SUNLIBB

Sustainable Liquid Biofuels from Biomass Biorefining

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Deliverable 5.2

"HT saccharification assay for maize and Miscanthus validated"

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Workpackage: **5**
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Deliverable 5.2: HT saccharification assay for maize and Miscanthus validated

The validation of the HT saccharification assay was performed during the first reporting period. The objective of this validation was to establish the conditions of pre-treatment, enzyme loading and hydrolysis for exposing genotype-determined differences in susceptibility to enzymatic digestion by cellulases.

To establish the optimal conditions we used increasing concentrations of enzyme at different incubation times for each crop. The optimal conditions were established at 1.2 FPU/g for maize and 3.3 FPU/g for Miscanthus. The optimal hydrolysis time for both crops is 8 h, in order to expose differences between genotypes.

Once the conditions of saccharification were established, the following step was to determine the pre-treatment to be applied to obtain the maximal differences between genotypes. The results clearly show that the pretreatment with water at 90 °C for 40 min allows a substantial hydrolysis of the biomass while showing clear differences in saccharification potential between lines. In conclusion, hot water pre-treatments were selected for screening of both maize and Miscanthus.

SUNLIBB deliverables

Del No: 5.2	**Deliverable Name: HT saccharification assay for maize and Miscanthus validated**			
WP: 5	**Lead partner:** P1	**Dissemination level:** PU	**Delivery date (project month):18**	**Actual delivery date:** 18

Objective:

Plant lignocellulosic biomass is widely considered to have the potential to reduce our reliance on petroleum for liquid transportation fuels and other chemicals, because it is cheap and abundant and contains energy-rich polysaccharides that make up approximately 75% of its mass. In theory, these polysaccharides can be broken down to produce sugar substrates (saccharification) from which a range of useful products, such as biofuels, bioplastics, fine and bulk chemicals, food and feed ingredients can be produced by fermentation. In this context, the US department of energy has set the target of replacing 30% of petroleum consumption with biomass. Even more value may be obtained by using integrated processing systems that allow multiple products to be produced from the same biomass feedstock: the biorefinery concept. Perhaps, the greatest barrier to realizing the potential of lignocellulose as an industrial feedstock lies in its indigestibility. Cellulose, the major component of lignocellulose is composed of polymers of pure glucose, but the crystalline nature of cellulose microfibrils makes this material resistant to chemical and enzymatic degradation. Cellulose microfibrils are embedded in matrix polysaccharides such as xylans and arabinoxylans, and the whole structure is interpenetrated and encased by the phenolic polymer lignin, another hard to digest polymer. In totality, this composite material forms an insoluble macromolecular mass providing a challenge to degradation in either the biological or industrial contexts. Improving the ease and yield of cell wall saccharification represents the major technical hurdle that must be overcome before the full vision of the plant-fuelled biorefinery can be realized.

The development of high-throughput (HT) methods of screening for phenotypic and biochemical alterations in plants has played an important role in identifying the functions of genes and enzymes in specific pathways in plants and other organisms. However, the analysis of large populations of plants for cell wall digestibility is time-consuming, labour-intensive and expensive. The availability of automated, sensitive and reliable methods to reveal differences in the digestibility of plant materials is essential for identifying and selecting genetic loci with the potential to improve lignocellulosic raw material quality.

The adaptation of biomass saccharification analysis to a HT format requires the miniaturization of processes In most of the configurations for the conversion of lignocellulosic biomass into sugars and other bioproducts, the biomass is first pre-treated, then hydrolyzed, and finally the resulting sugars utilized for fermentation or other chemical transformations. Therefore, a reliable analysis of saccharification properties should be able to reproduce these steps at a smaller scale in order to evaluate the saccharification in large and diverse sample populations. One of the

major challenges in developing such a system derives from the fact that lignocellulosic biomass is an insoluble and heterogeneous material that is difficult to handle at the milligram scale.

Handling and distribution of the biomass materials is facilitated by grinding into very small particles. However, size reduction represents the first step in the conversion process and is, in itself, a pre-treatment that may increase digestibility. At present, several alternatives have been used for dispensing biomass in an accurate and reproducible manner, ranging from the distribution of fabricated sheets of lignocellulose in microplate wells, to pipetting biomass slurry.

Considerable resources have been invested in HT screening systems to identify optimal pre-treatments and considerable information is available in this crucial aspect of biomass conversion. This information is essential in order to assist the development of efficient industrial platforms.

The improvement of feedstocks for conversion into biofuels is an equally challenging area involving breeding crops with reduced recalcitrance to saccharification. What is required in this context is a simple and rapid assay that can be applied to biomass preparations in order to identify cell wall traits that facilitate the conversion of biomass. Indeed, examples of low throughput characterization of biomass in this context can be found in the literature, but such approaches lack the throughput required to evaluate large populations of plants.

Here we report on an analytical approach to perform saccharification analysis in a 96 well plate format, and has been developed to allow the screening of lignocellulose digestibility of large populations of samples from various plant species. We have scaled down the reaction volumes for gentle pre-treatment, partial enzymatic hydrolysis and sugar determination, in order to allow large numbers to be assessed rapidly in an automated system. Our aim is to evaluate biomass samples from Miscanthus, maize and sugar cane, in order to screen mutant and breeding populations, and transgenic lines for variability in saccharification.

