Final report

1.1 Project details

Project title	A stable 2G bioethanol yeast strain formulation: Scalable dry C5 yeast production for commercial application
Project identification (program abbrev. and file)	64015-0638
Name of the programme which has funded the project	EUDP 2015-II
Project managing company/institution (name and address)	Terranol A/S c/o Aalborg University A.C. Meyers Vaenge 15 DK-2450
Project partners	Terranol A/S
CVR (central business register)	30895770
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1.2 Short description of project objective and results

English version

The development and demonstration of an efficient aerobic yeast propagation process followed by a process that results in a stable active dry yeast (ADY) strain product, has become important for the 2G bioethanol producers.

In this project a scalable, industrially relevant three-stage yeast propagation protocol, which increases the biomass by a factor of one hundred over a period of less than three days, yielding close to 100 grams dry weight per litre, has been developed and demonstrated. The propagation protocol, which has successfully been validated on a 120 litre scale, is designed in such a way that the last step induces the accumulation of intracellular trehalose, enabling the yeast to withstand severe desiccation. Terranol's yeast strain cV-110 is suitable for storing as an active dry yeast product, and satisfactory viability and functionality after one and a half year of storage has been demonstrated.

Dansk version

Udvikling og demonstration af en effektiv aerob gæropformeringsproces efterfulgt af en proces, der resulterer i et stabilt aktivt tør gærprodukt (ADY), er blevet vigtig for 2G bioethanol-producenter.

I dette projekt er der udviklet og demonstreret en skalerbar, industrielt relevant tre-trins gæropformeringsprotokol, som øger biomassen med en faktor hundrede over en periode på mindre end tre dage, hvilket giver næsten 100 gram tørvægt pr. liter. Opformeringsprotokollen, som med succes er valideret i 120 liter skala, er designet på en sådan måde, at det sidste trin inducerer akkumulering af intracellulær trehalose, så gæren kan modstå udtørring. Terranols gærstamme, cV-110, er velegnet til opbevaring som et aktivt tørgærprodukt, og der er demonstreret tilfredsstillende levedygtighed og funktionalitet efter et og et halvt års opbevaring.

1.3 Executive summary

In this project a scalable, industrially facile propagation and preservation process has been developed for a Terranol modified yeast strain with a favorable combination of efficient xylose fermentation and high robustness. Complete fermentation of available fermentable sugars in biomass hydrolysates in less than 48 hours has formerly been demonstrated in up to 270.000 L scale. However, the experience with such highly specialized strains has been that aerobic growth, yeast stability and functionality, and ability to withstand a drying procedure are often inadvertently hampered or destroyed during the development of such strains.

Thus, the development and demonstration of an efficient aerobic yeast propagation process with a high yield of biomass, followed by a robust preservation process that results in a stable active dry yeast strain product, has become as important as demonstration of the ability of the yeast strain to perform adequately in the desired production process. Failure of a developed strain to grow fast and with high yield aerobically in the propagation process is as disqualifying for the strain as failure to perform adequately in the production process.

Protocols for efficient yeast propagation on both industrial and synthetic media followed by a process that results in an active dry yeast strain product was developed and in collaboration with partners the performance of the resulting active dry yeast product has been tested in larger scale by using industrially relevant substrates and under industrial relevant conditions for 2G bioethanol production.

In one instance has the propagation strategy been scaled up by a factor of one hundred and evaluated on a 120 L scale by the partner Sekab E-technology AB, Örnsköldsvik, Sweden. The subsequent fermentation on wheat straw hydrolysate in 10.000 L scale was successful and gave the same result as was obtained in laboratory scale.

Long-term continuous fermentation experiments have demonstrated that the yeast is genetically stable. In addition, we were able to establish that the Terranol strain is suitable for storing as an active dry yeast product, and satisfactory viability and functionality after one and a half year of storage has been demonstrated.

The developed technology allows for easy delivery of dry yeast batches guaranteeing reproducible cell viability and performance after hydration. The active dry yeast product has been or is being evaluated by partners/customers in e.g. India, UK, Sweden, Finland, and Austria.

1.4 Project objectives

Terranol has previously developed a modified yeast strain which has a favorable combination of efficient xylose fermentation and high robustness. Complete fermentation of available fermentable sugars in biomass hydrolysates in less than 48 hours has been demonstrated in up to 270.000 L scale.

However, the gap in scale between inoculating a pilot or demonstration 2G bioethanol production fermenter and a full-size commercial fermenter with a specialized pentose fermenting yeast appears to be an underestimated challenge as such a development is largely done in an empirical trial and error way. The experience with highly specialized strains has been that aerobic growth, yeast cream stability and ability to withstand a drying procedure are often inadvertently changed, hampered or even destroyed in such strains. This because these characteristics have not been included in or tested along with the screening for and optimization of the desired production traits. Thus, some strains may be impossible to formulate as active dry yeast with good viability and rehydration properties.

