Final report

1.1 Project details

Project title	Development Of Chromogenic True-To-Nature Biomass Substrates For Bioconversion Process Optimization
Project identification (program abbrev. and file)	64016-0133
Name of the programme which has funded the project	EUDP
Project managing company/institution (name and address)	GlycoSpot ApS, Østmarken 9, 2860 Søborg
Project partners	GranBio, São Paulo, Brazil NMBU, Aas, Norway
CVR (central business register)	36487976
Date for submission	08.05.219

1.2 Short description of project objective and results

1.2.1 [English]

GlycoSpot has already demonstrated that multi-colored chromogenic polysaccharide and protein substrates can be used in cheap, convenient and high-throughput multiplexed assays. These substrates can be used to screen the activities of different carbohydrate-acting enzymes and provide insight into substrate availability within biomass. Within this project we developed the next generation of biomass substrates in which native chemical structure is modified as little as possible and naturally-occurring chemical substitutions are maintained. These novel True-To-Nature Complex Biomaterial Substrates (TTN-CBS) closely mimic natural biomass encountered in biorefining and new findings can be directly applied to enzyme screening with the aim to improve raw material utilization.

1.2.2 [Danish]

GlycoSpot har allerede vist, at flerfarvede kromogene polysaccharid- og proteinsubstrater kan anvendes billiget, praktiske høj-volumen multiplex analyser. Disse substrater kan bruges til at screene aktiviteterne i forskellige kulhydratvirkende enzymer og give indsigt i substrattilgængelighed inden for biomasse. I dette projekt har vi udviklet næste generation af biomassesubstrater, hvor den naturlige kemiske struktur ændres så lidt som muligt, således at de naturligt forekommende kemiske substitutioner opretholdes. Disse nye "True-To-Nature Complex Biomaterial Substrates" (TTN-CBS) nærmer sig naturlig biomasse som opstår ved biorefinering, og de nye resultater kan anvendes direkte til enzym screening med det formål at forbedre udnyttelsen af råmaterialet.

1.3 Executive summary

National and international policies for increased independency in energy provision and diminished greenhouse gas emissions support the creation of a market for 2nd generation (2G) bio-ethanol. However, production is not yet cost-effective and producers demand technologies to help reduce production costs and increase energy yield from biomass.

Enzymes are indispensable in bioethanol production and constitute a major cost. Better screening of enzyme performance in biomass degradation will lead to higher exploitation of known biomass energy sources, lower production costs, and faster identification of potential new biomass as energy sources.
Biomass and crops used for 2G bio-ethanol such as sugarcane bagasse, wheat and rice straw, and residues from forestry (lignocellulosic biomass) consist of multiple polysaccharides entangled in complex structures. They do not contain readily available sugars but have high concentrations of lignocelluloses, celluloses, and some also starch. They have to undergo pre-treatment and enzymatic hydrolysis processes in order to obtain sugars that can be converted into ethanol but the complex structures inhibits enzyme performance and makes choice and dosage of enzymes crucial in order to obtain maximum energy yield and minimize costs.

Current state-of-the-art enzyme screening assays such as AZCL substrates are designed to analyse enzyme or culture performance on substrates based on purified pure polysaccharides. Consequently, they can only provide an approximate picture of an enzyme's performance in biomass.

GlycoSpot has developed a range of new true-to-nature substrates for screening biomass quality and carbohydrate active enzymes' performance in biomass degradation in production of 2nd generation (2G) bio-ethanol:

Insoluble Chromogenic Biomass substrates (ICB)
The ICB substrates are synthetic variants of popular biomass types synthesized from raw plant material, chemically dyed in and modified rendering them chromogenic and insoluble, while maintaining resemblance with the original material.

In this project, we have successfully developed novel True-To-Nature Complex Biomaterial Substrates (TTN-CBS) and shown that they can provide novel information, compared to the established ICB substrates. Based on the results of the EUDP project, GlycoSpot is now primed to produce the TTN-CBS substrates in large scale and market them to customers in the bioethanol as well as enzyme development industries.