Results and discussion:

Overview of the platform/ variability of loading

a) Formatting of the plant materials in 96 well plates

In order to obtain a precise and uniform distribution of the plant materials for the digestion assays, a robotic platform was designed to grind, distribute and weigh dry plant samples (Figure 1A). This device consists of a robotic arm that is able to move vials containing plant samples between 6 different stations (Figure 1 B). The first step is a grinding station where the vials containing three ball bearings are shaken at approximately 5000 rpm. The desired particle size for each plant material can be achieved by varying the grinding time in this station. Once a suitable particle size is achieved, the grinding time is kept constant across all the samples of the experiment. The sample tubes are subsequently moved to a de-clogging station where the powder is mixed by inverting the vials. At the third station the base of the

vial is pierced to allow the dispensing of the powder into the designated locations in 96 well plates. Finally, the vials move to a 96 well plate placed on top of a balance and the robotic arm vibrates at variable speeds to dispense the powder up to a determined sample weight, with the final weight recorded for each sample. The standard weight of each sample used in this paper is 4 mg, and the accuracy of dispensing is shown in figure 1C. The plates are then sealed with a silicone cover to avoid evaporation during the pre-treatment and hydrolysis.

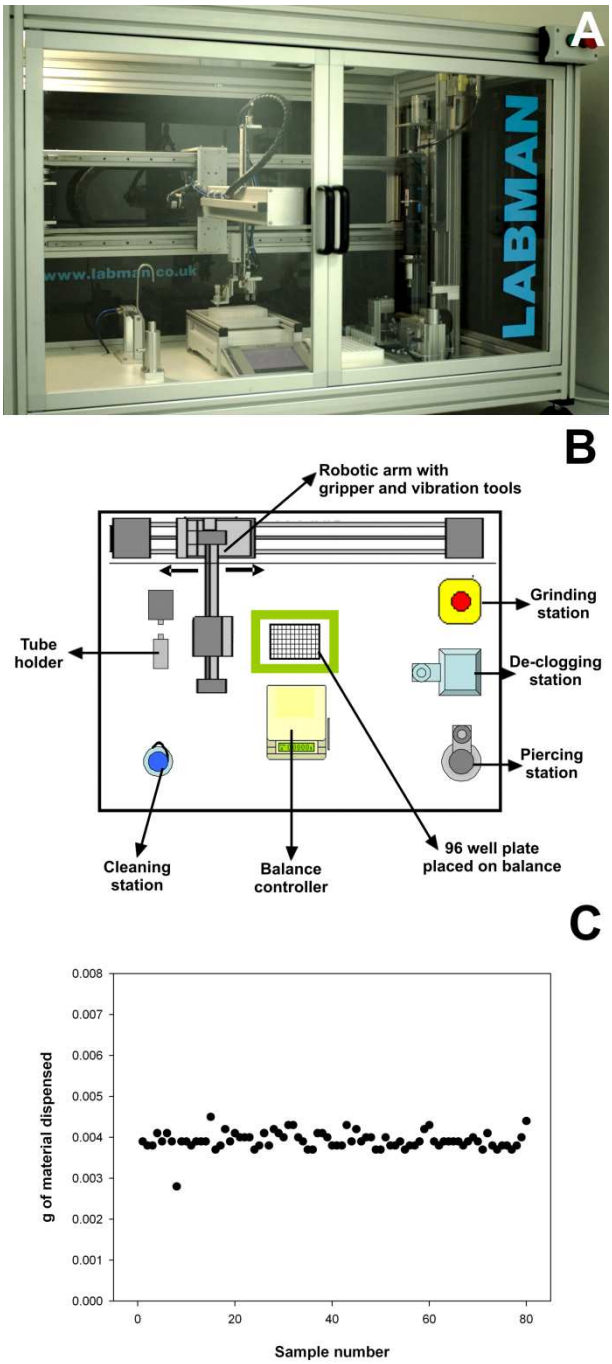

Figure 1: Robotic platform for grinding, dispensing and weighing samples. (A) General view of the robot. (B) Schematic plan of the robot showing the different stations. (C) Accuracy of the sample weights observed in the delivery of plant powder in a 96 well plate.

b) Pre-treatment, hydrolysis and sugar detection.

Once the samples are loaded as described above, the 96 well plates are processed by a robotic liquid handling system (Figure 2A). This system performs a mild pre-treatment of the plant materials by heating in the presence of acid or alkaline solutions, removal of the pre-treatment solution, enzymatic hydrolysis, and quantification of the released material as reducing sugar equivalents. It is important to remark that the aim of this system is to screen plant materials more than improve the pre-treatment conditions at a pilot or industrial scale and consequently we apply only mild heating to the biomass samples before hydrolysis as a form of pre-treatment. The sequence of steps involved in the saccharification analysis is shown in Figure 2B. The platform allows flexibility in the duration of all the steps. An overview of the labware and processing steps is shown in Figure 3.

