Final report

1.1 Project details

Project title	Demonstration of 2G ethanol production in full scale
Project identification (program abbrev. and file)	EUDP 2015II- J.nr. 64015-0642
Name of the programme which has funded the project	EUDP
Project managing company/institution (name and address)	MEC BioHeat&Power A/S Nupark 51, 7500 Holstebro
Project partners	KU – University of Copenhagen DTU – Technological University of Denmark Novozymes A/S
CVR (central business register)	25495977
Date for submission	30 September 2019

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1.2 Short description of project objective and results

English version

The perspective of the project according to the revised project description fra 12 January 2017 is to support the creation of a Danish full scale lignocellulosic biorefinery large enough to fulfil the EU requirements in The Renewable Energy Directive established mandatory targets for a 10% share for renewable energy in the transport sector by 2020 with a recommendation of 0.5% of the 10% is advanced biofuels as 2G ethanol. From 2021 to 2030 the share of advanced biofuels including 2G ethanol is to be gradually increased to 3,6 %. The project perspective is rooted in the fact that lignocellulosic ethanol is the most obvious choice of an advanced biofuel to fulfil the new EU directive given the well-developed state of the technology compared to other new technologies of advanced transport fuels. Furthermore biorefineries where biomass is fractionated in sugars, lignin and other components, can also be used in other productions than ethanol. Lignocellulosic sugars could for example be used for fermentation of lactic acid or other green chemicals.

Since advanced biofuels must be produced from residual biomass including lignocellulose, the perspective of the project is important. Therefore the main objectives of the project are:

- To explore the potential of alternative biomasses
- More efficient use of enzymes
- Improved yeast strands
- Build up generic knowledge on the subjects

The main results of the project are:

- A large variety of alternative biomasses have been screened and tested in pilot scale for the ethanol potential and potential for other energy production uses and here especially biogas
- Monitoring strategies and methods for more efficient use of enzymes have been explored
- Improved C5 yeast strain able to convert arabinose has been developed
- Generic knowledge on validated method for measurement of enzyme activity and the in line method for measuring feedstock productivity have been developed and compiled

Dansk version

Projektets perspektiv i henhold til det reviderede projektbeskrivelse fra 12 januar 2017 er at støtte skabelsen af en dansk fuld skala lignocellolostikt bioraffinaderi, der stort nok til at opfylde EU's krav i direktivet om vedvarende energi, hvor der er fastsat obligatoriske mål for en andel på 10 % for vedvarende energi i transportsektoren i 2020 med en anbefaling på 0,5% af de 10% er avancerede biobrændstoffer som 2G ethanol. Fra 2021 til 2030 skal andelen af avancerede biobrændstoffer, herunder 2G-ethanol, gradvist øges til 3,6 %. Projektets perspektiv er forankret i det faktum, at lignocelluloseholdigt ethanol er det mest oplagte valg af et avanceret biobrændstof til at opfylde det nye EU-direktiv i betragtning af teknologiens vel-

udviklede teknologi sammenlignet med andre nye teknologier inden for avancerede transportbrændstoffer. Desuden kan bioraffinaderier, hvor biomasse er fraktioneres i sukkerarter, lignin og andre komponenter, også anvendes i andre produktioner end ethanol. Lignocellulostisk sukker kan for eksempel anvendes til gæring af mælkesyre eller andre grønne kemikalier.

