SUNLIBB

Sustainable Liquid Biofuels from Biomass Biorefining

Grant Agreement no. 251132

Collaborative Project
EU 7th Framework Programme
ENERGY

Project duration: 1st October 2010 – 30th September 2014

Deliverable 5.1

"HT saccharification assay established in Brazil"

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Workpackage: **5**
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Clarifications on deliverables for the SUNLIBB 2nd reporting period
WORK PACKAGE 5
Biomass Deconstruction

As stated in the main body of the report, the deliverables of this Work Package were achieved on time, with the exception of the Deliverable 5.1"High Throughput (HT) saccharification assay established in Brazil". The delay in this deliverable was informed to the Project Officer and was due to delayed release of funds by the Brazilian funding agency. The HT platform is based at the University of Sao Paulo, Sao Carlos, SP.
The report for the second reporting period is structured on the basis of the 6 Tasks for this Work Package. Below is a list of the Deliverables 1-4 with a detailed explanation of procedures, results, discussion / conclusions

Deliverable 5.1: HT saccharification assay established in Brazil

The purpose of this deliverable was to set up a common methodology for the evaluation of saccharification as a major trait to phenotype populations in order to breed specifically for industrial applications of biomass. The experience of Partner 1 in development of this methodology would be useful to speed up and work synergistically with the Brazilian counterpart [1]. This deliverable should have been completed by month 12, but the delay in the release of funds necessary to purchase the relevant equipment produced a delay in the development of the platform in Brazil.
Professor Igor Polikarpov visited the HT platform in York and in coordination with Dr. Leonardo Gomez designed a project that was further defined during the collaboration around the characterization of novel biomass crops by Marisa Lima at the University of York (paper submitted and [2]).
The platform is based on a Tecan Evo 200 liquid handling station and has been designed for high throughput saccharification assays as well as for HT cloning of potential glycosyl hydrolases. This platform was deployed during August 2013 for the determination of the saccharification potential in a sugar cane mapping population.

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 is to establish the conditions of pretreatment, enzyme loading and hydrolysis for exposing the genotype-determined differences in the susceptibility to enzymatic digestion by cellulases.
To establish the optimal conditions we use increasing concentration of enzyme at different incubation times for each crop. Figure 1 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.
Once the conditions of saccharification are established, the following step is to determine the pre-treatment to be applied 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. In conclusion, hot water pre-treatments were selected for screening both maize and Miscanthus.

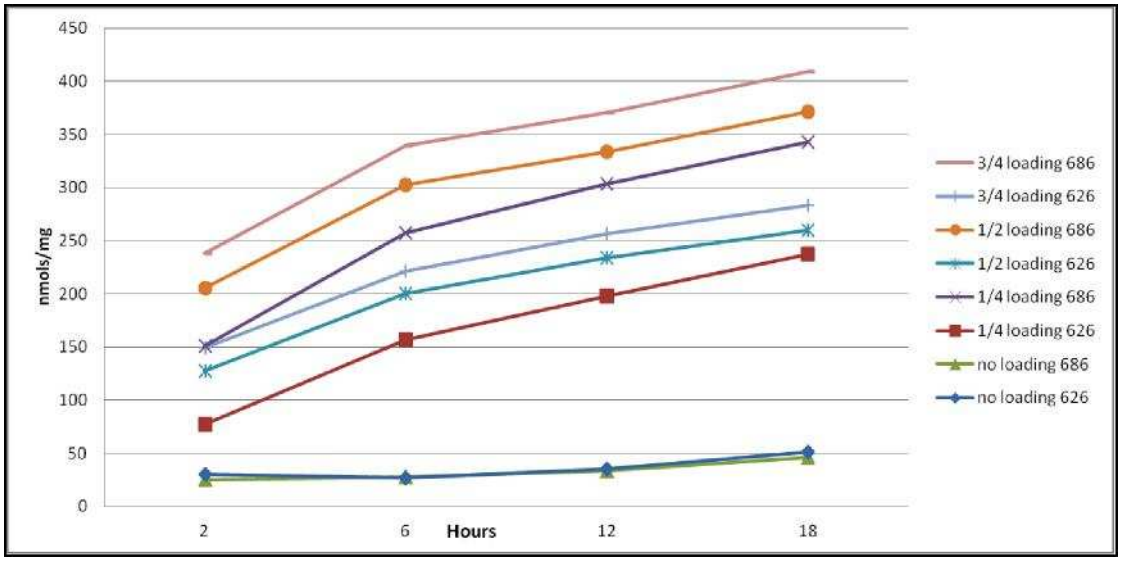

Figure 1 Deterrmination of optimal enzyme loading and incubation time for two maize genotypes

