Final report

1.1 Project details

Project title	Demonstration of a high yield fermentation process for 2G bioethanol production
Project identification (program abbrev. and file)	EUDP 2012-I Journal number 64012-0123
Name of the programme which has funded the project	EUDP Programme for Energy Technology and Demonstration
Project managing company/institution (name and address)	Terranol A/S c/o DTU Biosys, Building 223, DK-2800 Lyngby
Project partners	DONG Energy Power
CVR (central business register)	30895770
Date for submission	December 17, 2015

1.2 Short description of project objective and results

Terranol and DONG Energy have aimed to develop, implement and test a combination of 2G bioethanol production technologies for efficient pre-treatment, enzymatic hydrolysis and yeast fermentation. Proprietary technologies developed by the two companies have been joined in a successful demonstration of yeast based C6 and C5 sugar co-fermentation at DONG Energy's demonstration plant for Inbicon 2G ethanol technology in Kalundborg. The results in 270.000 litre scale are in accordance with results obtained in 2 litre laboratory scale. The Terranol yeast yields close to the theoretically maximum and is fast and robust in the Inbicon process. More than 90% of both C5 and C6 sugars was converted into ethanol within 48 hours using a low yeast pitch. Furthermore, a semi-continuous fermentation protocol developed by Terranol with partial draining and refilling was demonstrated in the Inbicon GMO approved pilot plant in Skærbæk in 300L scale. A very satisfactory ethanol yield of 93% was achieved

Terranol og DONG Energy formål var at udvikle, implementere og teste en kombination af 2G bioethanol produktionsteknologier til effektiv forbehandling, enzymatisk hydrolyse og gærfermentering. Beskyttedeteknologier udviklet af de to selskaber er blevet forenet i en vellykket demonstration af gærbaseret C6 og C5 sukker cofermentering på DONG Energys demonstrationsanlæg for Inbicon 2G ethanolteknologi i Kalundborg. Resultaterne i 270m3 skala er i overensstemmelse med resultaterne opnået i 2 liters laboratorieskala. Terranols gær giver et udbytte tæt på teoretisk maksimum og er hurtig og robust i Inbicons proces. Mere end 90% af både C5 og C6 sukkerarterne blev omdannet til ethanol indenfor 48 timer med anvendelse af en lav gærmængde. Ydermere blev en semi-kontinuert fermenteringsprotokol udviklet af Terranol med delvis tømning og genopfyldning demonstreret i Inbicons GMO godkendte pilotanlæg i Skærbæk i 300L skala. Et meget tilfredsstillende ethanoludbytte på 93% blev opnået.

1.3 Executive summary

The necessary GMO approval was in place well in advance of the large-scale demonstration, the so-called campaign, with Terranol's yeast, cV-40, in Inbicon's demonstration plant in Kalundborg. Novozymes was enlisted to deliver cV-40 yeast cream as an alternative solution for Inbicon's own production. In total 6 fed-batch fermentations of 270 m3 scale were completed and an ethanol yield of 90% was reached, calculated from the amount of monomeric sugars of wheat straw hydrolysate. There is a need to show a yield of 90% or more, in order for the yeast to be used in full-scale commercial projects and by inter alia optimizing the feed profile for the actual sugar concentration it is estimated that an improvement of a few per cent can be achieved, as this was demonstrated in laboratory scale.
Prior to the campaign there was a series of laboratory fermentations and preparation work for the determination and calculation of the full-scale fermentation conditions regarding pH optimum, pH adjusting agent, the addition of nutrient and feed profile. Parallel laboratory fermentations were simultaneously run during the campaign using a corresponding scaled-down protocol with the same substrate and yeast cream as in large scale. These copy fermentations in 2 litre scale progressed very similar to the corresponding on large scale, with a slightly higher ethanol yield of 93%. As the provided yeast cream was produced in one batch a few weeks before the campaign at Novozymes, it was necessary to follow the evolution of the yeast's vitality during the campaign period as yeast cream loose efficiency during storage. The loss of viability during the campaign was therefore continuously determined and the yeast dosage was adjusted over time to compensate for the loss.
The operation of Inbicon's demonstration plant has been suspended in 2015 and therefore we were not able to perform the part of the campaign, which was planned to demonstrate the semi-continuous fermentation protocol developed by Terranol with partial draining and refilling. Instead, this part of the project was carried out in the Inbicon GMO approved pilot plant in Skærbæk in 300L scale. A very satisfactory ethanol yield of 93% was achieved
In conclusion, the upscaling was successful and the results regarded as positive as the same ethanol yield was obtained in industrial scale as in laboratory scale within 48 hours. The performance of the yeast is thus considered well suited for commercial projects.