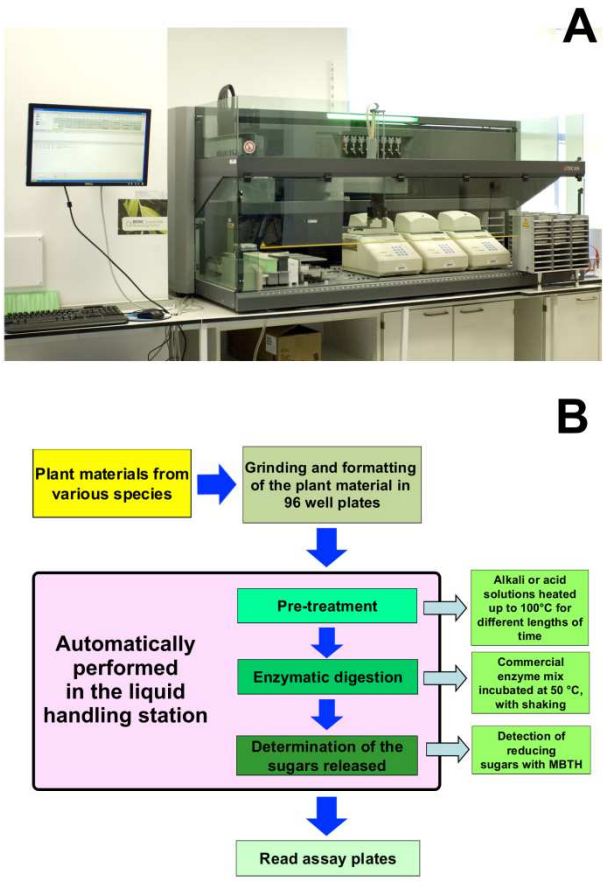

Figure 2: Liquid handling station. (A) General view of the liquid handling station. (B) Flow chart showing the steps involved in the saccharification analysis

The pre-treatment takes place on a heating block where the temperature can be regulated up to 100 °C (Figure 3). The pre-treatment is followed by several rinses (usually 6) with buffered solution, before the enzyme mix is dispensed into the wells. The rinses are carried out by adding buffer to the pre-treatment solution and subsequently removing 50% of the total volume after allowing the solids in the

sample to settle. The height of the aspiration is set at half of the liquid mass to avoid aspirating solids from the bottom of the well, and there is negligible loss of solids using this approach. These washes are repeated 6 times in order to bring the pH of the sample to the same value as the pH of the hydrolysis. The deep well plates are then incubated at 50°C with constant shaking in ovens designed for delivering an even heat in 96 well plates. After hydrolysis, aliquots of the digestate are removed from the deep well plates for colorimetric assay of the released reducing sugars.

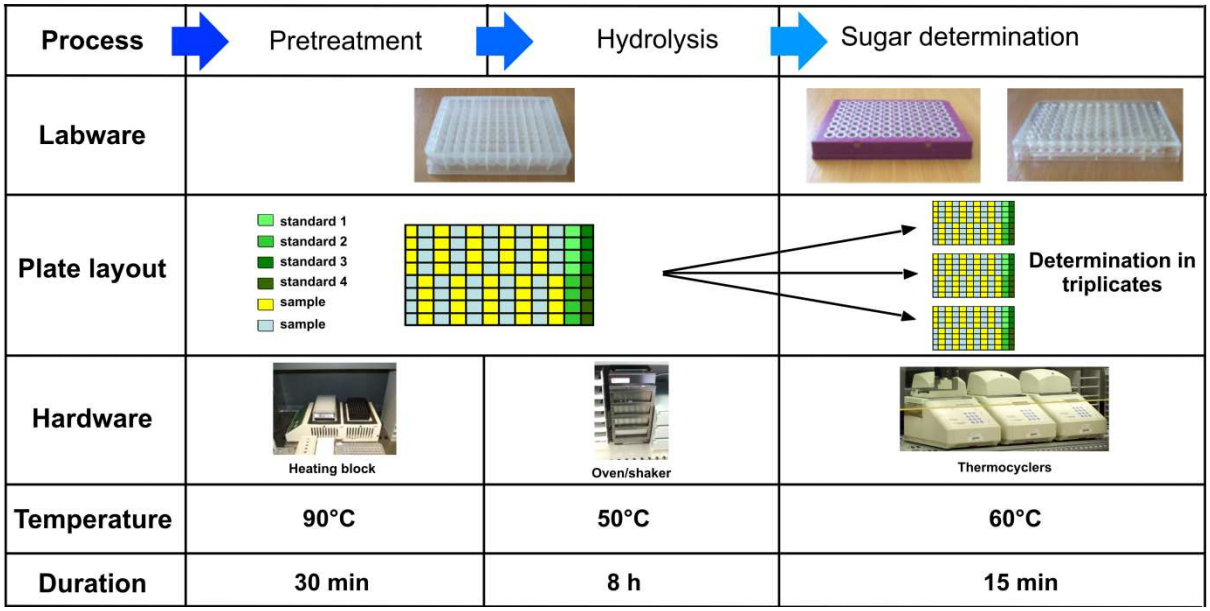

Figure 3: Labware, hardware and incubation conditions used in the saccharification analysis

c) Reducing sugar detection.

