In-situ digestion of wheat cell wall polysaccharides

in the context of MS imaging

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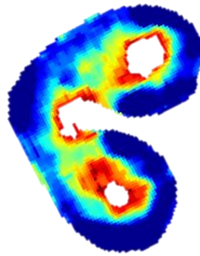


Arabinoxylan distribution
in wheat cell walls

PREFACE

Cell walls of the wheat endosperm are mostly composed of Arabinoxylans (AX) and mixed (1→3), (1→4)-β-glucans (BG) [1]. Here, we present an optimized protocol to degrade enzymatically these cell-wall polysaccharides into oligosaccharides, directly from the wheat grain cross sections. The main difficulty is to provide a sufficient amount of humidity for the enzyme to be active, while the amount of liquid at the surface of the tissue should stay low to prevent any delocalization of the released products. With this protocol, enzymatic degradation was shown to be efficient and delocalization of released oligosaccharides was estimated below 50µm [2].

Although it can be employed for other purposes, this in situ enzymatic digestion was primarily developed to obtain molecular images of the cross-sections of wheat endosperm by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry [2,3]. The cell wall polysaccharides are heterogeneous in structure, exhibit high masses and are entangled into complex networks. Thus, they are not amenable to direct analysis by mass spectrometry and they need to be degraded into smaller compounds as a first step. In this protocol, additional steps corresponding to the deposition of the MALDI matrix are also described.

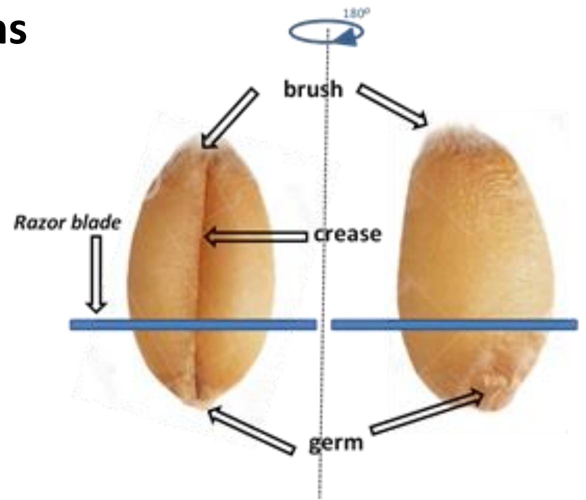
(1) SAULNIER, L., GUILLON, F. & CHATEIGNER-BOUTIN, A. L. (2012) Cell wall deposition and metabolism in wheat grain. *Journal of Cereal Science,* 56**,** 91-108.

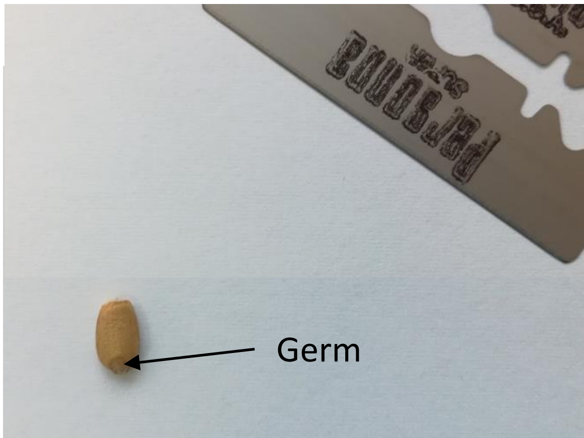
(2) VELICKOVIC, D., ROPARTZ, D., GUILLON, F., SAULNIER, L. & ROGNIAUX, H. (2014) New insights into the structural and spatial variability of cell-wall polysaccharides during wheat grain development, as revealed through MALDI mass spectrometry imaging. *J Exp Bot,* 65**,** 2079-91.