1.4 Project objectives

Terranol and DONG Energy have collaborated for a number of years and exchanged material, pretreated hydrolysed biomass and yeast, and shared test results. This project was therefore a natural next step in the effort to demonstrate a high yield fermentation process for 2G bioethanol production. Terranol and DONG Energy have aimed to develop, implement and test a combination of 2G bioethanol production technologies for efficient pre-treatment, enzymatic hydrolysis and yeast fermentation. Proprietary technologies developed by the two companies were joined in a demonstration of yeast based C6 and C5 sugar co-fermentation at DONG Energy's demonstration plant for Inbicon 2G ethanol technology in Kalundborg.

A total of four technical milestones were identified:

Firstly, as the use of a Genetically Modified Organism (GMO) was required for the completion of this project, Danish Regulatory Approval was needed. This is a somewhat lengthy process involving the complete description of the joint technology to be employed at the Inbicon facility. The necessary GMO approvals for the physical plant (factory and laboratory) and the yeast to be used, cV-40, were in place in good time before the demonstration campaign was started in October 2014.

Secondly, as the demonstration of the joint Terranol/Inbicon technology in "full scale" is a costly endeavor, all essential process parameters needed to be identified and optimized before commencing the demonstration at the Kalundborg facility. Process parameters prior to the October campaign in demonstration scale were completed, as were the extended feed phase process parameters.

Thirdly, a number of specific modifications of the Inbicon facility were needed before the demonstration of the high yield fermentation technology could take place. Two proposals and price estimates for the overall design of propagation equipment in the Kalundborg plant demonstrated that establishment costs would be very high; about 8 million DKK. Since this design would not be used in a full-scale plant and thus would not lead to focused, relevant building of experience it was in early 2014 decided that such an investment in Kalundborg was too expensive. Instead the possibility of external supply of yeast was explored and an agreement was finalized with Novozymes to supply Terranol yeast cream for the campaign.

Finally, this project should lead to demonstration of the very ambitious goal of 95% sugar conversion in 95% ethanol yield. This goal is identical to the one made by the US Department of Energy (DOE), which no one has been able to demonstrate so far. In the October campaign in Kalundborg an ethanol yield of monomeric sugars of 90% in the feed was achieved. In spite of the fact that an ethanol yield of 95% has not been reached, the result obtained is evaluated extremely positive. The results in 270m3 scale are in accordance with results obtained in 2 litre laboratory scale. The Terranol yeast yields close to the theoretically maximum and is fast and robust in the Inbicon process. More than 90% of both C5 and C6 sugars was converted into ethanol within 48 hours using a low yeast pitch. There is a need to demonstrate a yield of at least 90%, for the process to be facile for full-scale commercial projects. In particular by optimizing the feeding profile to follow closer the actual sugar concentration it is estimated that the yield can be increased by a few per cent in full scale, as it was shown in laboratory scale.