We applied a colorimetric method of sugar detection in order to reach the throughput required to analyze large populations. However, both the non-digested materials and the hydrolysate of selected samples can also be retained for further analysis as required. We assessed several methods for reducing sugar quantification, such as dinitrosalicylic acid (DNS), bicinchonimic acid (BCN), 2-cyanoacetamide, and 3-methyl-2benzothiazolinonehydrazone (MBTH). MBTH was selected as the most suitable method, being the easiest to automate and the least susceptible to interference from compounds such as proteins. We modified the MBTH method for use on the robotic platform so that it could accurately quantify sugars at the concentrations present in the biomass hydrolysates. For this we modified the highly sensitive method developed by Anton and Barrett (2002) to work with a final volume of 250 µl, suitable for a standard optical 96 well plate. Table 1 shows a comparison of the reaction conditions between the original method and the modification for HT.

Table 1: summary of the changes from the original MBTH method to be adapted to the HT-format

Reagent	Volume of reagent (Anton and Barrett)	Concentration (Anton and Barrett)	Volume of reagent (HT-determination)	Concentration (Anton and Barrett)
Sample	100 µl	--	75 µl	--
NaOH	100 µl	0.5 N	25 µl	1 N
MBTH mixture	100 µl	1.5 mg/ml MBTH 0.5 mg /ml DTT	50 µl	0.21 mg/ml MBTH 0.7 mg/ml DTT
Oxidising Reagent	200 µl	0.5% ($FeNH_4(SO_4)_2$) 0.5% Sulfamic acid 0.25 N HCl	100 µl	0.5% ($FeNH_4(SO_4)_2$) 0.5% Sulfamic acid 0.25 N HCl
H_2O	500 µl	--	---	--
Heating	80 °; 15 min	--	60 °C, 20 min	--
Range of detection	0–20 nmol		0-200 nmol	

The concentration of MBTH was optimized to increase the range of concentrations at which the detection of reducing sugars is linear. Figure 4 shows the linearity between different dilutions of MBTH reagent and a range of concentrations of glucose. In a similar manner we optimized the temperature and incubation times for an optimal absorbance reading, to 60 °C for 15 min (data not shown). The sensitivity of the automated method with different sugars was established by measuring the response and linearity of determinations, which was carried out by comparing the original method with that modified for the automated system. Figure 5 shows the calibration for glu, xyl, ara, and gal. Although the response in absorbance units is different for each monosaccharide, the quantification is linear in the range of concentrations at which the saccharification analysis is performed in the automated platform (between 0 and 150 nmol). The varied response in detection by MBTH for different monosaccharides contradicts the report from Anton and Barret regarding the use of the method for a range of sugars. We decided to refer to our

measurement of reducing sugar equivalents as glu equivalents based on standard curves of glu, as this is the major monosaccharide in the cell wall.

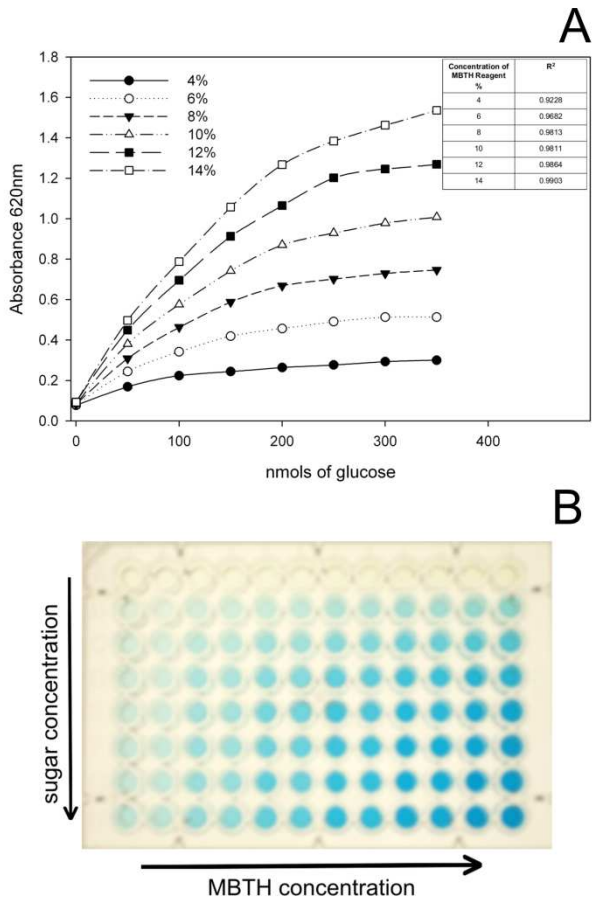

Figure 4: Adaptation of the MBTH method for use in automated analyses (A) Calibration curve for the detection of reducing sugars using increasing concentrations of MBTH. The insert in the figure shows the R^2 values obtained for the different MBTH concentrations between 0 and 200 nmol.