The substrates are integrated with an industry standard 96-well filter plate format into a high-throughput, easy to use enzyme-screening kit. Customers can utilize their standard laboratory equipment when using GlycoSpot kits, thus minimizing their switching cost.
In this project we have achieved the following results:

- we have developed a novel synthetic methodology for synthesis of fluorescently labeled polysaccharides susceptible to enzymatic degradation
- we have developed a novel synthetic methodology for synthesis of fluorescently labeled substrates from raw biomass feedstocks (exemplified with the following 6 biomass feeds: wheat straw, pre-treated sugar cane straw, sugar cane straw, sugar cane bagasse, energy cane straw, energy cane bagasse)
- we have conducted analysis of newly developed fluorescent biomass substrates and have proven that we have succeeded in:
    - developing a milder technique for functionalizing complex biomass feedstocks with fluorophores without removing naturally occurring chemical modifications
    - implementing them into a 96-well high-throughput screening format
    - analyzing different commercial enzymes and enzyme cocktails with the fluorescent substrates
    - generating degradation profiles for each of the 6 different feedstocks mentioned above reflecting relative activity/specificity/relative polysaccharide composition
    - investigated effects of xylan deacetylase enzymes prior to treatment of fluorescent biomass samples
- we have conducted the same type of analysis as mentioned under (3) on our chromogenic variants of the same 6 biomass feedstocks in order to compare differences stemming from natural chemical substitutions (e.g. acetyl groups) as well as different dye molecules used (chromophores vs. fluorophores)

1.4 Project objectives

1.4.1 Project objectives outline

Prior to beginning this project, GlycoSpot had developed and analyzed prototypes of different biomass substrates, e.g. ICB-wheat straw, ICB-sugarcane bagasse, ICB-hemp fiber, ICB-spruce, ICB-willow, ICB-filter paper.

Results of the analyses conducted on these substrates have revealed characteristic profiles of different samples such as an already known difference between "type I" and "type II" plant cell walls which validated the ICB substrate approach as an accurate approach reflected in as a proof-of-concept that is capable of reproducing already published results (Kračun et al, 2015, Biotechnology for Biofuels).

However, the lab tests also showed that important chemical groups, mainly esters and sugars such as uronic acids are degraded during the preparation of biomass substrates as a result of the harsh synthetic methods used for preparing the substrates, especially the high pH level needed for covalent binding of the dye.

The absence causes deviations in screening results. Even though the results still provide a far better image of substrate quality and enzyme performance towards complex biomass, the deviations combined with the familiarity with existing methods make customers reluctant to change from existing methods to ICB substrates. We needed to find ways to bind the dye to the biomass with a minimum of loss of chemical integrity and to identify the consequences of the remaining absences so that customers can account for any deviations in their analyses while at the same time obtain accurate data relevant to their processes.
The main work objectives of this project were:

- Development of alternative dying methods
- Evaluation of ICB substrates developed in WP1 in an actual bio-industrial setting
- Extrapolation of ICB-substrates to other types of biomass of highest commercial interest
- Implementation of ICB substrates developed in WP1 and WP3 into routine bioindustrial processes

In more detail – the actual work packages and the associated milestones and deliverables in the application were formulated as following:

1.4.2 Work Package structure from the original application

WP 1: Development of alternative dying methods
Activities:
A1.1: Choose model biomass (wheat straw / sugarcane bagasse / wood chips)
A1.2: Use different dyes for dying biomass
A1.3: Use different conditions for dying biomass
A1.4: Monitor the effects of dying using HPLC-MS to validate the most effective approach

Milestones:
M1: Developed accurate QC protocol using HPLC-MS (and potentially FTIR) to reliably quantify effects of biomass dying
M2: Implemented QC protocols for evaluation and identification of the best and most effective method of ICB-substrate production

WP 2: Evaluation of ICB substrates developed in WP1 in an actual bio-industrial setting
Activities:

A2.1: Set up controlled and closely monitored bioconversion process in a bio-industrial setting with ICB substrates made from the biomass used in the bioconversion process

A2.2: Monitor all aspects of the bio-industrial process such as pretreatment methods, process conditions in the bioreactor, saccharification degree in the bioreactor and the final yield of the biofuel

A2.3: Analyze and compare the results of lab ICB-substrate analyses and the results of the analyses of bio-industrial processing

A2.4: Calculate environmental and commercial impact of implementation of ICB substrate assays.

