain better resolution and green exocellulase isoforms, Figure 2c.

The differences in cellulase profiles between the examined *Trichoderma* sp. fermentation samples are clearly visible in Figure 2. The positions of all major isoforms are indicated by arrows in Figure 2, comparison clearly shows differences in the positions of cellobiase, endocellulase and exocellulase isoforms. Most notably, not all types of cellulases were detected in each sample.

It can be observed (Figure 2, samples 2, 3 and 4) that different *Trichoderma* sp. wt strains produced different cellulase types, indicating that the described zymographic technique can be used as a powerful means to compare cellulase producers' profiles between different fungal strains. Another important observation is that one *Trichoderma* sp. strain can produce different cellulase profiles depending on SmF conditions or types of substrate used in SmF, samples 1, 5 and 6 from Figure 2. It demonstrates that this zymographic method is a well-suited analytical procedure in fundamental research on improvement of fungal cellulase production.

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Each cellulase producer has its own cellulase profile that is significant from an industrial point of view. The comparison of cellulase – producing profiles among *Trichoderma* sp. or other fungi is very important for industrial characterization of an entire secretome, as well as for the more fundamental study of cellulase expression. Two dimensional (2D) electrophoresis and MALDI-TOF or LC-MS/MS protein identification give results suggesting potential use in analyses of cellulase profiles[21]. The results shown in this paper clearly indicate that the zymographic technique is more suitable than 2D electrophoresis, by being faster, capable to analyze multiple samples simultaneously and easier for manipulation and interpretation, as well as less expensive.

This zymographic technique enables a clear distinction between cellulase subtypes – endocellulase, exocellulase and cellobiase; thereby eliminating errors that are found in papers by use of only CMC as substrate, in common zymographic practice. It can be a helpful tool for monitoring and control in fundamental research, screening for particular fungal cellulase producers, for monitoring of the effects on cellulase production resulting from genetic manipulation or testing of new lignocellulosic substrates.

Acknowledgement

This study was supported by a grant from the Serbian Ministry of Education and Science (project grant number 172048). We thank Jasna Bojčevski for linguistic correction.

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Figure 1. Schematic representation of zymogram procedures for identification of cellulase subtypes. NC-nitrocellulose membrane, CMC gel-gel with copolymerized carboxymethyl cellulose, IEF gel-acrylamide gel after isoelectric focusing.