Reproducibility of the saccharification assay

We used Whatman N° 1 paper discs in order to test the variability within the 96 well plate-based automated assays. After pre-treatment with 1% H_2SO_4 for 30 min at 90°C, the discs were digested in the robotic liquid platform for 8 h, and the reducing sugars released determined in triplicate. The variation coefficient across 96 wells is 5.5%, as shown in Figure 6. This variation is considerably lower than the 9% variation observed when the saccharification of paper discs is performed manually (data not shown).

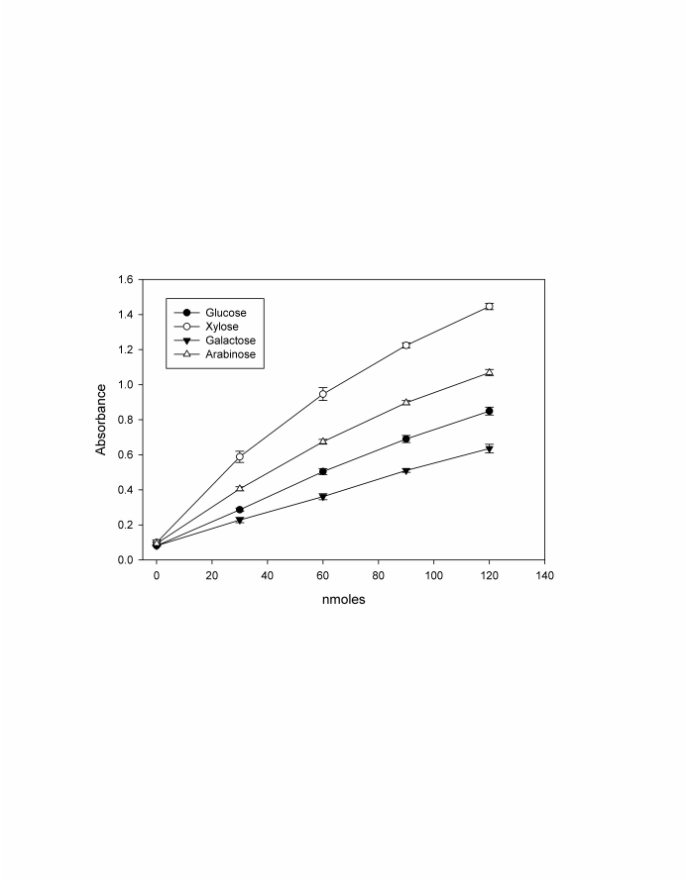

Figure 5: Calibration curve of the MBTH detection of different monosaccharides.

To establish the optimal conditions we use increasing concentration of enzyme at different incubation times for each crop. Figure 7 shows the kinetics of the release of reducing sugars from two different maize genotypes at different enzyme loadings and incubation times. The enzyme loadings of: 10, 3.3, 1.65 and 1.2 FPU/g of biomass were used. The optimal conditions were established at 1.2 FPU/g for maize and 3.3 FPU/g for Miscanthus. The optimal hydrolysis time for both crops is 8 h in order to expose differences between genotypes. This hydrolysis time allows the processing of 80 samples/day, making the analysis of large populations such as the Biomis or SUNLIBB populations achievable within the timeframe of the project.