Milestones:
M3: Established firm relationship between the results of lab ICB-substrate analyses, the results of bio-industrial processing of the biomass and the biofuel yield.
CM1: Customer business case supports commercial and environmental potential

WP 3: Extrapolation of ICB-substrates to other types of biomass of highest commercial interest

Activities:
A3.1: Identify and collect up to 6 most relevant biomass types for standardized / customized ICB substrates through dialogue with main stakeholders
A3.2: Prepare biomass substrates according to protocols developed in WP 1
A3.3: Monitor effects of dying to ensure that representation of the structure, consistency and properties the new ICB substrates is still reliable

A3.4: Possibly revision of QC protocols for optimization of process across multiple biomass types

Milestones:
M4: Preparation of 3 substrates of the commercially most relevant biomass types
M5: Identified best dying method for true-to-nature ICB substrates that accurately represent the structure, consistency and properties of natural biomass

WP 4: Implementation of ICB substrates developed in WP1 and WP3 into routine bioindustrial processes

Activities:
A4.1: Establish and test a feedback loop consisting of

1. Biomass selection by industrial partner
2. ICB-substrate production by GlycoSpot
3. ICB-substrate incorporation into the desired layout
4. ICB-substrate sent back to industrial partner

A4.2: Identify the most practical format for use of ICB-substrate which may include existing layouts in 96-well filter plates / filter tubes / microfluidic / lateral flow / or similar devices

A4.3: Prepare for operational semi-automatic production of selected kit types, fully integrated with shipping/handling and inventory management

Milestones:
M6: Best format(s) identified
CM2: Feedback loop successfully tested
CM3: Production plan for large scale production ready

WP 5: Communication

Activities:
A5.1: Participation in conferences and fairs
A5.2: Publications in professional and scientific media
A5.3: Presence in professional networks

Milestones:
M7: Participation in selected fairs and conferences
M8: Scientific article
M9: Technical article
M10: Social media strategy and presence in professional networks

WP 6: Project management
Activities:
A6.1: Overall control, coordination, and administration of the project
A6.2: Periodic and final reporting
A6.3: Establishment of collaboration with experienced 2G bio-ethanol developer
A6.4: Communication with partners and stakeholders
A6.5: Handling of legal issues (IPR, MTAs, NDAs, contracts etc.)

Milestones:
M6.1: Detailed work plan for full project
M6.2: Periodic and final reports
M6.3: Agreement of collaboration with experienced 2G bio-ethanol developer in place
M6.4: Patent applications and / or other IPR protection

1.4.3 Work package changes during the project

We encountered a setback in the project in Q1 of 2018. Our industrial partner, Granbio, failed to provide all of the samples and data that we required to complete our proposed study.
Granbio terminated their research-scale activities and outsourced some of their analytical activities to other companies. In January 2018, we expected to receive all of the raw biomass substrates with their respective pretreated variants, together with analytical data for liquid fractions and measurements of bioethanol yields for all of the samples. After consultation with the Granbio representative, we were informed that we can only receive raw material samples without the possibility of obtaining any accompanying data or materials (including pretreated materials, liquid fraction analysis data and bioethanol yield data). As indicated by Granbio, there was a chance to have Granbio pay for the pretreatment and analysis in their external laboratory, however – this possibility was ruled out by an executive decision from Granbio in the end of March 2018. In April 2018, Granbio agreed to send some of the raw materials and one single sample of pretreated energy cane in addition to some raw sugar cane and energy cane samples. Although this situation was not ideal, we accepted their offer and finally received all of these materials in the end of June 2018.
Because of the change of data and materials available to us, we had to change our research plan in order to fulfill the project success criteria.

The updates to the specifications in the original work package plan are the following:

WP1 is completed and milestones M1 and M2 have been reached.

WP2 cannot be completed because of the lack of support from Granbio (in terms of required materials and data) making milestone M3 impossible to meet. However, in our opinion, this still has no influence on the commercial milestone CM1, which we aim to reach after completion of the study and the EUDP-funded project and establish a relevant commercial and environmental potential through novel technologies developed during the project but with commercial endeavors funded by GlycoSpot.