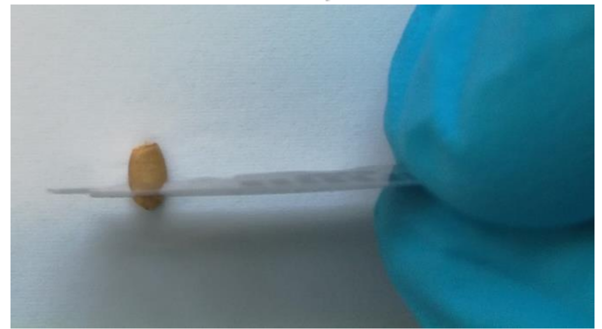
(3) VELICKOVIC, D., SAULNIER, L., LHOMME, M., DAMOND, A., GUILLON, F. & ROGNIAUX, H. (2016) Mass Spectrometric Imaging of Wheat (Triticum spp.) and Barley (Hordeum vulgare L.) Cultivars: Distribution of Major Cell Wall Polysaccharides According to Their Main Structural Features. *Journal of Agricultural and Food Chemistry,* 64**,** 6249-56.

I- Tissue preparation

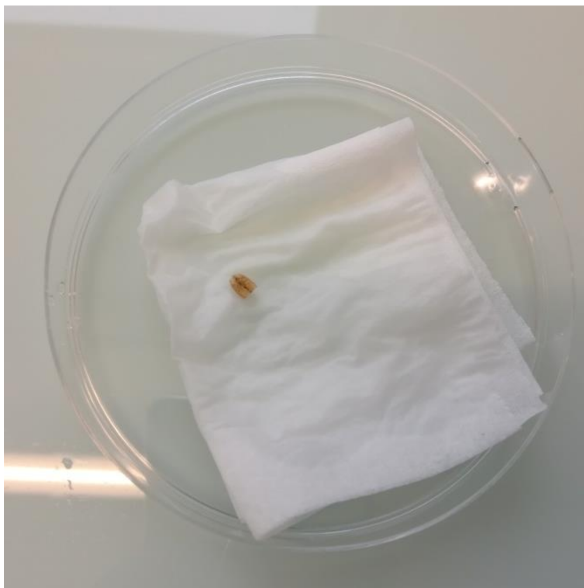
1) rehydration of wheat grains

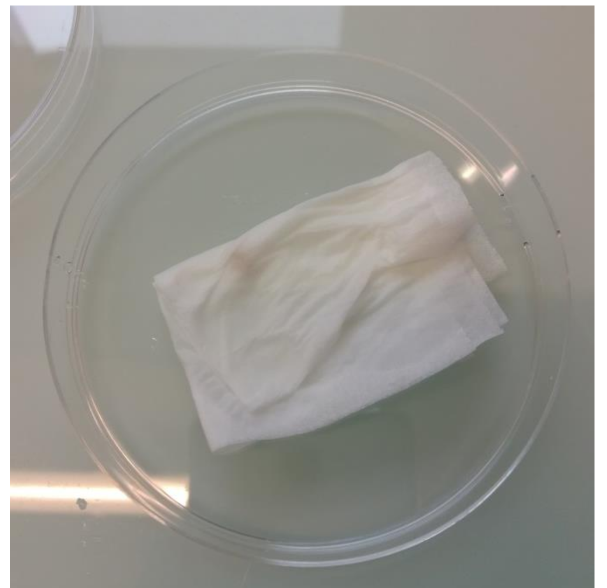






The germ part is removed with a razor blade, perpendicular to the crease zone (longitudinal axis) of the grain, and is discarded.