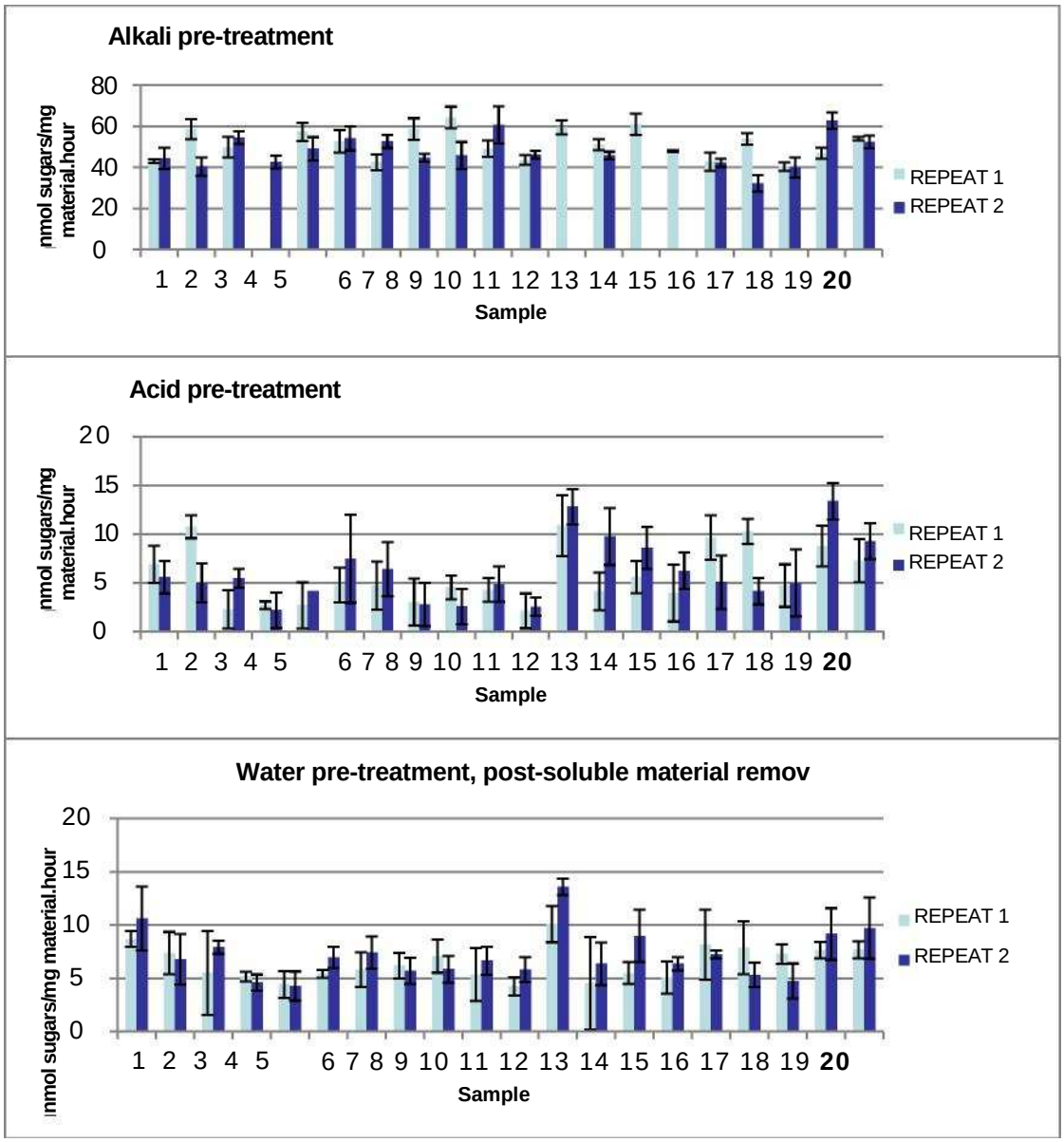

Figure 2 Different pre-treatments applied on twenty contrasting lines of Miscanthus.

