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A comparison of plate assay methods for detecting extracellular cellulase and xylanase activity

Fatma Meddeb-Mouelhi[a,b,*], Jessica Kelly Moisan[a,c], Marc Beauregard[a,c]

[a] Centre de recherche sur les matériaux lignocellulosiques, Université du Québec à Trois-Rivières, 3351 Boul. Des Forges, C.P. 500, Trois-Rivières, Québec G9A 5H7, Canada
[b] Buckman North America, 351 Joseph-Carrier, Vaudreuil-Dorion, Québec J7V 5V5, Canada
[c] PROTEO, Université Laval, 2705 Boul. Laurier, Québec, Québec G1V 4G2, Canada

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ABSTRACT

Identification of microorganisms for the production of carbohydrolytic enzymes is extremely important given the increased demand for these enzymes in many industries. To this end, dye–polysaccharide interactions which provide a visual indication of polymer hydrolysis (clear zones or halos) have been used for decades. For the detection of extracellular cellulase or xylanase activity many laboratories use Gram's iodine as the chromogenic dye, as it is a more rapid initial screening method compared to the use of other dyes. Here, we compared Gram's iodine and Congo red as indicators of polysaccharide hydrolysis. We attempted to detect cellulase activity using carboxymethylcellulose, and xylanase activity using birchwood xylan, in fourteen uncharacterized bacteria isolated from wood chips. Our results indicate that Gram's iodine may lead to identification of false positives in a typical screening protocol and that Congo red allows for avoidance of such pitfall. Congo red allowed detection of cellulase activity from live microbial colonies but not Gram's iodine. To confirm this, detection of enzymatic activity was also assessed using cell-free enzyme preparations. Congo red was found to be reliable in detecting cellulase activity with isolated enzymes preparations. Under the same conditions, neither of these dyes detected xylanase activity, despite independent evidence of xylanase activity for one of the preparations. We detected xylanase activity for this particular enzyme preparation using a coloured derivative of xylan (Remazol Brillant Blue R-xylan adduct) that respond to xylan hydrolysis. Our results suggest that methods that rely on interactions between a dye (Congo red or Gram's iodine) and a polymeric substrate (carboxymethylcellulose or birchwood xylan) for indirect detection of hydrolysis may require the use of relevant controls and independent confirmation of enzymatic activities.

1. Introduction

Hydrolysis of carbohydrate polymers represents one of the most important biotechnologies in the 21st century given the increasing demands for cellulose biomass-derived fuels. The key enzymes in this process (cellulases and hemicellulases) are produced by a wide variety of microorganisms [1] and their importance has prompted the development of various methods for high throughput screening and selection of microbial producers. Common screening techniques involve plate assays, where the target polymer substrate or a derivative has been incorporated into a basal growth medium. Bacteria with extracellular cellulolytic activity are typically detected on agar media plate containing carboxymethylcellulose (CMC) or xylan as substrates. These substrates are polysaccharide organic compounds consisting of a linear chain of β (1-4) linked D-glucose units and xylopyranose residues, respectively. Substrate hydrolysis can be revealed by clearing zones formed around the growing colonies after dye staining by Gram's iodine [2–8] or Congo red [2–4,7].

However, in several papers cited above no negative controls were included during screening (*i.e.* assays on plates that do not contain substrate). Assuming that clearing zones are exclusively associated with cellulase activity is somewhat risky, considering earlier publication of an artefact due to agar composition and/or degradation, and consequent detection of false positives [9]. Congo red has also been used for the detection of hydrolysis zones, as well

* Corresponding author at: Centre de recherche sur les matériaux lignocellulosiques, Université du Québec à Trois-Rivières, C.P. 500, Trois-Rivières, Québec G9A 5H7, Canada. Tel.: +1 819 376 5011x3354; fax: +1 819 376 5084.
E-mail address: fatma.meddeb@uqtr.ca (F. Meddeb-Mouelhi).