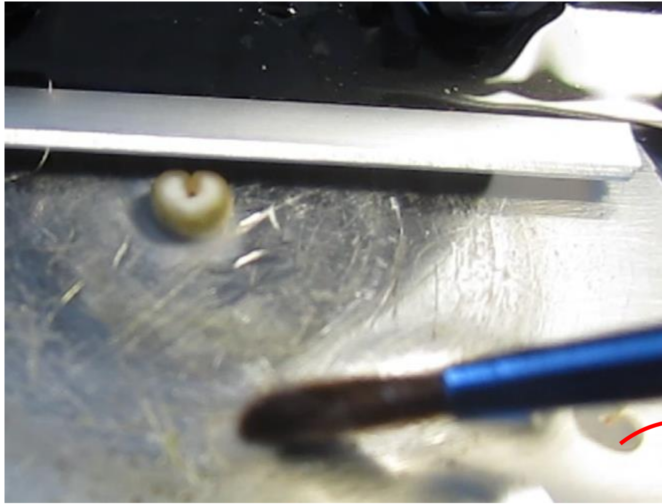
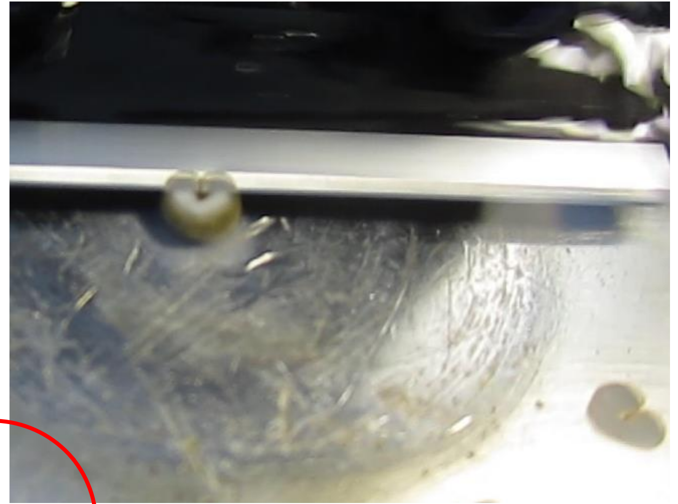
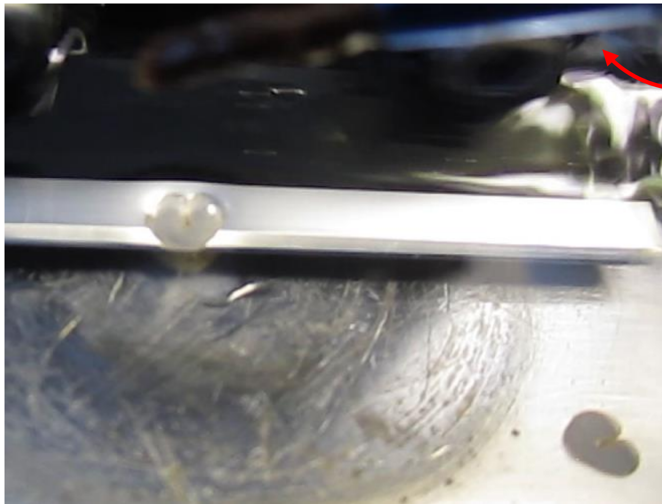
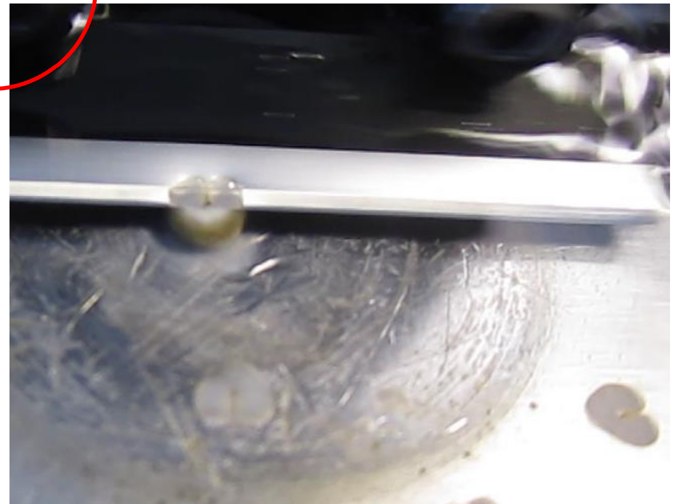


The grain is then placed in a Petri dish on top of a Kimwipes moistened with dH_2O, and left at +4°C for 24h.

Whole rehydration procedure is skipped for young grains, which are naturally hydrated.

I- Tissue preparation

2-preparing 70µm-thin cross sections of wheat grains

This step is done by using a Vibratome instrument. The grain is fixed with some glue onto the magnetic plate of the Vibratome. Orientation of the grain should be such that the longitudinal axis of the grain is placed perpendicular to the magnetic plate. Settings of the Vibratome instrument are as follows: frequency: 60; amplitude: 1.2; speed: 12.