WP3 is completed, after the samples had been designated to be sugar cane straw, sugar cane bagasse, energy cane straw, energy cane bagasse, wheat straw and pretreated energy cane (straw and bagasse). The major change is the switch from comparing variance between exclusively South American 2G bioethanol feedstocks to comparing South American (sugar cane, energy cane) and European (wheat straw) feedstocks – which is equally relevant for

the study, and will hopefully help us market the new TTN-CBS technology in additional markets.
The samples were processed, homogenized and depigmented. Subsequently, fluorescent and chromogenic variants of these substrates were synthesized using the already existing method for chromogenic substrates and the novel method developed within this project for fluorescent substrates. This marked the completion of M4 and M5.

WP4 has changed as we do not anticipate actual use of our newly developed technology by Granbio as a test partner, as we had originally planned. However, Granbio is still very interested in the data that we will produce as a result of our updated study plan. Therefore, we had to unilaterally decide to implement our substrates into our tried and tested 96-well filter plate format with a modification of using opaque supernatant receiver plates (to minimize optical interference during fluorescence measurements). This marked the completion of M6 (November 2018). However, commercial milestones CM2 and CM3 are anticipated to have limited impact.

WP5 is underway with the project being presented at the EUBCE 2018 conference held in May in Copenhagen with an oral presentation, commercial booth at the conference and a technical article submitted to EUBCE that was indexed by SCOPUS and published in July 2018 (DOI: 10.5071/26thEUBCE2018-3CO.11.1) and marked completion of M9. We are currently in the stage of drafting a manuscript based on our results that will constitute a scientific publication that we intend to submit before the end of Q2 2019 (M8).
M10 is completed.

In summary, all milestones and commercial milestones are met or will be met by the end of Q2 2019, with the exception of M3, which cannot be met as initially planned due to a strategic restructuring of activities at one of our partners, Granbio. However, GlycoSpot will continue to pursue the goals detailed as part of M3 with industries other than Granbio after the project's funding period.

1.5 Project results and dissemination of results

1.5.1 Main results outline

1. **<u>SYNTHETIC PROCEDURE DESIGNED FOR PRESERVATION OF NATURALLY OCCURRING ESTERS ON POLYSACCHARIDES IN BIOMASS FEEDSTOCKS</u>**
    a. exploration of synthetic approaches
    b. preservation of acetyl groups using the selected synthetic approach
        i. polysaccharide models
            1. polysaccharide model (dextran) – synthesis proof-of-concept
            2. acetylated polysaccharide model (xylan from birch) – ester preservation proof-of-concept
        ii. biomass models (with focus on xylan degradation profiles tracked by MS, LC)
            1. wheat straw
            2. pretreated sugarcane bagasse
            3. sugarcane bagasse
            4. energy cane bagasse
            5. sugar cane straw
            6. energy cane straw

2. **<u>COMPARISON WITH CHROMOGENIC SUBSTRATES</u>**
    a. MS data showing absence of esters in chromogenic substrates
3. **<u>ENZYME SCREENING WITH PANEL OF COMMERCIAL ENZYMES AND ENZYME COCKTAILS</u>**
    a. comparison of profiles of all feedstocks in terms of chromogenic vs. fluorogenic substrates
        i. comparison of profiles of all feedstocks in terms of retained esters in fluorogenic substrates (performance with xylanases chromogenic vs. fluorogenic, what does it mean etc.)

b. comparison of wheat straw vs sugar cane vs energy cane (comparison of European and South American bioenergy feedstocks).

1.5.2 Detailed results and discussion

1.5.2.1 Synthetic procedure designed for preservation of naturally occurring esters on polysaccharides in biomass feedstocks

In addition to existing monochlorotriazine dyes previously used, but now under different conditions (organic solvents with base catalysis), new dyes have been employed, namely fluorescein thiosemicarbazide (FTSC) and fluorescein isothiocyanate (FITC). An additional dye, namely *N*-methylisatoic anhydride, was also under consideration but has not been used.

Dyeing conditions have been changed to organic solvents with base catalysis to avoid hydrolysis of esters and other base-labile naturally-occurring substituents. An aqueous reaction environment has been employed in the case of FTSC where coupling was attempted through controlled periodate oxidation, Schiff-base formation and reduction.

Before using the model biomass, dyeing methods were evaluated using model polysaccharides. Efficiency of dyeing with the aforementioned dyes was tracked by controlled enzymatic degradation and analysis using MALDI-TOF-mass spectrometry conducted at the facilities of the partner NMBU. The MS spectra confirm that dyeing is successful (with FITC).