Da avancerede biobrændstoffer skal fremstilles af rest biomasse, herunder lignocellulose, er projektets perspektiv vigtigt. Projektets hovedformål er derfor:

- At undersøge potentialet for alternative biomasse
- Mere effektiv anvendelse af enzymer
- Forbedrede gærtråde
- Opbygge generisk viden om emnerne

Projektets hovedresultater er:

- En lang række alternative biomasser er blevet screenet og testet i pilotskala for ethanol potentialet og potentialet for andre anvendelser i energiproduktion og her især biogas
- Overvågningsstrategier og metoder til mere effektiv anvendelse af enzymer er blevet udforsket
- Forbedret C5 gær stamme i stand til at konvertere Arabinose er blevet udviklet
- Generisk viden om valideret metode til måling af enzymaktivitet og linje metode til måling af råvare produktivitet er blevet udviklet og kompileret

1.3 Executive summary

The project was after revision in January 2017 organised in 3 work packages (WP) around three main themes:

Biomass/Pretreatment – Screening and test of local biomasses

WP1:

Wheat straw has a high cost in Denmark. Alternative biomasses already on-site at the MEC biogas plant could be good options to reduce the biomass cost. Some of these reduced cost feedstock options include fibres from digestate, which are today transported for hundreds of kilometres before spreading on farmland, deep litter that creates operational problems in the biogas plant, and whey permeate. In this WP, alternative biomasses from the area of Måbjerg sourced by MEC BHP are analysed and screened and tested by KU for ethanol potential. KU will analyse the composition of the alternate biomasses prior and after pretreatment. The purpose is to elucidate optimisation potential as well as potential inhibitors. For the inhibitors, the alternative biomasses will be studied separately and in combinations. Most probably there will be a need for a washing step for both digestate fibres and deep litter to reduce ash content before using the cheap biomasses in the process. MEC BHP will develop and design equipment for the washing process. WP1 will also look in to optimize biogas production, when vinasses and other residues from the etha-

nol plant are mixed with manure and from the water from the washing of digestate fibres and deep litter.

Results and utilization

A large variety of alternative biomasses have been screened and tested in pilot scale for the ethanol potential and potential for other energy production uses and here especially biogas.

Tests both of pilot and laboratory scale showed that straw from barley, rye and grass in the standard pre-treatment are high-performance alternatives to wheat straw, both in single (C6) and in mixed fermentation (C5 + C6) scenarios. The result on rye straw is new as it was not part of the studies carried out by the MEC consortium in 2013 and the high level of performance shows a potential for expanding the field area. All in all, the tests indicate that the traditional commodity base of 2G ethanol production wheat straw can be expanded markedly, especially with other straw types.

Yeast – Strain developments, propagation and fermentation strategy development for advanced yeast, and development of methods for monitoring yeast health during propagation and fermentation

WP3:

Strain optimization: The commercial advanced yeast available today have potential for further improvement in several ways, such as improved efficiency (>2%) and speed (~20%) of conversion of glucose and xylose. In this WP, screening of yeast strains derived from current commercial C5 yeast will be done on first model and then later real 2G hydrolysates while selecting for improved sugar utilization rate (20%), increase in tolerance to acetic acid (10%), and robustness towards lower pH.

Propagation strategy (DTU) and fermentation strategy developments for advanced yeast: Advanced C6/C5 yeast is more expensive than regular C6 yeast used in 1G bioethanol production and more sensitive to choice of carbon source and propagation conditions. Accordingly, this WP will include a conceptual design of yeast propagation strategy as well as testing of the propagation efficiency dependency on various grades of biomass hydrolysate and other process-relevant parameters such as type of carbon source (in MEC the obvious choice is beet molasses), nutrient supplementation and aeration level. The fermentation step can also be further optimized, by controlling the inhibitor pressure in the start of fed batch fermentation, and optimization of the feed profile. The optimized propagation strategy and fed batch fermentation will be tested in pilot scale.

Development of methods for monitoring yeast health during propagation and fermentation (DTU): The project will also include the development of a tool for qualifying and validating the health (e.g. viability, vitality and contamination degree) of the propagated yeast for supporting fast start-up, and for trouble-shooting fermentations.

Results and utilization

Improved C5 yeast strain able to convert arabinose has been developed in order to increase the ethanol yield. During the project period, yeast strings were generated that could utilize C5 sugars arabinosis and xylose under airobic conditions. At the

same time, heterological transporters were identified that allow the exploitation of arabinosis in small concentrations.