Deliverable 5.3: Characterisation of lignin products released by enzymatic and metaloxide catalysis

It has been suggested that one can improve enzyme biodegradation efficiency by applying photocatalytic processes involving the illumination of semiconductor materials that have large band gaps. For instance, a significant enhancement of degradation efficiency of trinitrotoluene (TNT) by laccase from *Phanerochaete chrysosporium* was observed by application of titanium dioxide (TiO_2)-assisted photocatalytic pretreatment. Furthermore, it has been determined how the decolorization efficiency of immobilized laccase using TiO_2 photocatalytic pretreatment is affected by (1) support materials, (2) mediators, and (3) colors. TiO_2 or titania is a promising photocatalyst and has particular potential for lignin degradation. There has been increasing interest in the environmental applications of titania because it is an inexpensive, strongly oxidizing stable chemical. It is also capable of the photo-oxidative destruction of most organic pollutants, and the degradation of organic substrates is initiated when TiO_2 absorbs ultraviolet (UV) light. We believe to be the first published account of a combined process that uses both photocatalytic processes (TiO_2 and UV light) and biocatalytic processes (laccase) together to degrade high-molecular-weight lignin, with the hypothesis being that there would be more degradation from a combined process [3]. We conducted multiple-replicated experiments (single and dual stage) to make this assessment. For comparison, experiments using only one of the test materials, either TiO_2 or laccase, are also conducted, and the role of $_{H2O2}$ is examined. Of further interest is the analysis of intermediate compounds resulting from lignin breakdown, because these may have potential biorefinery applications. Overall, we detail which configurations offer opportunities for laccase and/or titania in providing an environmentally friendly alternative to chlorine and mild operating conditions or as an upstream process in a biorefinery chain.

P11 have determined that is possible to obtain a wide variety of products from lignin breakdown via either enzymatic routes of from low temperature catalysis via transition metal oxides, or a combination of these approaches. As reported previously, a portfolio of products is generated, including organic acids, with succinic acid being a major product of this type (Table 1).

Deliverable 5.4: Identification of QTL for cellulose digestibility in maize

This deliverable was accomplished in a close collaboration with WP1 and is also reported in the context of that WP.
The objective of this action was to identify physical positions in the genome of maize that alter the saccharification potential. In the present project we use Recombinant Inbred Lines to define these positions. The phenotyping of the population was performed by determining the saccharification potential of each of the lines under exactly the same conditions in an automated protocol.
P6 delivered biomass samples from a mapping population (Recombinant Inbred Lines derived from the cross F288xF271) to P1. The set up of the saccharification Analysis was performed as described in Deliverable 5.2. Saccharification potential has been quantified by P1 with different pre-treatments on this population (alkaline and water). One single QTL for saccharification has been detected on chromosome 1 on maize (see Figure 3). The confirmation of this QTL is under way at present. The physical positions of this QTL were also sent to P4 in order to identify the genes present at this locus (according to the reference maize genome from the accession B73). QTL for silage digestibility have been previously detected on the F288xF271 RIL population. P6 selected a set of NILs in order to validate the

effects of these QTLs and to see their impact on saccharification potential. These selected lines have been cultivated in field trials and harvested. These materials will be sent to P1 for further phenotyping.

Table 2: Intermediate compounds as TMS derivatives in ethyl acetate from lignin (control), lignin degradation by laccase, titania, and titania with laccase in the single and dual-step processes in the presence of H_2O_2.