Analysis of products using size-exclusion chromatography (SEC) and HPLC has also been conducted. NMBU has also produced a large amount of acetylated xylan from birch, which is used as an exemplary model substrate due to the large amount of naturally-occurring acetyl groups.

Initial experiments focused on model polysaccharides such as dextran, pectic galactan, konjac glucomannan and acetylated xylan from birch (the latter kindly provided by NMBU, Norway). A time-lapse experiment was performed with labeled dextran (TTN-PBS dextran) over the course of 50 minutes where a sample was taken every 10 minutes and deposited onto a thin-layer chromatography (TLC) plate. The plate was then developed with an eluent to separate the labeled oligosaccharide spots and illuminated with a long-wave UV lamp for visualization. The reaction mixtures as well as unmodified substrates have been subjected to MALDI-ToF-MS and size-exclusion chromatography (SEC) analysis (performed at NMBU, Norway). The results of the TLC analysis are shown in **Figure 1**.

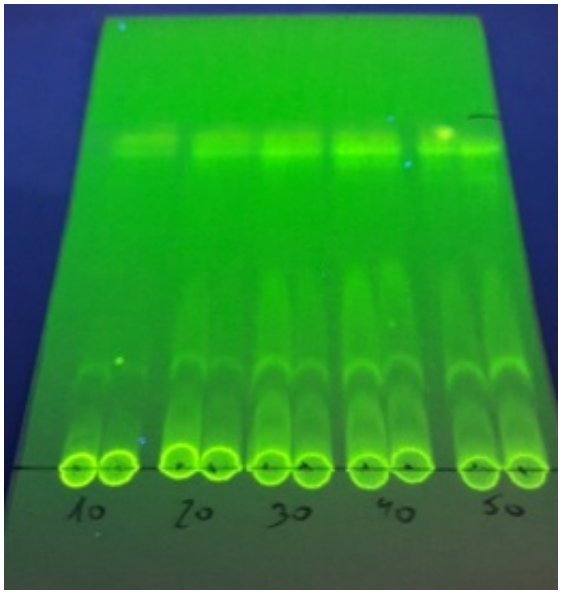

Figure 1. *TLC analysis of a time-course experiment with degradation of TTN-PBS dextran with dextranase of the course of 50 minutes with a duplicate of each 10-minute interval sample spotted. The plate was visualized with a long-wave UV lamp. Note the noticeable increase in more mobile fluorescent oligosaccharide fragments over time, especially in the first 30 minutes of the experiment.*

As a proof-of-concept for the feasibility of the novel synthetic method as applied to biomass samples, we have produced TTN-CBS substrates from wheat straw samples that were then degraded with a commercially available cellulase and xylanase.

The substrates have been incorporated in our already tried and tested 96-well filter plate format as previously published (Kračun and Schückel *et al,* 2015, Biotechnology for Biofuels20158:70).
The observed trend in degradation was similar for both the CBS and the TTN-CBS sample, however, some interesting differences were observed as shown in **Figure 2**.

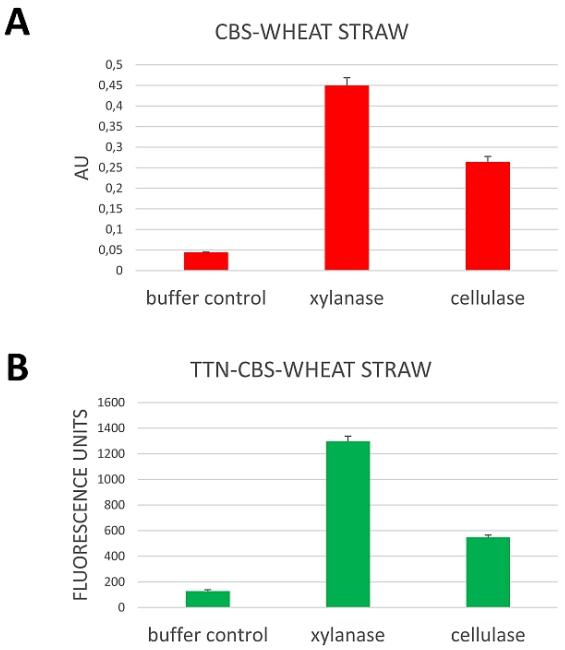

Figure 2. *Proof-of-concept comparison of degradation profiles of A) standard chromogenic CBS-wheat straw, produced using existing protocols and B) fluorogenic TTN-CBS-wheat straw produced using the new protocols developed in this project. The degradation of CBS- and TTN-CBS-wheat straw was measured in 96-well plates by measuring absorbance or fluorescence of the filtrate after the reaction, respectively. The buffer control values are normally subtracted from the overall signals as part of standard analysis.*

Based on this study we can increase the predictability of bioethanol yield from raw materials directly and support the implementation of GlycoSpot technology in the routine bioethanol refinery production process.