At the same time more efficient use of enzymes have been developed and tested on order to overcome inhibitors for yeast use for the production of 2G ethanol. This will contribute to greater efficiency in the fermentation process and higher ethanol yield.

Enzyme assay - Development of assays for determination of enzyme activity and feedstock processivity

WP4:

The enzymatic hydrolysis yield is the key parameter for an optimal ethanol yield, why precise and correct determination of the enzyme activity is of high importance. During the last 10 years large enzyme suppliers as Novozymes and DuPont have developed and introduced more and more advanced enzyme formulations for 2G ethanol processes. Today advanced yeasts fermenting C5 and C6 sugars are commercial available, which means that ethanol yield depends on conversion of both cellulose and hemicellulose. There is a need for a standardized enzyme activity assay, which can accurately predict the ability of a given enzyme batch to convert all carbohydrates from pretreated biomass into monomeric sugars. Likewise, there is a variation in the biomass supply that affects the biomass convertibility and which therefore needs to be adjusted for. The variation originates from crop varieties, storage and handling as well as growth factors e.g. precipitation and fertilizers. This variation needs to be countered by adjustment of the processing conditions e.g. pretreatment time or enzyme dosage. For this purpose, a very fast assay for in-line measurement of the biomass processivity is needed. In this project, KU - in close collaboration with the remaining project partners - will develop and validate two methods to be applied at the large-scale production of enzymes and 2G ethanol. 1) Determination of total enzyme activity for C5 and C6 hydrolysis. 2) In-line measurement of pretreated biomass processivity.

Results and utilization

A quick enzyme assay consisting of an automated powder dispensing system to prepare 96-well plates for hydrolysis, combined with a powerful colorimetric assay to analyze released sugars was successfully developed. Although, in the current state, the method meets the timely requirement, it fails to meet the requirement of reliability. In order to improve reliability and enhance utilization the next step would be to increase the working volume to 1ml, since volume was considered the biggest culprit of deviation.

1.4 Project objectives

In January 2017 the project was after a dialogue and with the approval of the EUDP administration and board revised due to a number of events. First, it proved not possible to realise the full scale MEC Biorefinery within the expected time frame. That meant that the original WP2 Improve the hydrolysis yield by control of oxygen tension and tailor made enzymes had to be abandoned. At the same time the tests of alternative biomasses under WP1 foreseen to be conducted at the MEC ethanol plant had to be relocated to KU regarding the lab tests and as it was later decided to Lund University as regards the pilot tests.

Secondly, in November 2016 Orsted (at that time DONG) decided to close down its 2G ethanol activities. The effect of this was that the other partners took over DONG's tasks in the project and that the project management was handed over from DONG to MEC BHP.

After the revision the project has developed according to plan. Only minor obstacles has been encountered and *all milestones and objectives have been reached as it appears from the enclosed Gantt diagram*.

1.5 Project results and dissemination of results

This section gives an overview of the main activities and results of the project. For a schematic overview please refer to the enclosed *Gantt diagram*. For more information on project details please refer to the enclosed *project documents* which in the annex has been grouped according to milestone and work package.

Biomass/Pretreatment – Screening and test of local biomasses (WP1):

The work has been concentrated on collecting different types of alternative local biomass and pre-processing the collected local samples for use in the project's testing of biomass yields and general applicability in ethanol production and other energy production.

The conclusion of the laboratory tests was that barley straw and seedgrass straw have a higher sugar content than wheat straw. In addition, high yields of sugar have been found in RF ryegrass fibers after experiments with protein extraction. The experiment with protein extraction is taking place at Foulum, Aarhus University and is not part of this project.The fiber fraction from MEC-BioGas after decaying, on the other hand, has a very low sugar content, which makes it unsuitable for ethanol production, but the energy use is likely to be used as fuel in cogeneration. Work is being done, among other things. making biofiber a stock-stable product that can then be stored and used for bio-fertilizers.