The development and demonstration of such scalable methods and protocols to obtain the necessary yeast amounts, either through contract manufacturing or through onsite production, is an important contribution for the emerging 2G bioethanol industry to be brought into reality.

Thus, the development and demonstration of an efficient aerobic yeast propagation process with a high yield of biomass, followed by a robust preservation process that results in a stable active dry yeast product, has become as important as demonstration of the ability of the yeast strain to perform adequately in the desired production process. Failure of a developed strain to grow fast and with high yield in the propagation process is as disqualifying for the strain as failure to perform adequately in the production process.

This need has in this project been demonstrated by developing a scalable, industrially facile propagation and preservation process tailored to the latest developed Terranol strain.

It is of crucial importance that scalability of the techniques used in small scale is ensured when choosing technology. The process equipment and methods used for lab scale development must be chosen to reflect as exactly as possible large-scale equipment to be amenable to direct upscaling.

Thus, the overall objectives of the project were:

1. To develop a scalable propagation and formulation of Terranol's C5-yeast into a stable active dry yeast product.
2. To document maintained production quality after 10 x propagation of the dry yeast in 10 m3 demonstration scale.

Five technical milestones were agreed upon at project start. They were all reached and are described in detail in paragraph 1.5:

M1 All equipment delivered and installed
M2 Downstream ADY production line established and tested
M3 Optimized dry yeast production process completed
M4 Final onsite 10x propagation procedure established and ready for demonstration
M5 Overall optimized method established, demonstrated and described

The project was initiated January 2016 and has since been granted extension and budget change. This was partly due to loss of key personnel and relocation of the company. Also, for various reasons partners supposed to test the active dry yeast product in larger scale were not able to fulfill their intentions and new partners were consequently found. These unforeseen challenges were, however, met and the project has reached its targets.

1.5 Project results and dissemination of results

The results of the work in each work package is described in detail below.

WP1: Aerobic propagation to high density in industrial media

The aim of WP1 was to aerobically propagate yeast to high density and focus was initially to establish a propagation procedure of strain cV-110 on the industrial media beet molasses. An active dry yeast formulation is intended for pitching starter cultures for potential long-time cultivations and also to be used as company samples for commercial purposes. Due to these reasons the produced dry yeast needs to be of very high quality i.e. dry yeast with a high viability, excellent functionality, minimal contamination, and reproducible performance. Focus for development of a propagation procedure was thus shifted towards synthetic media instead of beet molasses. A commonly used media "Verduyn/Delft media" was chosen as propagation results are more reproducible employing this medium than results obtained on beet molasses. It was also considered that the prize per produced unit dry weight of yeast is comparable on the two media. Experimentation resulted in the following protocol: 1.1 gDW/L of fresh yeast is pitched in a reactor containing 900 ml of diluted media – 50 g/L glucose. Once the sugar has been fermented, and the ethanol consumed, feeding is initiated. The feeding is fixed, and in such a manner that the

yeast will exhibit full respiratory metabolism. The feed is regulated every 24 hours in such a way that respiratory metabolism in the culture is stimulated, which leads to a higher cell yield. The feed is defined media with a sugar concentration i.e. 300 g/L in 300 ml. Depending on the sugar concentration of the feed, cell yields of 90 gDW/L has been obtained. However, a propagation protocol that yields up to 160 gDW/L was also developed, but a trade-off must be made regarding the long-term viability vs. the cell yield – see WP2.

WP2: Optimization of intracellular trehalose accumulation

It is a known fact that to protect itself from severe desiccation, such as encountered during the production of dry yeast, the yeast *S. cerevisiae* will accumulate intracellular trehalose. Optimization of the intracellular accumulation of this compound has thus been a key objective. Experiments employing different strategies have been evaluated e.g. salt induction, sorbitol induction, and nitrogen starvation. However, in our hands, the most effective strategy has been to induce the trehalose accumulation by slowly adding the carbon source e.g. glucose, ensuring absolute respiratory metabolism. Just the slightest overfeed, causing fermentative metabolism, will lead to depletion of the trehalose storage. Applying the developed protocol, trehalose concentrations as high as 15% (g/g) were achieved and according to literature this is among the best available.