1.5.2.2 Enzyme screening with panel of commercial enzymes and enzyme cocktails

Fluorogenic as well as chromogenic substrates were synthesized from the available feedstocks obtained from Granbio and the University of Copenhagen:

1. wheat straw (KU)
2. pretreated sugarcane bagasse (GranBio)
3. sugarcane bagasse (GranBio)
4. energy cane bagasse (GranBio)
5. sugar cane straw (GranBio)
6. energy cane straw (GranBio)

The substrates were incorporated into the GlycoSpot's high-throughput 96-well filter plate format and screened with a panel of commercial enzymes with activities against cellulose and hemicelluloses, with a special focus on xylanases, and including one targeting starch. Enzyme cocktails such as Driselase and Macerase were included as well as commercial cocktails tailored for the biorefining industry (CellTec 2 and NS22246 from Novozymes).
Half of the plates were treated with the degrading enzymes directly and the other half were pre-treated (in plates) with the xylan deacetylase (X-DA in later text) prior to treatment with degrading enzymes. This study was designed to explore the impact of enzymatic deacetylation of xylan on its subsequent enzymatic degradation within fluorogenic substrates themselves. This effect should also be apparent by comparing chromogenic (no-ester, due to the harsh dyeing conditions) and fluorogenic substrates (where the conditions are milder, preserving the esters).

A difference in degradation profiles of chromogenic versus fluorogenic substrates is clear from **Figure 3** and **Figure 4**.

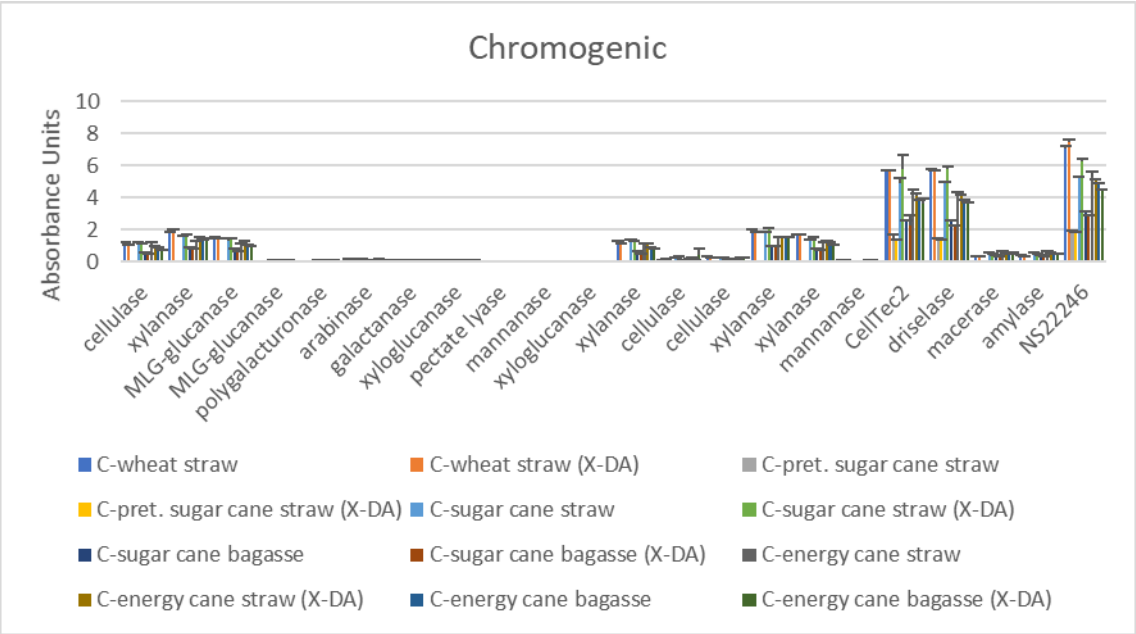

Figure 3. *Results of enzyme profiling of chromogenic substrates produced from the 6 different feedstocks.*