Similarly, the tests have shown that straw from deep litter is unsuitable for ethanol production due to its low sugar content. Therefore, there was no need in the project to design a special "washing machine" for washing the slurry from the straw.

The laboratory tests have been supplemented by pilot-scale pre-treatment as planned. In the original project, the pilot test was to have taken place at the ethanol factory in Maabjerg, but when this was not realized, they were instead carried out at Lund University.

The pilot tests were conducted for the alternative biomasses for wheat straw, which had performed best in the laboratory tests: barley straw, hatching straw and grass straw.The pilot tests confirmed the results of the laboratory tests, namely that straw from barley, rye and grass in the standard pretreatment are high-yielding alternatives to wheat straw in both single (C6) and mixed fermentation (C5 + C6) scenarios.

The result regarding the rye grass straw is new, as rye grass straw was not included in the studies conducted by the MEC consortium in 2013, and the high level of performance shows a potential for expansion of the field area. All in all, the tests

indicate that the traditional commodity base of 2G ethanol production wheat straw can be expanded markedly, especially with other straw types.

Yeast – Strain developments, propagation and fermentation strategy development for advanced yeast, and development of methods for monitoring yeast health during propagation and fermentation (WP3):

Strain developments, propagation and fermentation strategy development for advanced yeast

Novozymes (NZ) has launched a C5/C6 sugar utilizing yeast, which is used in commercial 2G ethanol processes and is characterized by an already high xylose uptake rate, fermentation speed and high acetic acid tolerance. To further improve the economy of ethanol fermentation processes from 2. generation biomasses, it is of high interest to continue the optimization of the currently available C5/C6 sugar fermenting yeasts. Within the EUDP project, NZ therefore set the ambitious target to generate a yeast that was improved compared to the current benchmark NS22202. The traits in scope for optimization was:

- Broadening sugar utilization profile to also include the C5 sugar arabinose, which is present in 2G biomasses, but currently not utilized.
- Increase the tolerance towards the yeast inhibitor acetic acid, which is also present in high amounts in 2G biomass fermentations.

Extensive work was done to develop a yeast with increased tolerance towards the yeast inhibitor acetic acid compared to the current state-of-the art C5/C6 utilizing yeast from NZ. The results showed that NZ were able to generate yeast strains with dramatically improved performance than the benchmark yeast (NS22202) under high acetic-acid concentrations.

In addition to improving acetic acid tolerance, NZ furthermore incorporated an arabinose-utilizing pathway into this yeast background.

After demonstration of arabinose utilization under high arabinose concentrations, the new strains were tested under more challenging conditions reflecting industrial 2G biomass processes with lower arabinose and higher acetic acid concentrations. The results showed that while the new strains were able to utilize arabinose, the trait came at a cost of reduced xylose utilization and lower final ethanol titers.

The results showed that additional work was needed to improve xylose utilization while maintaining arabinose utilization in this strain library.

The xylose-utilizing S. cerevisiae strain NZXYL was used as a new host strain to introduce an arabinose utilization pathway, based on a high-throughput (HTP) screening of various combinations of genes encoding the specific arabinose pathway. Not all genes tested created a functional pathway but certain combinations of genes for the multi-enzyme pathway created a functional arabinose utilization pathway in S. cerevisiae.

In addition to evaluating strains to consumption of xylose or arabinose as the sole carbon source NZ also evaluated the strains for xylose and arabinose consumption when both C5 sugars were present in the media. After establishing aerobic arabinose and xylose utilization NZ next tested the consumption of these C5 sugars in the presence of glucose. To determine whether addition of a sugar transporter specific to C5 sugars could enhance the ability of the strains to completely utilize the available sugars when glucose was present at high levels putative transporters

were introduced. A subset of strains consumed more arabinose with the added transporter, demonstrating heterologous transporter expression may allow for utilization of arabinose even at low concentrations.