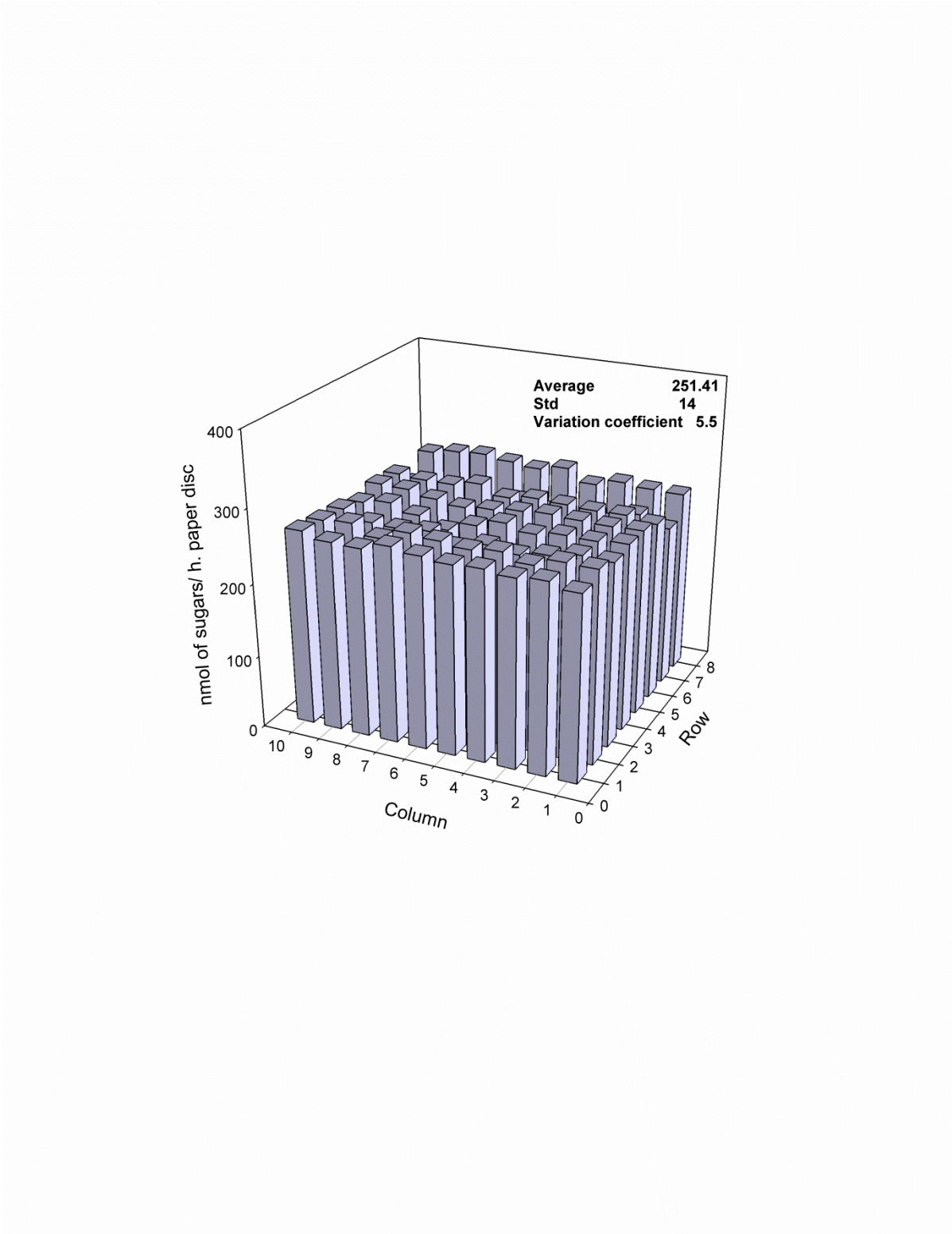

Figure 6: Quantification of the reducing sugars released in the hydrolysis of paper discs in a 96 well plate.

Validation of assay conditions for Maize and Miscanthus

The objective of this validation was to establish the conditions of pre-treatment, enzyme loading and hydrolysis for exposing the genotype- determined differences in the susceptibility to enzymatic digestion by cellulases.

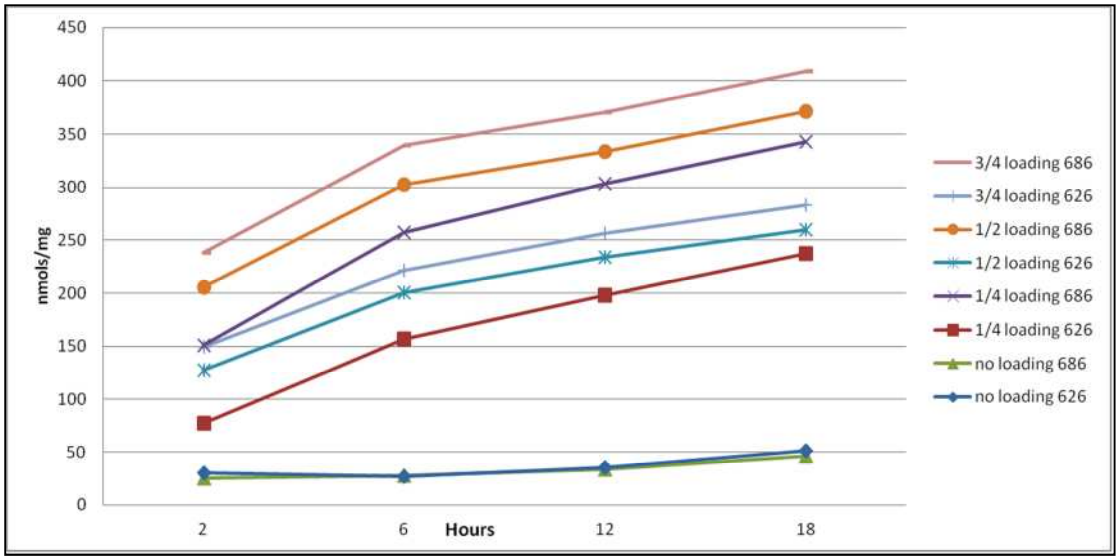

Figure 7: Determination of the enzyme loading and hydrolysis time for two maize cultivars along an 18 hours period

Three different pretreatments were applied to each material to obtain the maximal differences between genotypes. Figure 2 shows the effect of acid, alkaline and water pre-treatment on the saccharification of 20 Miscanthus genotypes selected from the SUNLIBB population generated at Wageningen. The results clearly show that the pre-treatment with water at 90 °C for 40 min allows a substantial hydrolysis of the biomass while showing clear differences in saccharification potential between lines. Water pre-treatment also enhanced the differences in saccharification potential in maize (data not shown).

