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An Improved and Simplified Radial Gel Diffusion Assay for the Detection of Xylanase Activity

Raviraj M. Kalunke, Ilaria Moscetti, Silvio Tundo and Renato D'Ovidio*

Dipartimento di Scienze Agrarie e Forestali (DAFNE), Università degli Studi della Tuscia, Viterbo, Italy
*For correspondence: dovidio@unitus.it

[Abstract] Xylanase (E.C. 3.2.1.8) degrades β-1, 4 xylan by cleaving β-1, 4 glycosidic linkages randomly, resulting in the generation of xylose and xylo-oligosaccharides. Xylanases are produced by organisms including fungi, bacteria, yeast, marine algae, protozoans, snails, crustaceans and insects. Xylanases present considerable industrial interest for their use in paper manufacturing, improvement of animal feed digestibility, and clarification of fruit juices. In addition, this enzyme is the component of cell wall-degrading enzymes (CWDEs) during plant–pathogen interaction. Thus, considering their various applications in plant defence and also in industry, the characterization of xylanase activity becomes an important aspect. Conventionally, xylanase activity is determined by radial gel diffusion assay using Congo red staining (Emami and Hack, 2001) and by DNSA assay which is a colorimetric method for xylanase activity (McLauchlan *et al*., 1999; Kutasi *et al*., 2001). Comparatively, radial gel diffusion assay using Congo red staining is a qualitative assay whereas DNSA method is a quantitative assay. Moreover, Congo red is a chemical considered as hazardous category 1B (Carcinogenicity) and category 12 (Reproductive toxicity) by the 2012 OSHA Hazard Communication Standard (29 CFR 1910.1200). In the present study, the proposed method enables qualitative detection of xylanase activity using ethanol precipitation in the radial gel diffusion assay which is safer and simpler. The ethanol precipitation in agar plate has been adapted from the method for detecting xylanase activity in polyacrylamide gels (Royer and Nakas, 1990).

Materials and Reagents

1. 90 x 15 mm Petri plates (SARSTEDT AG, catalog number: 82.1473001)
2. 0.5-cm-diameter drinking straw
3. 150 ml Erlenmeyer flask
4. *Aspergillus niger* xylanase (Xylanase M4) (Megazyme) or *Trichoderma longibrachiatum* xylanase (Xylanase M3) (Megazyme)
5. Disodium hydrogen phosphate (Sigma-Aldrich, catalog number: S5136)
6. Citric acid (Sigma-Aldrich, catalog number: C1909)
7. Birch wood xylan (Sigma-Aldrich, catalog number: X0502)
8. Agarose (AppliChem GmbH, catalog number: A8963.0500)
9. Absolute ethanol (VWR International, catalog number: 20821.321)
10. McIlvaine's buffer (pH 5.0) (see Recipes)
11. 95% (v/v) ethanol (see Recipes)

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Equipment

1. Microwave
2. Hallow flat end with 0.5-cm-diameter drinking straw
3. 30 °C incubator (Mini BATT 805) (230 V, 50 Hz, 270 W) (Asal Srl, model: 805)
4. Scanner camera (Epson, model: perfection V30)

Procedure

1. Mix 1.0% (w/v) birch wood xylan with 1.0% (w/v) agarose in McIlvaine's buffer (15 ml/Petri dish) and boil in 150 ml Erlenmeyer flask (approximately 10 min) until the birch wood xylan is dissolved completely; mix thoroughly and then dispense it in a Petri plate (44 mm base diameter x 12 mm depth).
2. Allow the birch wood xylan–agarose solution to solidify in the Petri dish and then prepare a 0.5-cm-diameter well on it. The hole in agarose is poked using a hallow flat end of a 0.5-cm-diameter drinking straw.
3. Place 30 µl of *A. niger* or *T. longibrachiatum* xylanase solution (total volume to be adjusted with McIlvaine's buffer) in 0.5-cm-diameter wells of birch wood xylan-agarose Petri plate.
4. Incubate the Petri plate at 30 °C for 16 h.
5. Overlay the plate with 95% ethanol and keep the plate at room temperature for 30 min to reveal the halo representing the degradative activity of xylanase.
6. Measure the diameter of the halo using a scale.
7. Take the image using a digital camera or scan the Petri plate using a scanner to keep the record of the results (Figure 1).

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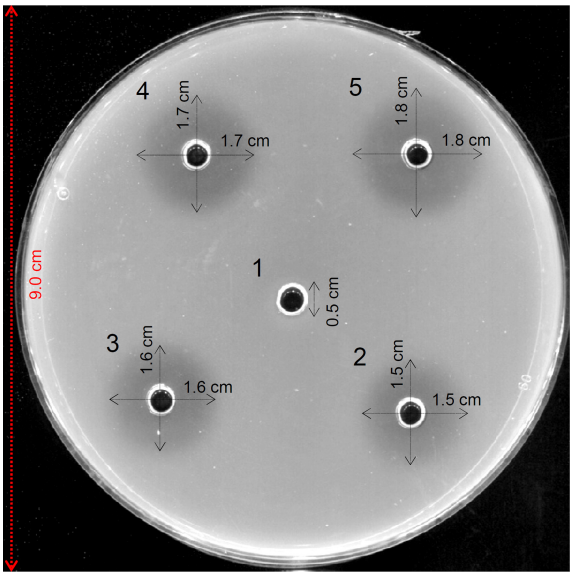

Figure 1. Example of agarose diffusion assays for detection of xylanase activity. Haloes represent xylanase activity. 1: Buffer (negative control); 2: *Aspergillus niger* xylanase M4 (0.006U); 3: *A. niger* xylanase M4 (0.012U); 4: *A. niger* xylanase M4 (0.018U); 5: *A. niger* xylanase M4 (0.024U).

Recipes

1. McIlvaine's buffer (pH 5.0)
   0.2 M disodium hydrogen phosphate
   0.1 M citric acid
2. 95% (v/v) absolute ethanol
   Mix 95 ml absolute ethanol with 5% (v/v) distilled water

Acknowledgments

This protocol has been adapted from the previously published by Kalunke *et al*. (2013). This protocol was designed to determine xylanase inhibition for wheat transgenic plants overexpressing the xylanase inhibitor TAXI-III.

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