Improved C5 yeast strain able to convert arabinose has therefore been developed in order to increase the ethanol yield. During the project period, yeast strings were generated that could utilize C5 sugars arabinosis and xylose under airobic conditions. At the same time, heterological transporters were identified that allow the exploitation of arabinosis in small concentrations.

The results contain confidential and proprietary information that cannot be published without prior permission from Novozymes.

Development of methods for monitoring yeast health during propagation and fermentation

The production of 2G bioethanol is often limited by the inhibitors generated during the pretreatment. The tolerance of yeast to such compounds can be improved by exposing it to controlled concentrations of inhibitors in an adaptation step. In this project, flow cytometry was used to study how different physiological features of yeast change during the adaptation step.

The cell membrane integrity, potential and cytosolic ROS concentration were monitored during 9 fermentations with different concentrations of inhibitors. The results showed how these features are affected due to the inhibitors and how they change as the culture adapts to the media. These experiments also gave valuable insights on how the individual and synergic effect of the inhibitors affect the cell culture.

The objective of the work was to elucidate the individual and combined effects that inhibitors typically found in hydrolysate have on the physiology of Saccharomyces cerevisiae during the course of a fermentation. 3 inhibitors (vanillin, furfural and acetic acid) representing the main inhibitory groups found in 2G feedstocks (phenolic compounds, furan derivates and weak acids respectively). A 23 full factorialdesign with a central point and low and high concentrations of each inhibitor was used for the experimental design. Samples for HPLC, OD600 and flow cytometry were taken every 1.5 hours during the course of the fermentations.

The results showed the dynamics of adaptation and the changes on the targeted physiological features during the different fermentations.

Whilst cells growing in the presence of only vanillin or furfural showed an slightly extended lag phases (of 2 to 3 hours), the cells growing in the presence of acetic acid showed a very extended lag phase of 10 hours. During the lag phase the cell culture experienced a decrease in the membrane potential, and an increase in the concentration of cytosolic ROS, which indicates high metabolic stress in the cells. However, as the fermentation took place, detoxification and adaptation occurred, restoring the initial values for the membrane potential and cytosolic ROS, and resulting in the start of the cell growth. The viability of the cell culture (measured by the membrane integrity) remained close 100% during all the fermentations. When two inhibitors were used simultaneously, two different behaviours were observed.

In the combination vanillin-furfural, an extended lag phase of 5 hours was observed. This lag phase was larger than the one obtained when each inhibitor was used individually (the detoxification took longer time).

As previously observed, the membrane potential and the cytosolic ROS were altered, indicating a state cellular stress. The combinations including acetic acid resulted in a complete inhibition of the cell growth and in persistent high levels of ROS and low membrane potential. Despite the high metabolic stress no loss of viability was detected in these experiments. The combined effect of the three inhibitors resulted in high metabolic stress and in an increasing loss of cell viability.

Phenolic compounds and furan derivates inhibit the glycolysis at different steps, limiting the production of ATP and NADH+H. Without these compounds the cells cannot grow and struggle to survive (showing high cellular stress). However, if the concentration of inhibitors is not high enough, the cells are able to detoxify them and initiate a normal growth phase.

Acetic acid has an inhibitory effect closely related to the pH of the media. When the pH outside the media is below the pKa of acetic acid, it moves the equilibria towards the protonated form. The protonated acetic acid can freely penetrate the cell membrane and enter the cell, where it finds a pH higher to its pKa. In consequence it gets deprotonated and the internal pH of the cells decreases, causing a situation of cellular stress. In order to keep the internal pH stable, the cells actively pump out the protons using the ATP generated in the glycolysis. When the glycolysis is completely inhibited (either due to high concentrations of one inhibitor or to a combination of two inhibitors) the availability of ATP becomes very limited, hindering the cell to maintain the internal pH and causing loss of viability.