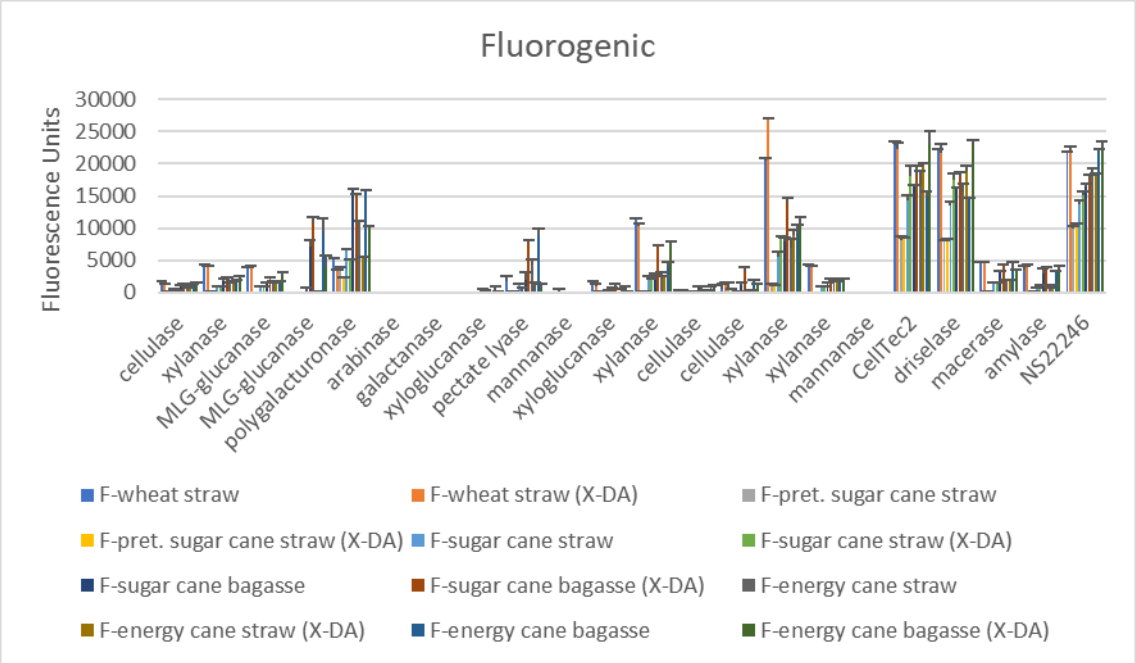

Figure 4. *Results of enzyme profiling of fluorogenic substrates produced from the 6 different feedstocks.*

Additionally, actual supernatants from the reaction were used to establish that acetyl groups were in fact preserved in fluorogenic substrates.