Time	Product compounds	Percentage of product compounds (%)						
(min)		Lignin	Lignin with laccase	Lignin(H_2O_2)	photocatalytic reaction	biocatalytic reaction	Single-step reaction	Dual-step reaction
4.57	Ethylene glycol	5.47± 0.75	4.39±0.90	4.37±0.12	6.29±0.09	7.11±0.48	6.94±0.56	3.80±0.56
5.54	lactic acid	1.04±0.07	0.25±0.00	1.87±0.03	3.60±0.15	0.70±0.05	0.92±0.02	0.48±0.02
5.85	Acetic acid	4.67±1.18	1.58±0.17	2.98±0.17	4.05±0.15	4.43±0.19	3.65±0.64	2.51±0.11
6.05	Ethylethylene glycol	1.37±0.97	0.65±0.02	1.57±0.07	0.79±0.03	2.54±0.46	2.59±0.26	1.01±0.05
6.85	2,6-Dimethoxyquinone	1.44±0.43	1.42±0.06	0.73±0.06	2.34±0.10	2.26±0.02	2.57±0.52	0.25±0.01
7.61	N,N,4-Trimethylbenzenamine	1.68±0.05	1.32±0.03	1.92±0.10	1.03±0.02	1.97±0.31	2.65±0.01	0.84±0.03
7.90	Malonic acid (tms)	0	0	0	11.51±0.43	0	0	0
9.14	Phosphoric acid	1.18±0.57	1.93±0.05	0.20±0.01	19.20±1.42	1.16±0.18	1.18±0.26	3.02±0.56
9.41	Mannopyranose, 1-O-(trimethylsilyl)-, 2,3:4,6-dibutaneboronate	1.26±0.10	1.77±0.05	1.27±0.25	0	1.72±0.26	5.16±0.94	2.55±0.11
9.65	Succinic acid (tms)	1.10±0.22	0	0	9.97±0.26	0	0	0.45±0.05
9.70	Dodecamethylcyclohexasiloxane	3.13±0.26	4.24±0.13	1.63±0.09	0	5.23±0.67	6.30±0.29	0.77±0.11
9.85	2-methylsuccinate	0	0.62±0.06	0	0	0		0
10.01	Tetraethyleneglycol monomethylether	1.79±0.43	0.62±0.06	1.87±0.07	4.26±1.03	1.22±0.09	1.35±0.27	1.47±0.11
10.16	Fumaric acid	0.28±0.04	0	0	0	0	0	0
10.21	glycerol	0.35±0.03	0.23±0.02	0.21±0.03	0.70±0.23	0.72±0.20	0.95±0.04	0.29±0.07
11.61	2-Furanone, 3,4-dihydroxytetrahydro	0	0.38±0.03	0	0	0	0	0
11.62	(R*,S*)-3,4-Dihydroxybutanoic acid	0	0	0	0.32±0.02	0	0	0
12.78	Butylated Hydroxytoluene	0.77±0.11	0.44±0.03	1.77±0.16	2.07±0.11	1.68±0.03	0.22±0.02	0.21±0.02
13.14	Vanillin	0.37±0.05	0.38±0.06	0.57±0.07	0.32±0.02	0.54±0.01	0.73±0.04	0.36±0.01
13.24	2,4,6-Tri-t-butylbenzenethiol	0.55±0.06	0.78±0.01	1.89±0.12	0.82±0.00	1.93±0.08	0.98±0.05	0.94±0.03
13.95	Veratric acid	1.34±0.08	0.84±0.07	1.06±0.01	1.22±0.09	1.22±0.03	1.84±0.13	0.89±0.07
14.41	D-Xylofuranose	0.48±0.06	0.59±0.03	0.46±0.05	0.31±0.12	0.56±0.02	0.76±0.04	0.61±0.01
14.65	D-Ribonolactone	0	5.04±0.30	0.22±0.07	0.29±0.02	1.17±0.02	0.54±0.02	1.31±0.34
15.65	3,4-dimethoxybenzoate	1.93±0.58	0.85±0.04	1.82±0.27	2.15±0.44	2.42±0.00	2.39±0.04	1.25±0.01
15.76	β-D-Galactopyranoside, methyl 2,3-bis-O-(trimethylsilyl)-, cyclic butylboronate	0	0.53±0.03	0	0	0	0	0
16.94	Methyl a-D-glucopyranoside	0	2.60±0.53	0	0	1.41±0.32	1.58±0.12	0.33±0.05

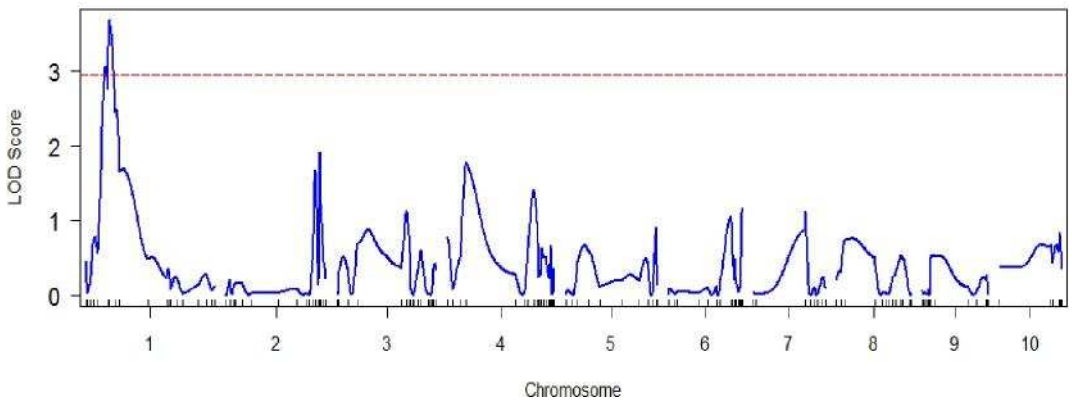

Figure 3: QTL detection for saccharification potential in the F288xF271 RIL population using water pretreatment.

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3. Kamwilaisak K, Wright PC: **Investigating Laccase and Titanium Dioxide for Lignin Degradation.** *Energy & Fuels* 2012, **26:**2400-2406.