The dynamics of physiological change and adaptation of yeast to different inhibitors, and the mechanisms of inhibition have been studied in this project. The knowledge gained through this project will be used to optimize the propagation step in order to develop robust cell cultures able to increase the yield and productivity of 2G bioethanol processes.

Enzyme assay - Development of assays for determination of enzyme activity and feedstock processivity (WP4):

This work package was made as part of "Demonstration of 2G ethanol production in full scale, MEC". A key parameter to high ethanol yield is enzymatic hydrolysis of the feedstock. Due to high enzyme cost, the optimal enzyme dosage is essential for an economically viable 2G ethanol production. The MEC 2G biorefinery will mainly use local feedstock (mostly straw), and should be able to deal with changing feedstocks depending on availability. An optimal enzyme dosage based on the specific feedstock is required to minimize cost while maintaining a high ethanol yield. Knowledge on the activity and performance of enzyme mixes are thus essential. Enzyme performance of cellulolytic enzyme mixes are commonly defined by the filter paper based FPU. Besides having known reproducibility issues, the FPU assay does not cover the full activity spectrum of modern enzyme mixes. This part of the project was focusing on development of a quick and reliable enzyme activity assay to detect several relevant enzyme activities such as xylanase, endoglucanase, beta-glucosidase activity as well as an overall cellulolytic activity towards different cellulosic materials. The goal is to be able to perform the assay within 2 normal working days with standard deviations less than 15 %.

In order for this assay to be successful, the most abundant biomass constituents must be included in the assay. Therefore, the most abundant hemicelluloses types is in focus, that being firstly xylan with all substitution (acetylated-glucurono-arabino-xylan), and secondly mixed linkage glucan – a hemmicellulosic glucan type that contain both β-1-4 and β-1-3 linkages.

Eight cellulolytic enzyme mixes (E1-8) from Novozymes were selected to represent a range of enzyme activities and performances. The protein concentrations of the enzyme mixes were analyzed on a FLASH 2000 HT elemental analyzer (Thermo Scientific). Aliquots of 200 µl of each enzyme mix was oven dried (40oC) overnight before analysis. All samples were made in duplicates. Pine and maple samples with known C/N content were used as instrument standards. Nitrogen content was converted into protein with a conversion factor of 6.25, presuming the average nitrogen content of proteins are 16% and all nitrogen are bound to amino acids.

The cellulolytic activity of enzyme mixes were tested by the filter paper unit assay performed in microtiter plates. The filter paper unit in microtiter plates is defined based on the amount of enzyme required to release 80µg glucose from a piece of Whatman no. 1 filter paper during 1 hour of hydrolysis. Released sugars was measured with DNS reducing sugar assay.

Enzyme concentrations and glucose release were plotted, and a straight line between two enzyme concentrations that released less and more than 80 µg, respectively, defined the exact enzyme concentration used to calculate the specific FPU. Suitable enzyme concentrations were found through trial and error.

In total 3 plates were made which included 4 dilutions (D1-4) per enzyme (E1-8), a blank (without DNS) for each dilution as well as a glucose standard (0.16 – 10 g l-1) and a water blank. Substrates for enzyme hydrolysis was the acquired

Wheat straw was hydrothermally pretreatet and together with Carboxymethyl cellulose and Sigmacell cellulose type 101 transferred into microtiter wells using an automated powder dispensing system. The substrate feeding was done with the automated feeding robot "Marvin".

Enzymatic hydrolysis was done in 96 well microtiter plates. Enzymes were immediately inactivated by adding 10 µl of 1.5 M NaOH after hydrolysis. Inactivation was followed by 10 min centrifugation at 4000 G. Hydrolyzed pretreated wheat straw were filtered through a filter plate gravimetrically for 30 min at 4000 G.

A plate design was made to assess the reproducibility of the enzyme assay containing 2 types of substrates and the 8 above-mentioned enzyme mixes. A plate reference was included in all plates used for hydrolysis in order to compare the results between plates.