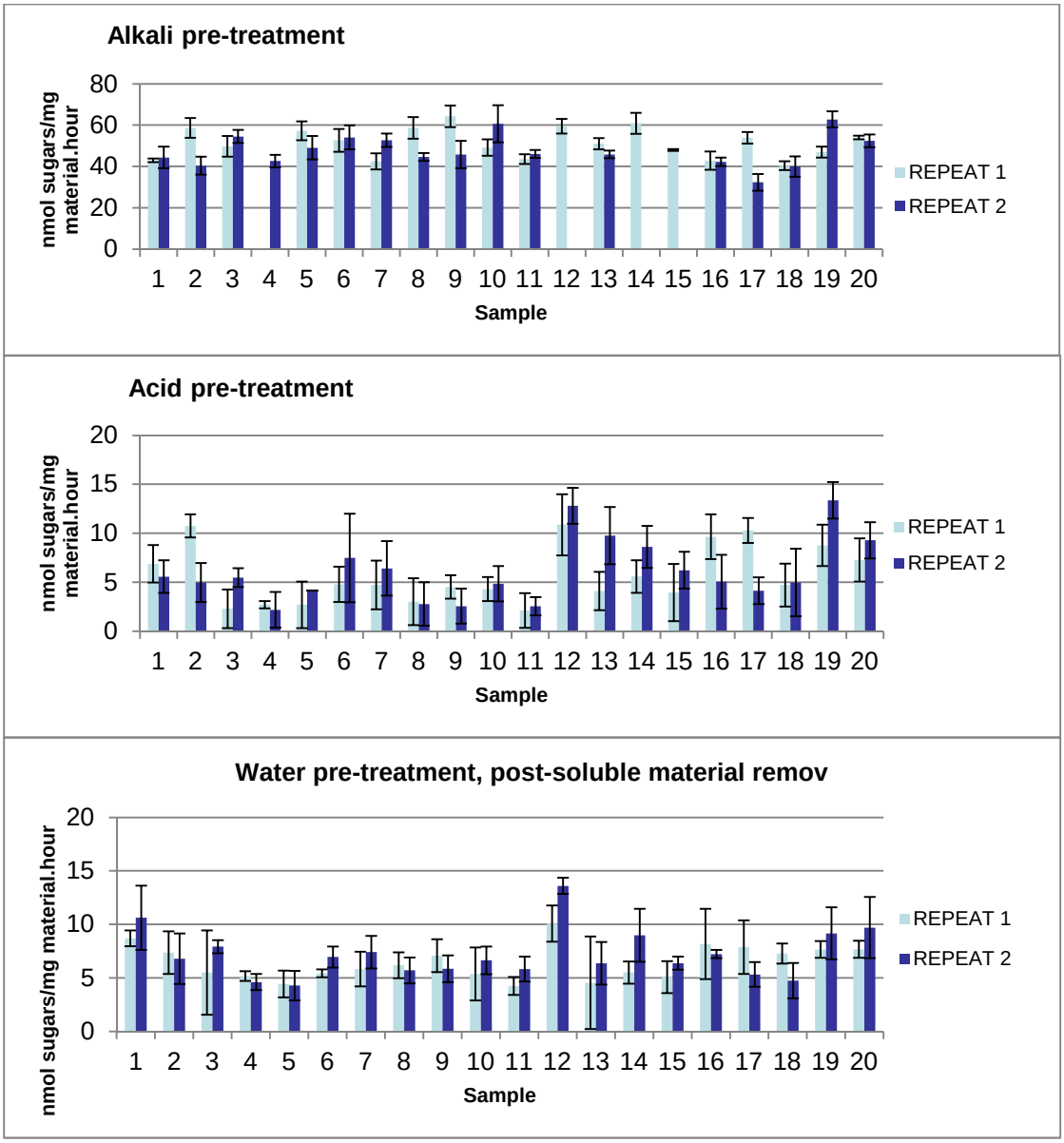

Figure 8: Saccharification potential determined in twenty selected genotypes of Miscanthus under alkaline, acid, and water pre-treatment.

The saccharification assay developed in the SUNLIBB Consortium has allowed us to screen large populations of maize and Miscanthus, identifying a novel QTL for this trait in maize (deliverable 5.4).

Figure 9 shows the saccharification potential in the BIOMIS Miscanthus population, where different genotypes show a significant degree of variation. This phenotyping will prove extremely valuable for further breeding programs. The outcomes of the consortium will be extended towards the phenotyping of the SUNLIBB population in 2014.

The method was used for the screening of several maize populations. Figure 10 shows the saccharification potential of the RILs population LU2007 and LU2010 from INRA (see WP1 for details). The analysis was concentrated in LU2007, where a QTL for saccharification was found and described in Deliverable 5.4