Dong's management decided in the end of year 2014 to shut down the operation of the Inbicon facility in Kalundborg during 2015 and therefore the second planned demonstration campaign, where Terranol's developed semi-continuous fermentation protocol should have been demonstrated, could not run in Kalundborg. In this process, 75% of the fermentation broth is transferred to an empty tank after the feeding is completed. Here the fermentation finishes the last sugar while the filling from 25% to 100% in the first tank is done by a secondary feed phase, without adding new yeast.
Instead, this part of the project, following approval by EUDP, was performed in the Inbicon GMO approved pilot plant in Skærbæk in 300L scale with the Terranol yeast strain, cV-110. A nice yield of 93% was obtained in the refill test in pilot scale.

As the 2G bioethanol industry in general is still challenged by the global economic crisis and lack of policy frameworks for the support of the industry's first full-scale plant, the commercial milestones concerning signing of first end-user contract and initiation of building of a full-scale project based on joint developed technology could not be met despite the good technical results obtained in this project.

1.5 Project results and dissemination of results

The development work leading to the final demonstrated process was divided into 8 work packages: WP1, WP2 and WP6 concern the development of the basic batch and fed-batch fermentation process, followed by the further development of this into an extended process in semi continuous or continuous mode, and determination of process critical parameters. WP3 concerns test, choice and implementation of a process monitoring system suitable in large scale. WP4 and WP6 cover factory modifications and regulatory work necessary for obtaining permission for production with a genetically modified organism. In WP5 a procedure for initial production of yeast inoculum should be implemented in large scale, and finally WP8 comprise two large scale demonstration campaigns with time allowed between the two campaigns to identify eventual deviations and causes of these between small and large scale performance and which corrective actions to take in the second campaign.

A detailed description of the individual work packages (WP) and results is given below.

WP1: Initial process characterizations and fed-batch optimization

Task 1.1: Strain/substrate characterization (sugar cont., pH, initial yeast pitch etc.)

A significant difference between the process conditions in laboratory and demonstration scale is that the coarse substrate containing fiber residues and other particles of different sizes cannot be pumped around in laboratory scale. The substrate for fed-batch laboratory fermentations must first be filtered so that approximately 10% particulate material is removed. Batch fermentations with and without solid particles showed that comparable results could be obtained taking into account the different amount of liquid per. weight unit of hydrolysate with and without solid particles.

The process conditions in demonstration scale was determined on the basis of the results of a number of fed-batch fermentations in laboratory scale under varying conditions with respect to pH, temperature and varying additions of nitrogen source.

Seen from a process economic point of view it has been important to optimize these factors, since the process must necessarily be industrially viable, but at the same time as beneficial for the yeast as possible.

Task 1.2: Determination of ideal point for initiation of fed-batch

Experimentally, we have determined that the optimal time for starting supply of substrate occurs when we can measure that the CO2 concentration in the exhaust gas has peaked and just starts to fall in the batch phase. However, CO2 measurement cannot be carried out and used in a similar fashion online in demonstration scale as in laboratory scale and exact time of substrate supply in demonstration scale was therefore determined in laboratory scale with the representative substrate and the established process conditions.

Task 1.3: Determination of optimal glucose concentration (feed-rate) in fed-batch,
and
Task 1.4: Fed-batch fermentation with predetermined values.

An optimal constant feed rate and a rate of substrate supply with linear increase has been adapted from an experimentally determined optimal substrate supply profile. This is determined by an automated substrate supply that can maintain a predetermined glucose concentration. The control program for substrate supply is based on continuous calculation of the monomer sugar concentration in the fermenter, based on the initial concentration, an online measured and calculated total CO2 production as well as the amount of the fed hydrolysate in the feed phase. The results using the linear increased feed rate are comparable with results using the sophisticated feedback control, while a constant feed rate gives a slightly lower yield.