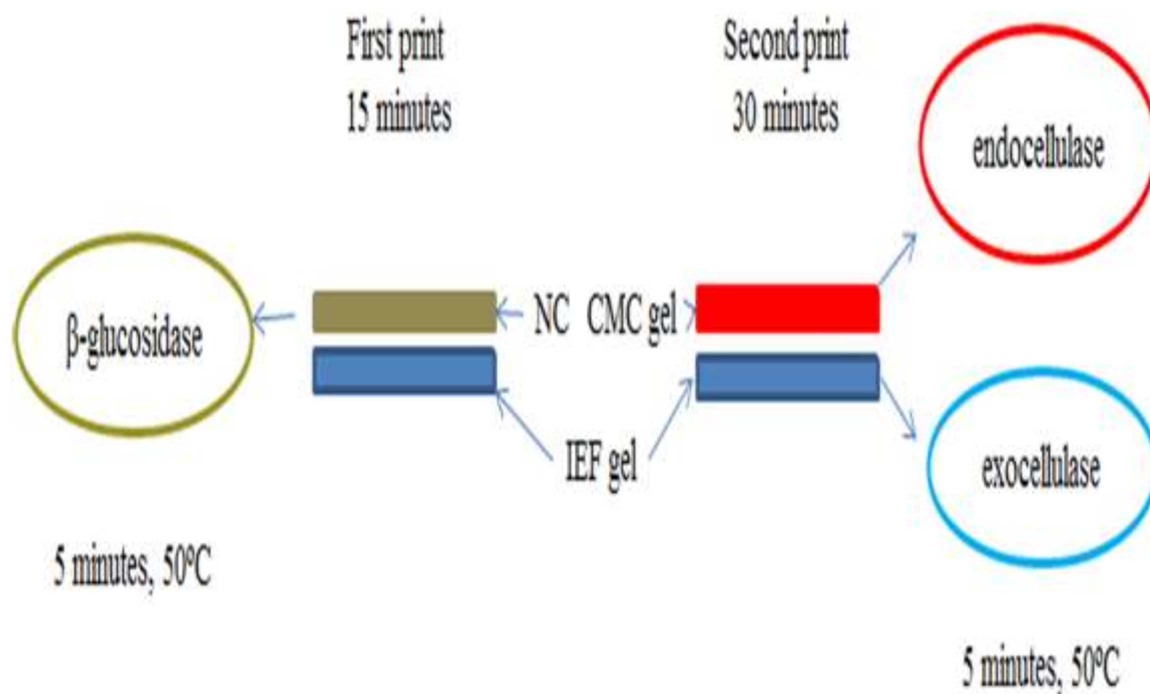

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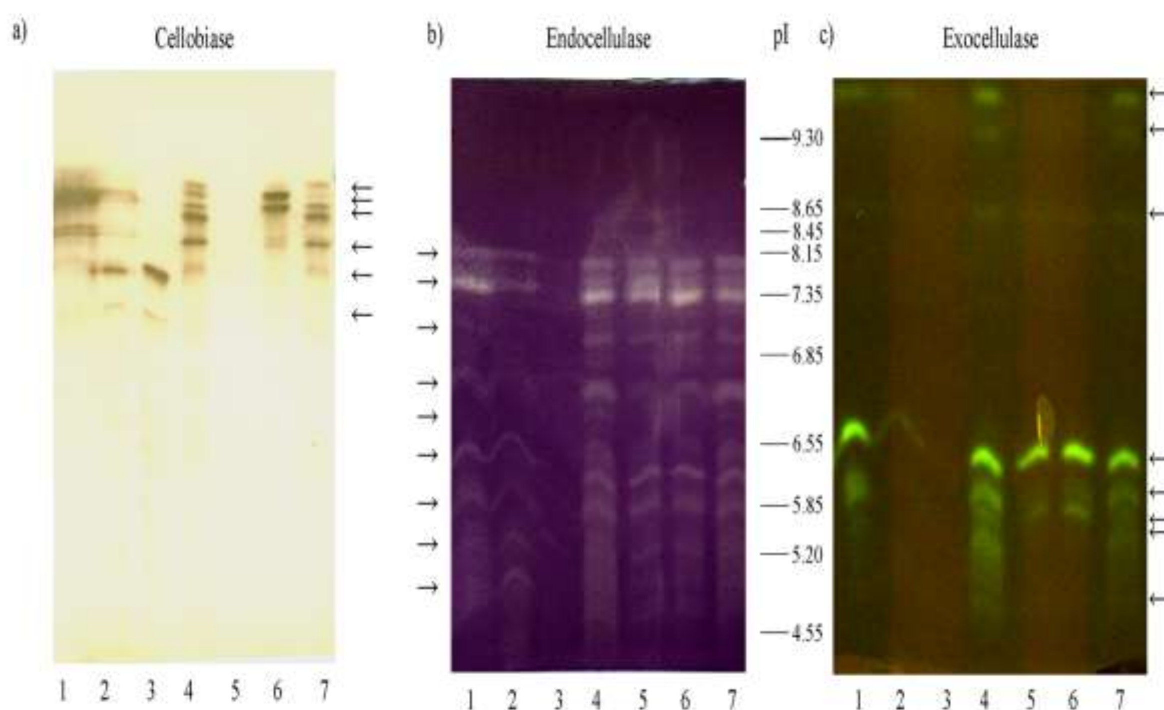
Figure 2.Simultaneous zymographic detection of cellulase isoforms after IEF. a) Zymogram for β-glucosidase on NC membrane using esculine as substrate; b) zymogram for endocellulase on PAA gel with copolymerized CMC; c) zymogram for exocellulase on IEF PAA gel using MUC -β-D-cellobioside as substrate and D-glucono-δ-lactone as cellobiase inhibitor, and UV transilluminator for visualization; pI – position of pI markers (Sigma, Broad range from 3.5-10); arrows indicate the positions of major isoforms for all enzymes. 1-7 samples of *Trichoderma* sp. fermentation extracts (described in the text).



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