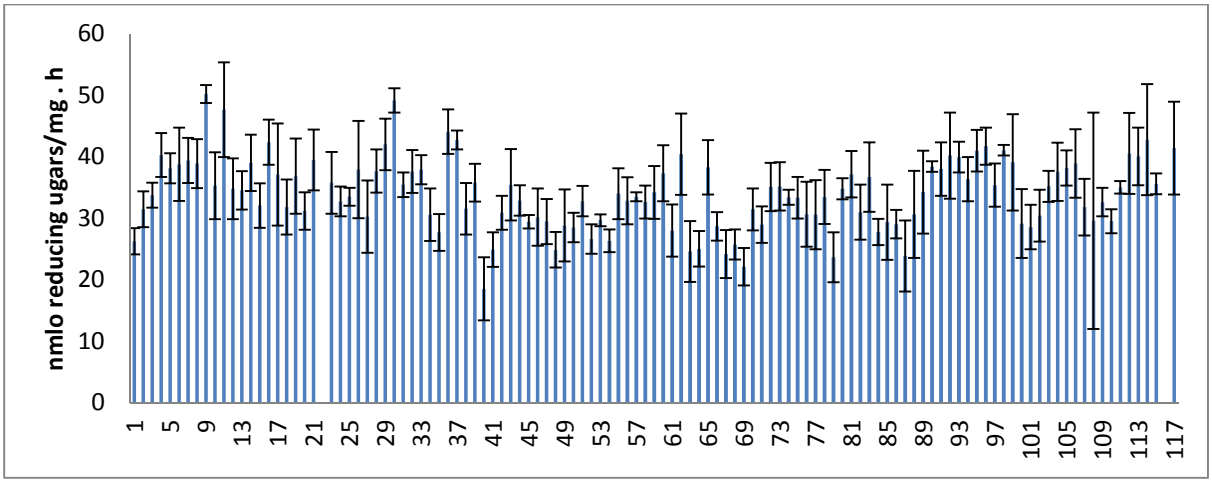

Figure 9: Saccharification potential of the BIOMIS population of Miscanthus

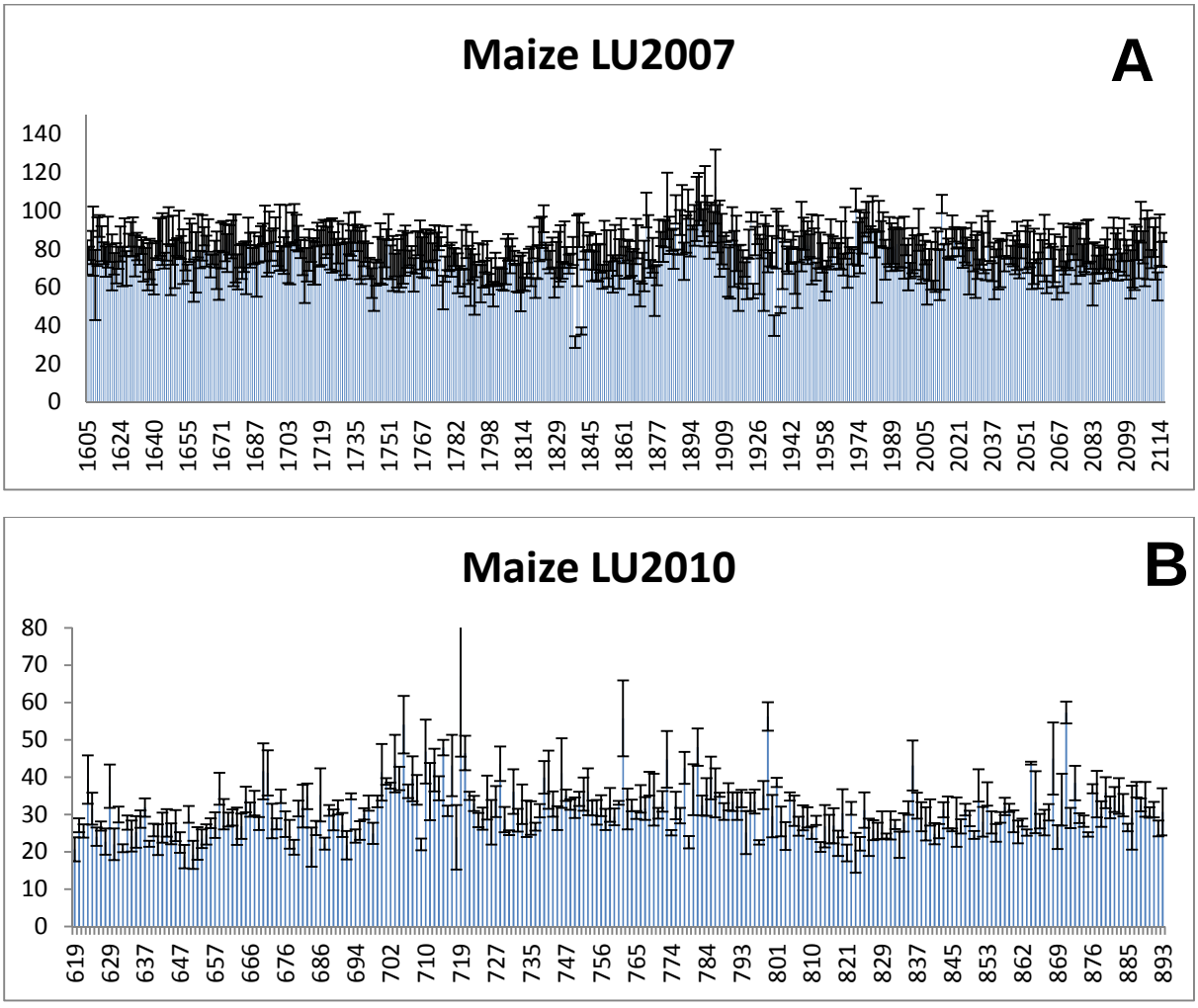

Figure 10: Saccharification potential in two maize populations