In full scale in the October campaign a predetermined constant feed rate was used. This was chosen, as it was important to have a solution easy to handle for the operators. However, it would be a great advantage in full-scale plants to be able to control the fermentation dynamically by a feedback system in order to maintain low glucose levels in the feeding phase of the process. This would potentially result in an improvement in yield and a reduced fermentation time.
To accomplish this, Terranol has developed a new control program for substrate supply optimized for the purpose of co-fermentation of glucose and xylose. Instead of the laboratory control program that continuously during the process calculates

the concentration of sugars and adapts substrate supply, then control of substrate supply is instead based on changes in CO_2 production. When the CO_2 production begins to decline due to low sugar concentration, i.e. a CO_2 development top can be detected; a predetermined amount of substrate is further fed to the reactor with maximum pump speed. This results in an increase of CO_2 production. When the fermentation rate again decreases, that is, a new peak is detected, a new portion of substrate is fed. This control strategy has been demonstrated to be very robust in a number of fed-batch fermentations, using different hydrolysate and sugar concentration, and the advantage of this strategy is that it is not necessary to know in advance the concentration of sugars in the wheat hydrolysate. A potential online measurement of CO_2 in large scale would be to measure the amount of generated exhaust gas from the fermenter. This measurement is a simple and inexpensive method that is realistic to implement in a full-scale production facility. We have in laboratory scale demonstrated that the above strategy and this measurement method can work together.

WP2: Extension of the feed phase

Task 2.1: Optimal semicontinuous fermentation
Task 2.2: Two-tank continuous fermentation
Task 2.3: Determination of most optimal combination of process sub-elements

In the set-up with extension of the feed phase 75% of the fermentation tank volume is tapped off, when the tank is filled, and this is then transferred to a simple storage tank to complete the fermentation. The first tank is then restarted with a new feed profile and without the need to add additional yeast. Under downscaled conditions in the lab, we have shown that the concept works since it is possible to reduce the added amount of yeast per produced ethanol unit by approximately 45% in just a single refill, while the ethanol yield is increased by a few per cent, as a greater proportion of the total fermentation takes place in the feed phase. Furthermore, it is demonstrated that the total ethanol production rate can be increased by about 15%. This fermentation strategy was planned as the last demonstration in Kalundborg, but unfortunately it was not possible to complete this part in Kalundborg, since the operation of the plant was put on hold in all of 2015. Instead, this part of the project was conducted in 300L scale in Skærbæk. Pilot demonstration of this refill strategy was successful and resulted in an ethanol yield of 93%.

WP3: Online glucose monitoring

Task 3.1: Implementation of NIR spectroscopy in laboratory settings
Task 3.2: Validation of real-time NIR spectroscopy vs. traditional HPLC

Results of Inbicon´s offline NIR measurements on Inbicon fermentation substrate have been assessed and compared with HPLC measurements. The spread on the NIR data is found to be too large and these measurements are therefore too imprecise to be used for online glucose control. Instead, we have tested a different feeding strategy based on CO_2 development (see Task 1.3 and 1.4) and tested measurements of speed of sound and refraction as an alternative to NIR. The spread of speed of sound measurements was too large between substrates of varying quality, whereas measurements of refraction look more promising.

Measurements of refraction were used to assess the initial batch phase under full-scale demonstration in the Inbicon Kalundborg plant in October 2014.

WP4: Regulatory/Inbicon

Task 4.1: Strain description
Task 4.2: Facility description
Task 4.3 Joint application to authorities

Task 4.4: Public consultation process

Two GMO approvals to carry out large-scale experiments with GM yeast in Kalundborg were required by Inbicon.

The necessary GMO approvals for the physical plant (factory and laboratory) and the yeasts required to be used, cV-40, were in place in good time before the demonstration campaign was started in week 41, 2014.

WP5: Initial yeast propagation/Inbicon

Task 5.1: Implementation of propagation equipment in Kalundborg
Task 5.2: Validation of propagation method
Task 5.3: Propagation of yeast

Terranol focused from the start of the project on developing a propagation protocol, which would ensure the production of yeast on a cheap substrate with a high yield per litre without the yeast during the process lost or diminished ability to xylose fermentation.