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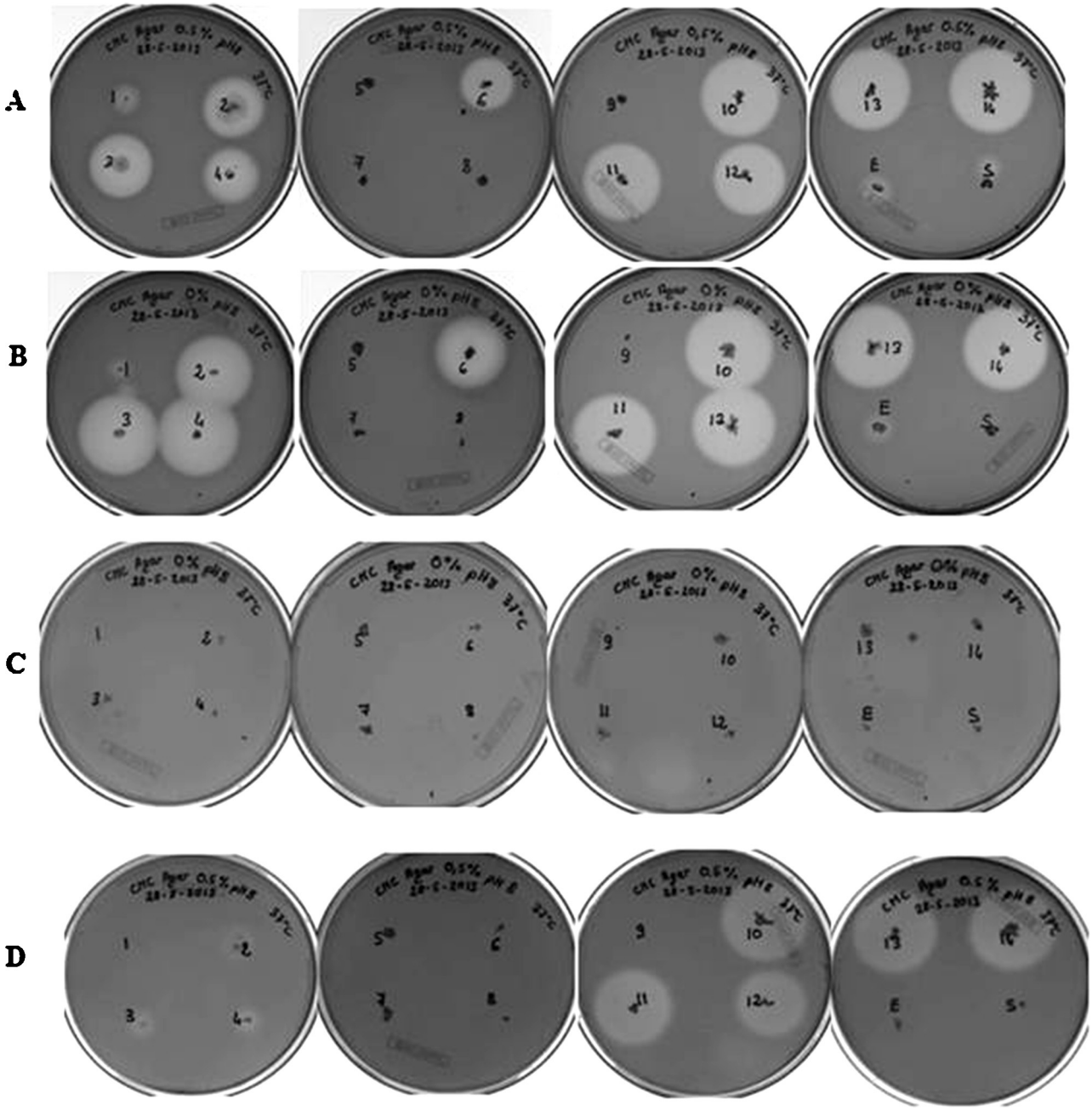

Fig. 1. Detection of enzyme activity from isolated bacterial strains on agar plates. *E. coli* (E) and *Salmonella enteritidis* (S) were used as negative controls (extreme right plates). (A) Plates with substrate flooded with Gram's iodine; (B) same but without substrate CMC; (C) plates without substrate flooded with Congo red; (D) same with CMC as substrate.

as other coloured substrates where no intermediate dye is needed for detection, such as Remazol Brilliant Blue R-xylan (RBB-xylan) [10].

Here we have compared these dyes in the absence or presence of various substrates, using live microorganisms and isolated enzymes. Depending on conditions used, halos were detected despite the absence of substrate.

2. Materials and methods

2.1. Substrates

Carboxymethyl cellulose (CMC) (cat. no# C5678), and birchwood xylan (cat. no# X0502) were purchased from Sigma. LE agarose (1062631) was obtained from Qiagen. Bacto agar (cat. no# 214010) and yeast extract (cat. no# 212750) were from Becton, Dickinson and Company (BD). Remazol Brilliant Blue R dye (cat. no# R8001) was purchased from Sigma. All other chemicals used in this study were reagent grade. Birchwood xylan was dyed with Remazol Brillant Blue R (RBB) following the procedure previously described by Biely et al. [10]. with some modifications.

2.2. Source of microorganisms and culture media

Microorganisms producing carbohydrolytic enzymes were isolated from tree wood chips. Wood chips (1 g) were mixed in 100 ml of sterile distilled water, then 0.1 ml was removed and spread on the surface of nutrient agar medium (0.3% beef extract, 0.5% peptone, 0.5% NaCl, and 1.5% agar at pH 8.0). Plates were incubated at 37 °C for 48 h. Morphologically different colony types were picked and re-streaked to obtain pure cultures.

2.3. Screening of cellulolytic enzyme-producing bacteria

Single colonies from fresh LB agar plates were patched onto minimal (Mm) agar plates (0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.1% KCl, 0.05% yeast extract and