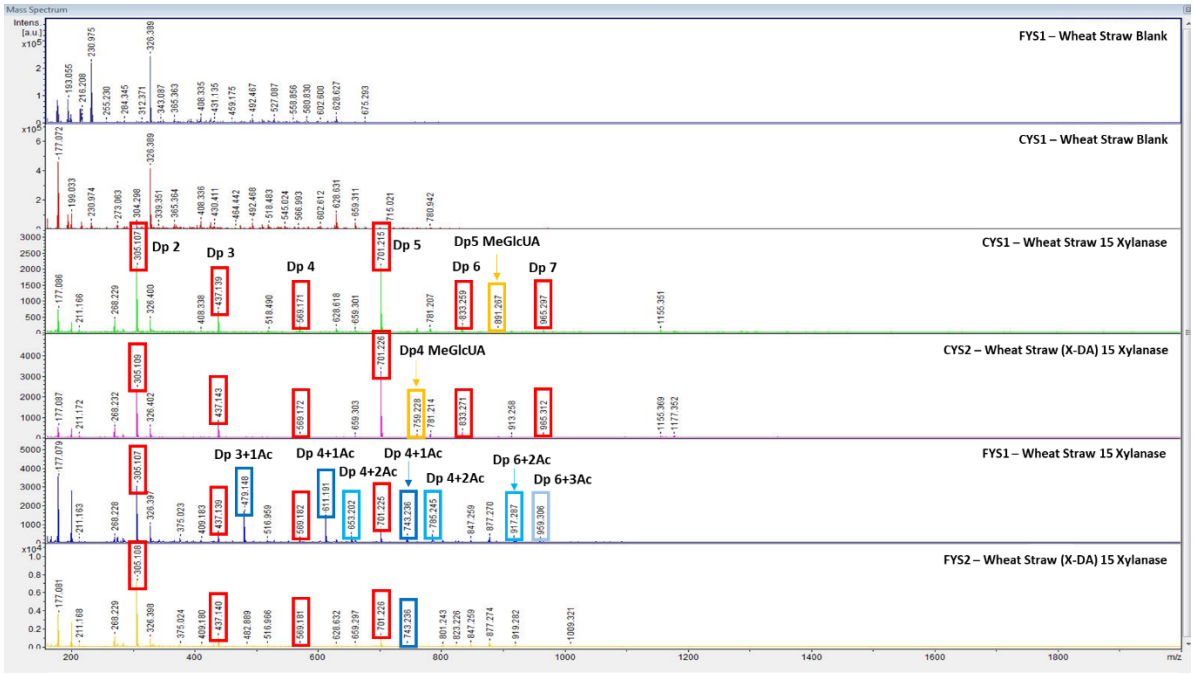

Figure 5. *Mass spectrometry spectra of supernatants from enzyme screening. Spectra of wheat straw in chromogenic and fluorogenic form. Non-acetylated peaks in red, acetylated peaks in blue (Dp = degree of polymerization, Ac = acetyl).*

By using MALDI-TOF-MS mass spectrometry, as shown in **Figure 5**, there is a complete absence of any ester groups in chromogenic samples (panels 3 and 4 from top) as expected because of the highly alkaline conditions used for synthesis. With regard to fluorogenic substrates – not only are acetyl groups present (panel 5 from top) but there is also a clear difference between samples that were treated and were not treated with xylan-deacetylase (X-DA, panel 6 from top).

1.6 Utilization of project results

During the project the market has shifted and where we 2 – 3 years ago saw a profitable market in Europe and South America, these markets are struggling and not open to new investments. However, India has developed new technologies and are looking in to the biorefining area with the help of the large enzyme manufactures. We are currently looking for a business partner to open the potential in India, and the plan is to have a concept ready for 2020 with a screening kit there can be used by regular staff in the plant directly on the production floor without the need for additional laboratory equipment.

We have chosen not to claim any new IP now, as the core technology is already protected by our current patent. When the concept is ready, we will reevaluate the situation together with our Patent attorney.

During the project we have had discussions with Ørsted regarding the REnescience project (municipal waste to energy program), but it has not been easy to get real samples from real working reactors, do to delay in the REnescience project. We have worked on model waist and demonstrated the benefit of measuring activities, and we will explore this opportunity further.

We will see how we can use the new technology in other applications we are working on for the food, feed and detergent area. It is still to early to project any spin off from the project, but it seems to be multiple entry points to in other markets there need to be explored further.

1.7 Project conclusion and perspective

The most important conclusions from the EUDP project are:

- GlycoSpot now has technologies for labeling biomass that is much milder than existing methods
- This means the TTN-CBS biomass substrates are more realistic and thus more predictive of conditions used in large-scale production of biomass.
- GlycoSpot expect that these new products will enter the market in 2020 and that the new product line has the potential to increase company revenue by at least 30%.
- The results also show that treatment conditions are extremely important for the analysis of the data and underlines that GlycoSpot are market leaders in true-to-life biomass substrates. The technical dissemination of these finding should underpin this position and will be a future tool in marketing of our products.

Overall, the EUDP project has been very successful and the results obtained have actually outperformed our expectations.

1.8 Annex

1.8.1 Relevant links

1. Technical Article

Kracun, SK, "Development of Chromogenic True-to-Nature Biomass Substrates for Bioconversion Process Optimization", **2018**, Conference: European Biomass Conference and Exhibition (EUBCE) 2018At: Copenhagen, Denmark, 979 – 983 (http://www.etaflorence.it/proceedings/?detail=14956)