With glucose as carbon source, and yeast extract, urea, and other salts as additional nutrient source an aerobic fed-batch fermentation protocol was developed, which after 25-30 generations of growth gives an overall yield of more than 50 g /l, and a yield relative to the amount of carbon, of 1 g of yeast (dry weight) per 2.4 g sugar consumed. This is close to the theoretical maximum. In this media part of the necessary amino acids is supplied from the yeast extract.
Based on this process and by substitution of the yeast extract/glucose medium with the far cheaper sugar beet molasses from Nordic Sugar, with addition of phosphate, vitamins and urea and/or ammonia as nitrogen source, an industrially applicable process was developed that provides a final concentration of 50 g dry weight of yeast/litre with a yield relative to consumed sugar of 1 g of yeast per 3 g of sugar, or about 85% of the theoretical maximum. This is slightly lower than what could be obtained in rich medium, which is due to that the yeast itself produces all the amino acids, and the propagation takes thus somewhat longer, but because of the cheap substrate it will be a much more economically advantageous process.

Inbicon, however, decided to cancel the investment in a propagation facility in Kalundborg following an assessment in relation to other needs for investment and development of Inbicon technology. As a necessary alternative solution a cooperation agreement with Novozymes was established in order to produce sufficient amount of Terranol's cV-40 yeast in their manufacturing facility in Kalundborg for the October campaign. The yeast was delivered as yeast cream in pallet tanks for the Inbicon plant. The three parties jointly planned the production of yeast at Novozymes. Inbicon and Novozymes covered the necessary authority approval of transportation of GMO material between their addresses in Kalundborg. Inbicon organized how to keep the cream yeast cooled in a cooling container at their site during the campaign, how to transport the pallet tanks internally in the plant when it was to be used, and how to stir and pump the cream yeast from pallet tanks to fermentors. This included correspondence and meetings with the GMO authorities, for an approval of the chosen solutions.

Propagation of cV-40 for fermentation in pilot scale in Skærbæk was performed on rich medium employing a simplified protocol, which begins with a high inoculation of yeast and a substrate feed, which started immediately after inoculation and then feeding at a constant rate through 48 hours. Inbicon adjusted the protocol, with a slightly lower flow rate for the first 20 hours, followed by a slightly higher flow for 20 hours.

WP6: Factory modifications

Task 6.0a *Modifications to receive and use externally generated yeast cream in the* *facility* (derived and introduced as a result of change in relation to yeast propagation).

Task 6.1 Modification of pump systems for controlled fed batch
Task 6.3 Connection of tank system according to developed feed method

It was necessary to implement a system for receiving, cooling and internal transportation of pallet tanks with cream yeast. Likewise, a stirring and pumping system from the pallet tanks, as well as tube connections to the fermenters were installed.

Task 6.2 Implementation of real time NIR

Since NIR was found to be too imprecise and therefore not useful, a real time system was not implemented.

WP7: Critical substrate parameter determination/Terranol

Task 7.1: Fine-tuning in laboratory setting with best future strain according to previous WPs
Task 7.2: Determination of process robustness to feedstock variations

Covered in the description in section 1.3 and 1.4

WP8: Large scale demonstration

Task 8.1: 1st full scale fermentation
Task 8.2. Data processing and subsequent protocols modifications

A demonstration scale campaign with Terranol's yeast, cV-40 in Inbicon's demonstration plant in Kalundborg in October 2014 was completed. Novozymes supplied cV-40 yeast cream and despite of challenges of delivery in pallet tanks with predetermined amount of yeast, GMO transport between the Novozymes and Inbicon plants, it went fine.

Alongside large-scale demonstration parallel laboratory scale fermentations were run using the same protocol and substrate and both freshly prepared yeast and stored yeast produced by Novozymes in industrial scale. Fed-batch fermentations were satisfactory and the difference between the results of laboratory scale and demonstration scale and between the two types of yeast was small. This is remarkable considering that this has been scaled up by a factor of 100,000 without any intermediate step.