A series of 400µm-thick slices are cut and discarded, until the surface of the grain becomes flat and parallel to the razor blade of the Vibratome instrument. Thin sections (approximately 70µm) are then cut consecutively and placed immediately into Eppendorf tubes filled with 70% ethanol. They are stored at 4°C until further processing.

I- Tissue preparation

3) removing starch

This step is intended to avoid any hydrolysis of starch by endogenous enzyme activity, which would produce some glucans of similar masses as those expected from the hydrolysis of cell walls BG (additionally, when MALDI mass spectrometry is used for further analysis of the tissue, these compounds generate a strong suppressive effect on the signal).

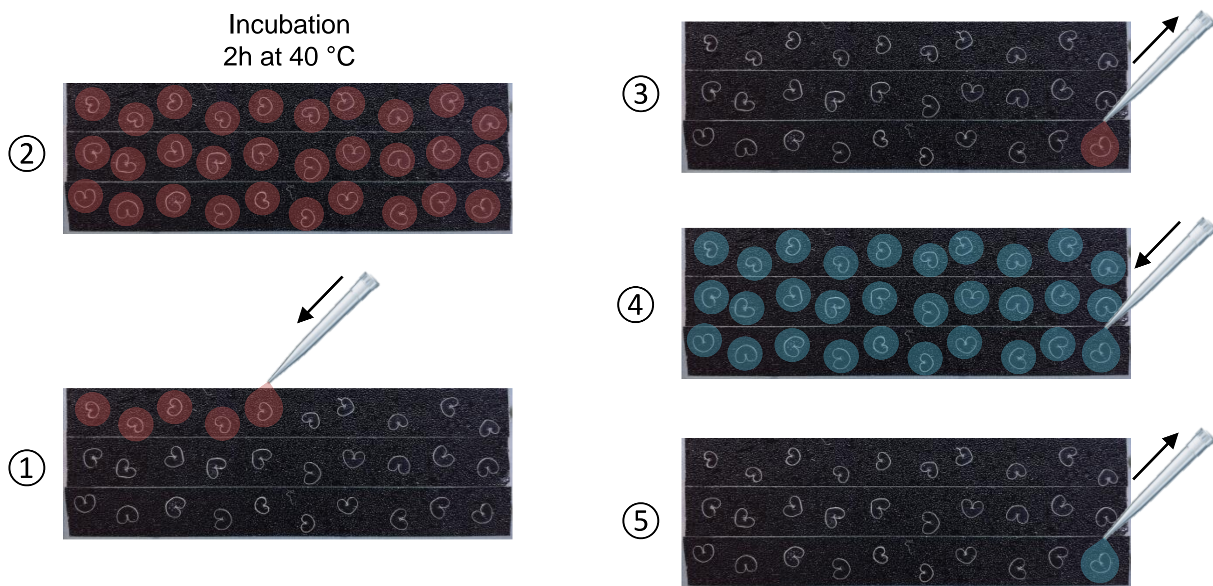
There are two ways to do this step. The first is the simplest and the fastest. The second is more time-consuming but allows better preserving the shape of the cuts.

First way: The wheat cross section is grabbed using a paint brush and transferred into a clean Eppendorf tube, filled with 0.5mL of 1mg/mL α-Amylase solution. This is allowed to incubate at 40°C for 24h on a Thermo-shaker. Rinse thoroughly by transferring the wheat cross section into a clean Eppendorf tube filled with 1mL of dH_2O. Repeat this step twice. Wash the brush in water after each sample transfer.

I- Tissue preparation

3) removing starch

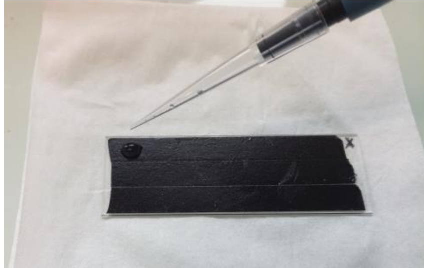
Second way: First step is to place the cross-sections onto the ITO glass plate, covered with adhesive carbon tape, as described in the following step of the procedure (I-4), but without the drying part. Then, put a droplet (30 µL) of 1 mg/mL α-Amylase solution on each tissue sections (①). After 2-3 hours of incubation at 40 °C (②), the α-Amylase solution is carefully withdrawn (③) and the tissue sections are rinsed with a droplet (30 µL) of water (④ and ⑤). Repeat this step until the starch is completely removed from the tissue. To prevent any contamination with oligosaccharides originating from the degraded starch, the tissue sections are finally rinsed 3 times with water droplets.