The PASC hydrolysis showed much lower sugar yields than expected (more than 5 times lower). The production of PASC involves swelling and some depolymerization of cellulose, which also releases some oligomers. The PASC was not washed and gave high background signal, which could explain part of the apparent low sugar yield.

The amount of released sugars during hydrolysis was analyzed using p-hydroxybenzoic acid hydrazide (PAHBAH) and HPLC to compare the results.

The standard sugar concentrations were used to predict sugar samples with known concentrations (figure 3.6). Sugar concentrations between 0.13 mM and 0.5 mM were predicted with an accuracy of 92-105% with very low standard deviations. The PAHBAH assay was further reviewed by comparing released sugars from 24H hydrolysis of Sigmacell type 101, quantified by HPLC and PAHBAH. Since the hydrolysis was run for 24 hours, there should be close to none soluble oligomers left which would only be detected by PAHBAH. Despite the long hydrolysis time the PAHBAH assay predicted slightly higher sugar concentrations compared to HPLC.

The largest difference (and the only example of not-overlapping standard deviations) was observed for enzyme mix E8 where the PAHBAH assay on average predicted 36% higher sugar concentrations compared to HPLC. The advantage of using PAHBAH compared to HPLC is analysis speed of multiple samples whereas the advantage of HPLC is precision. Analysis time for HPLC was 13 min per sample that amounts to a total analysis time of 6 hours including necessary blanks to analyze 27 samples. In comparison, it takes ~30 min to prepare and analyze a 96 well plate with PAHBAH reagent. Even though PAHBAH did not have the same reactivity towards all tested monosaccharides and demonstrated mediocre accuracy, PAHBAH was the preferred reducing sugar assay due to its fast, non-toxic and easy to handle procedure.

Enzyme hydrolysis followed by sugar quantification of 5 different substrates were done to assess the reliability of the new enzyme assay.

A quick enzyme assay consisting of an automated powder dispensing system to prepare 96-well plates for hydrolysis, combined with a powerful colorimetric assay to analyze released sugars was successfully developed. Although, in the current state, the method meets the timely requirement, it fails to meet the requirement of reliability.

In summary the project succeeded in realising its objectives and gave answers and produced deliverables to the problems stated in the original project proposal which the funding has been based on.

The project results are expected to generate increased turnover, exports, employment in the future as the 2G ethanol technology is deployed.

The project results has been disseminated through four scientific articles and a number of conferences and meetings especially SBFC. Document references are:

"Zhang_et_al-The multi-feedstock biorefin-ery_2018-GCB_Bioenergy";
"2017_04_Pau Cabaneros Lopez_review online monitoring fermentation processes";" Real time monitoring alternatives"; "Gong_Effects of preheating on briquetting and subsequent_2019"; "SBFC presentation 2018_Sune Thomsen_Final" and "SBFC poster 2017 sune_2".

1.6 Utilization of project results

The project results will be used both commercially and as a base for further research and development. No immediate plans for taking out patents exist but the results of the project can very well together with other research results form the base for future patenting for example as regards to yeast strains.

Results been transferred to other institutions through dissemination of articles, participation in conferences, teaching and peer discussions. The projects has not been the singular source for education of Phd's but contributed as part to it.

Below the commercial and scientific possibilities are elaborated a little further.

1) Commercial uses

Knowledge on the energy content of alternative biomasses help to widen the base of affordable 2G feedstock from wheat straw as the core base which has often been pointed to as a weakness of the 2G technology. The project confirmed the potential of grass and barley straw and added rye straw to the list of potent feedstocks to supplement. The project did also show that a number of other potential feedstocks such as deep litter and biofiber from biogas production which not did pass the test for use in ethanol can have uses in other kinds of energy production as regards biofiber for example in the production of district heating and power in cogeneration.

The developed yeast strings have the obvious commercial potential for both the project partner Novozymes and the companys customers of increasing the use of sugars and here arabinose from the straw and thereby increasing the ethanol yield of the feedstock.