As the provided yeast cream was produced in one batch already a few weeks before the campaign start at Novozymes, it was also necessary to follow the yeast's vitality during the campaign period as yeast cream loses efficiency during storage. The loss of viability was monitored regularly during the campaign, and the dosage was adjusted with a compensation factor over time.

A total of 6 fed-batch fermentations of 270 m3 scale were completed during the campaign and 90% ethanol yield of monomeric sugars in the feed was reached. For comparison, fermentations reached 93% in parallel laboratory scale experiments. It is estimated that the ethanol yield may be increased by inter alia optimizing the consumption profile to the actual sugar concentration, as it was shown in laboratory scale.

Task 8.3: 2nd full-scale fermentation

Dong's management decided in autumn 2014 to shut down the operation of the

Inbicon facility in Kalundborg in 2015 and therefore the second planned demonstration campaign could not run in Kalundborg, where Terranol's developed semi-continuous fermentation protocol with replenishment should have been demonstrated. In this process 75% of the fermentation broth is directly after the first fed batch period transferred to an empty tank, where the fermentation is finished, after which the filling continues from 25% to 100% in the first tank, without adding a new yeast. Instead, this part of the project, following approval by EUDP, was performed in the Inbicon GMO approved pilot plant in Skærbæk in 300L scale with the Terranol yeast strain, cV-110. An excellent yield of 93% was obtained in the refill test in pilot scale.

Both Inbicon and Terranol were invited to speak at the conference "37th Symposium on Biotechnology for Fuels and Chemicals, San Diego, USA, April 27-30, 2015", which was an excellent opportunity to share the joint results.

1.6 Utilization of project results

The Terranol yeast performs so well that it can be considered for commercial Inbicon projects. These still awaits decision on the first full scale plant in Europe, awaiting a political frame that make them palatable for investors. The most worked-through Inbicon full scale project in Europe is the Måbjerg plant near Holstebro in Denmark, and the Terranol yeast is a good yeast candidate for the project. Other competing C6+C5 sugar consuming yeasts are from the companies Dutch DSM, French Lesaffre, or Danish Novozymes.
If the Måbjerg plant is realized, it will contribute to Denmark's reductions of CO_2 from the transportation sector.
No patents are planned to be drawn from the present project, but it contributed considerably to the joint understanding of the effect of the Inbicon process on the yeast, and the yeast performance in Inbicon substrates.

1.7 Project conclusion and perspective

Terranol and DONG Energy have demonstrated an efficient process for second generation ethanol production. Proprietary technologies for efficient pretreatment, enzymatic hydrolysis, and fermentation, developed by the two companies, have been joined in a successful demonstration of yeast based C6 and C5 sugar co-fermentation in industrial scale.

To obtain high ethanol yields it is important to ferment not only the easily accessible C6 sugars (glucose), but also the more difficult C5 sugars (e.g. xylose), and a yeast that ferments C5 sugars is often essential in order to obtain a cost-efficient production of 2G bioethanol. Terranol has designed genetically optimized C6/C5 yeasts demonstrated to fulfil the requirements in industrial settings with respect to robustness, performance and productivity. This enables increased yields of ethanol production. In October/November 2014 the C6/C5 yeast of Terranol was tested in industrial scale at DONG Energy's demonstration plant for Inbicon 2G ethanol technology in Kalundborg, Denmark. The test results in 270 m^3 scale were very good and in accordance with results based on optimized fed-batch fermentation development obtained in 2 litre laboratory scale. More than 90% of both C5 and C6 sugars was converted into ethanol within 48 hours using a low yeast pitch.

Furthermore, a protocol for semi-continuous fermentation developed by Terranol with partial draining and refilling increased the ethanol yield to 93% in the Inbicon GMO approved 300L pilot plant in Skærbæk.

To conclude, the Terranol yeast yielded close to the theoretically maximum and was fast and robust in the Inbicon process. These are important factors to establish positive business cases for commercial 2G ethanol projects. The performance of the yeast is thus considered well suited for commercial projects.