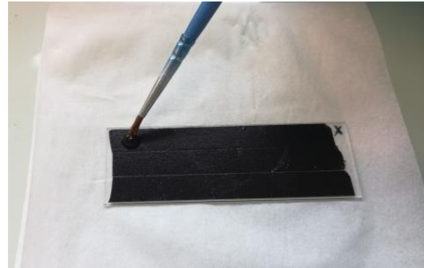
Enzymatic starch degradation
α-amylase/water deposition
until complete starch removal

I- Tissue preparation

4) placing the cross-section onto an ITO glass plate

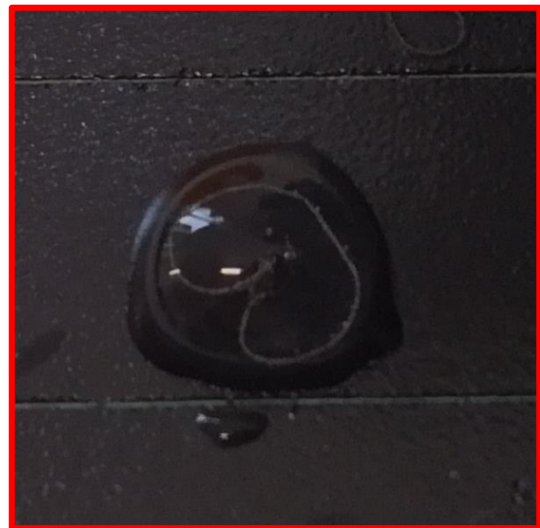


water drop



cross section deposition

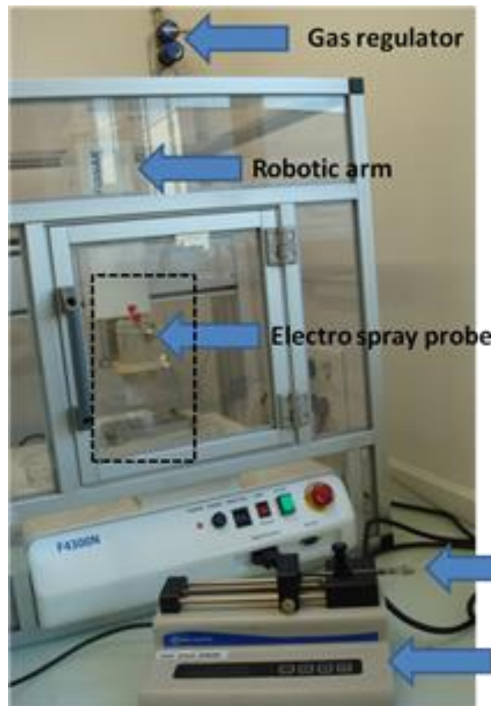
removing the excess of water

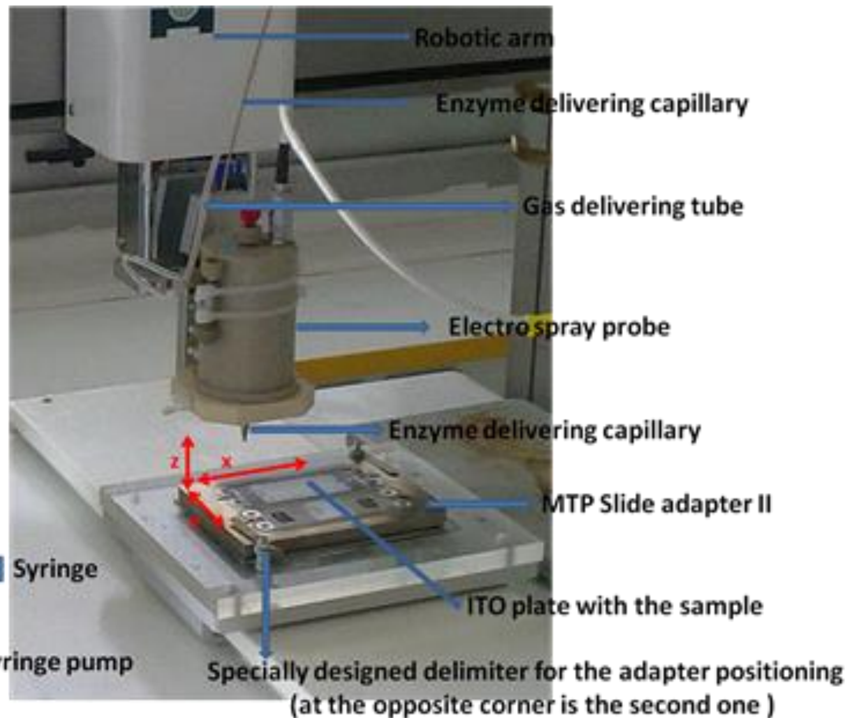


Prepare the ITO glass plate that will receive the cross section: (i) place a piece of adhesive carbon tape at the location where the cross section will be deposited on the ITO glass plate; (ii) with a pipette, put a droplet of water on the tape. Then, with a paint brush, grab the cross section from the Eppendorf tube. Lean the cross section at the surface of the water droplet, allowing it to detach from the paint brush. Proceed very gently, so to avoid any disruption or any distortion of the cross section. Gently remove excess water with a Kimwipes tissue. Allow sample to dry at RT (approximately 30 min).