In the same line, the knowledge created regarding the dynamics of physiological change and adaptation of yeast to different inhibitors, and the mechanisms of inhibition will be used to optimize the propagation step in order to develop robust cell cultures able to increase the yield and productivity of 2G bioethanol processes.

2) Research

The above mentioned commercially usable results will also be the base for further research and development regarding feedstock and process optimization.

Furthermore the developed enzyme assay has to be subject to further research and testing to improve its reliability.

The next step would be to increase the working volume to 1ml, since volume was considered the biggest culprit of deviation. Further, the isolation procedure of acetylated-glucurono-arabino-xylan (AGAX), a common hemicellulose in grasses, as well as mix linkage glucan, should be further developed and scaled up, which would increase the number of relevant enzyme activities to test. The role of lytic polysaccharide monooxygenases (LPMOs) could also be investigated by adding reducing agent such as ascorbic acid to the hydrolysis.

The project results thereby contribute directly to realize energy policy objectives on sustainable and renewable transportation technology as stipulated in the new EU Renewable Energy Directive (RED II) and to reach national and EU targets for CO2 reduction.

1.7 Project conclusion and perspective

The project has by its results contributed to and demonstrated that lignocellulosic ethanol is an obvious choice of an advanced biofuel to fulfil the new EU directive given the well-developed state of the technology compared to other new technologies of advanced transport fuels. Furthermore a future perspective is that the biorefineries where biomass is fractionated in sugars, lignin and other components, can also be used in other productions than ethanol. Lignocellulosic sugars could for example be used for fermentation of lactic acid or other green chemicals.

Annex

In the annex the attached project documents are referred to the relevant milestone(s) in the also attached Gantt diagram.

Milestones and document references

Milestones	Documents
M1 Screening report on alternative biomasses availability, MEC (WP1)	EUDP WP1 Screening report marts 2017
M2 Screening report on alternative biomasses test in pilot, DE (WP1) CM1 Risk analysis and ethanol production cost reduction with the alternative biomasses (WP1)	REPORT on pretreated straws (EUDP MEC_WP1_KU_2019) Zhang_et_al-The multi-feedstock biorefinery_2018-GCB_Bioenergy
M7 Report on strain construction and performance validation, NZ (WP3) CM3 C5 yeast strain developed able to convert arabinose (WP3)	Technical delivery report to EUDP_Novozymes Sept. 2019 Final ***Novozymes confidential and proprietary information that cannot be published without prior permission from Novozymes***
M8 Report on factors influencing yeast propagation and available on-line yeast monitoring techniques, DTU (WP3)	2017_04_Pau Cabaneros Lopez_review online monitoring fermentation processes
M9 Chapter in PhD thesis on experimentally determined optimum yeast propagation conditions, DTU (WP3) M10 Chapter in PhD thesis describing the potential and the design specifications of the developed monitoring strategies, DTU (WP3)	Real time monitoring alternatives PRESENTATION MEC PAU Figure1 Figure2 Figure3
M11 Specification of standard substrates for C5 and C6 enzyme assay, KU (WP4) M12 Specification sheet of measurements parameters for feedstock productivity, KU (WP4)	KU deliverables 2017: Specification of standard substrates for C5 and C6 enzyme assay Specification sheet of measurements parameters for feedstock productivity
M13 Report on the method validation, correlation with fermentation yields, KU (WP4) CM4 Validated method for measurement of enzyme ectivity (WP4)	MEC - WP4 - Enzyme assay - 20-06-2019_sune
M14 Design and performance report on the in line method for feedstock productivity, KU (WP4)	Gong_Effects of preheating on briquetting and subsequent_2019 SBFC presentation 2018_Sune Thomsen_Final SBFC poster 2017 sune_2 Thomsen_Weiss_Zhang_Felby_Manuscript_2019_Sep