Mount the ITO glass plate onto the MTP Slide Adapter II (Bruker).

II- In-situ degradation of the cell wall polysaccharides

1) application of the hydrolytic enzyme on the cross section





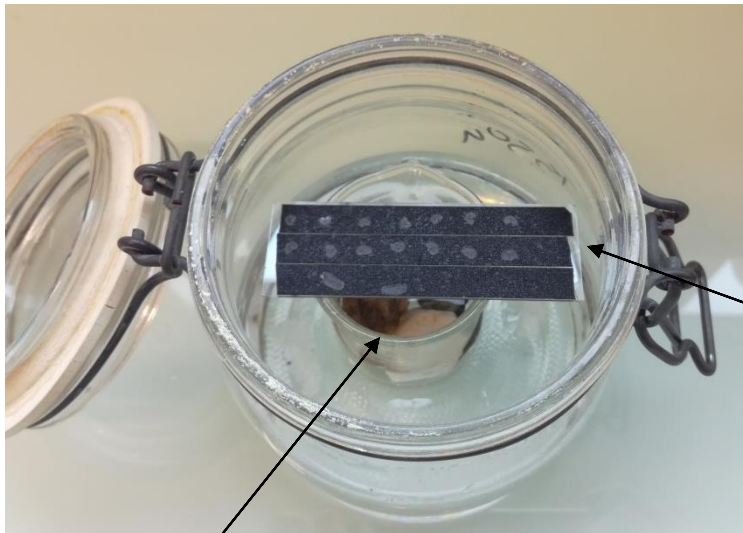
This step uses a homemade robot (see note 1 for the design). The aim is to deliver a controlled volume of enzyme onto the tissue by using an airbrush device, so that fine droplets of enzyme are applied on the tissue, thereby limiting the diffusion of the oligosaccharides released upon digestion over the tissue.

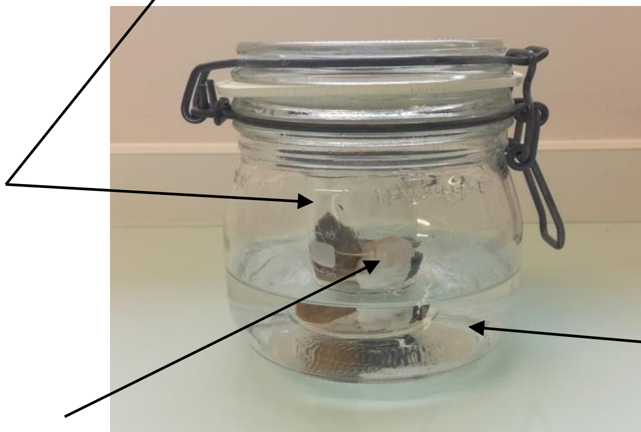
Spraying of the enzyme is achieved by connecting the Electrospray probe mounted on the robotic arm to a syringe pump delivering the enzymatic solution at a constant flow rate of 600µL/h. Spraying is assisted pneumatically with nitrogen (1.5×10^5 Pa). The distance between the needle tip of the Electrospray probe and the ITO plate is 3 cm (Z-axis). A X,Y deposition pattern following a "brush rectangle" is used. The movement speed of the robot head was set at 5 mm/s.

Other parameters (X and Y axis start and end coordinates, volume of the enzyme placed into the syringe, number of spraying cycles) are set to ensure that the robot consistently deposits 0.3µL of enzyme (4.6 U/mL Endo-1,4-β-xylanase or 2U/mL Lichenase) per mm^2 of sprayed area (corresponding to 0.0014 U xylanase and 0.0006 U lichenase per mm^2 tissue). Enzymes can be applied individually, consecutively or in mixture.

Note: Endo-1,4-β-xylanase is used for AX hydrolysis, while Lichenase is used for mixed-linkage BG hydrolysis.

II- In-situ degradation of the cell wall polysaccharides
2) incubation





The wheat cross sections recovered by the enzyme in the previous step are allowed to incubate at 40ºC for 4h. To prevent the liquid from evaporating too fast and the enzyme becomes inactive, a wet atmosphere is maintained by placing the ITO plate into a sealed incubation chamber filled with 150 mL of saturated K_2SO_4 and pre-incubated at 40ºC. The ITO plate is placed on top of a 50mL glass beaker weighted with stones and installed into the glass incubator.

After incubation, remove the tissues from the incubation chamber and let dry in the open air (approximately 15 min).

III- Deposition of the DMA-DHB MALDI matrix

This step is performed with the homemade robot. Three spraying cycles of matrix are made consecutively with the same parameters as those used for enzyme application (same X, Y and Z axis start and end coordinates, same flow rate). The amount of matrix deposited is 10.5 mg/mm^2.

This MALDI matrix (DMA/DHB) was demonstrated to be suitable for oligosaccharides detection [(3)].

Preparation of the matrix solution:
Weigh 100mg of DHB. Dissolve in 1mL of 50% acetonitrile.
Add 20µL of DMA.

This step poses a danger during the following solution preparation steps: Preparation of the matrix solution containing N,N-Dimethylaniline. This product is described as a Carcinogenic, Mutagenic and Reprotoxic (CMR) substance. Wear gloves, laboratory overcoat and laboratory glasses. Do not use this product during pregnancy. Proceed under a fume hood.

(3) ROPARTZ, D., BODET, P. E., PRZYBYLSKI, C., GONNET, F., DANIEL, R., FER, M., HELBERT, W., BERTRAND, D. & ROGNIAUX, H. (2011) Performance evaluation on a wide set of matrix-assisted laser desorption ionization matrices for the detection of oligosaccharides in a high-throughput mass spectrometric screening of carbohydrate depolymerizing enzymes. *Rapid communications in mass spectrometry,* 25**,** 2059-2070.

Note 1

The basic idea of the home-made robot is to put an airbrush-type device on a X,Y,Z robotic arm, and be able to control the liquid flow rate through the airbrush. To do so, we have dismounted an Electrospray probe from an old LCQ Advantage mass spectrometer (Thermo Fisher) and adjusted this probe to a FISNAR 4300N robot. Flow rate through the probe is controlled by a syringe pump, delivering a typical flow rate of 1-100 µL/min. Nitrogen is used as a co-axial nebulizing gas, at a pressure of 1.5×10^5 Pa.
The FISNAR 4300N robot is controlled by a Teach Pendant device, which enables to program the motion patterns for the enzyme deposition. A program consists in defining the start and end positions (defined by X, Y, Z coordinates), the pattern and the speed of the robotic arm movement. "Brush rectangle" pattern is one of the available patterns and is the one that we used in our experiments.