The viability of the dried yeast has so far been recorded to 75% after more than twenty months of storage in the fridge. During the efforts to maximize both yield and viability it was realized that the trehalose induction strategy needed further revision: the cellular yield was increased by almost 70% (g), but the trehalose content decreased to around 8% (g/g) with a decreased viability of 50% after 8 months as a result. It is this trade-off which needs to be considered as mentioned in WP1. Thus, optimization of the trehalose content in yeast production has been further developed. A number of experiments using "heat-shock" (incubation of the yeast at 42°C) before the drying process has been conducted and a further boost of the trehalose content can be obtained if an appropriate time interval is used. However, the above stability test using a propagation protocol that produces yeast with 15% trehalose shows that no additional content is necessary to dry yeast with long shelf-life. Therefore, there was no need to include an emulator for stabilizing the yeast in the drying process as originally intended cf. WP8.

WP3: Purchase and installation of industrial type downstream equipment

It is crucial that scalability of those techniques used on a smaller scale is ensured when selecting technology. The selection of process equipment and methods used for laboratory scale development should reflect as accurately as possible industrial large-scale equipment, so that a direct upscale can be convincingly described and subsequently performed.

In the industrial scale manufacturing process for active dry yeast a vacuum drum filter, an extruder, a fluid bed dryer and vacuum/controlled atmosphere sealer are used. Therefore, laboratory size equipment that reflects similar scaled-down equipment was needed.

Different available high-pressure laboratory equipment was tested for use in pressing the yeast into a solid cake prior to extrusion and drying. Unfortunately, this

equipment was not suitable for the task, and the search went on for an alternative. It was found that by applying everyday household equipment a protocol could be mimicked to press the yeast and by combining centrifugation and biomass drying, we were able to form an intermediate product in lab scale that closely resembles the intermediate product that results from the use of a vacuum drum filter process.

We have worked with an equipment supplier to identify a suitable extruder for our purpose and commercially available yeast was used for initial experiments at the supplier's premises. A compact table model co-combining extruder and spheronizer was chosen, that can operate with batches between 10-100 g of material.

With regard to the fluid bed dryer a programmable model from the company "Sherwood" was chosen. This equipment can deliver a homogeneous sample quickly and reproducibly by controlling airflow, temperature and drying time. The fluid bed dryer operates by pumping warmed air through a glass container. To prevent possible contamination, the air intake must be equipped with sterile filters. We have designed a custom-made funnel system for mounting HEPA filters onto the drier and testing of this equipment using commercial, pressed yeast cake proved that it could be easily handled and was fully suitable for the task.

The final atmosphere change and packing method of the ADY influences the shelf life of the ADY. A suitable vacuum packer was identified, and various packing materials were tried out until an appropriate type was found.

The necessary process equipment was installed, and each process equipment was individually tested and found suitable for the purpose. The entire process from yeast propagation to vacuum packaging was tested in its entirety, validated and proven well suited for establishing the optimal conditions for each unit operation.

WP4: Setting up the harvesting and filtering process

The harvesting of yeast cells was performed by concentrating the yeast to between 15 and 20 % dry matter and cells were subsequently washed to remove residual broth. The following filtering process is in industrial scale often done by drum filtration with continuous removal of the yeast cells from the drum filter, a process that is not practically usable in lab scale. Thus, to adhere to an industrially relevant process and ensure scalability, the filtering process was replaced with a centrifugation and pressing process followed by a drying process. The exact yeast dry weight value was then adjusted to facilitate the following extrusion process and the further drying process. This operation results in an intermediate product that closely resembles the intermediate product that is the result of vacuum drum filtration.

WP5: Extrusion process development

The extrusion determines particle size and shape of the yeast granules that is dried in the fluidized bed dryer, thereby determining required airspeed and air temperature profile in the drying process. An extrusion process has been established and can be further optimized according to feedback from future partner trials.

WP6: Fluidized bed process established

Fluidized bed drying has become the technology of choice for commercial yeast drying, as the process is gentler with a shorter process time than traditional techniques like belt, rotary or spray drying procedures. Use of fluidized bed drying results in higher and more consistent cell survival rate giving a better and more reliable product quality. Scalability is excellent, and instruments with programmable process parameters are available in small scale.

As described above a fluid bed dryer was purchased and a fluid bed drying technique has been established. The optimal combined parameters for specialized strains are dependent on the strain characteristics, thus no straightforward complete strategy was at hand. Critical factors such as particle size, temperature & temperature variation, airflow, timing and final moisture content have been determined. These are all key factors determining product shelf life, ease of rehydration and cell viability after rehydration.

WP7: Yeast viability determination

A method for yeast viability with high accuracy and reproducibility, based on anaerobic fermentation capacity during a three-hour incubation, has been established and tested. The protocol is as follows:

0.5 g dried yeast, which has been rehydrated in sterile tap water for one hour at room temperature, is used to pitch a 20 ml YPD, 5% glucose (w/v) culture. The culture is contained in a sterile 65 ml sealed glass vial and incubated at 30°C and 150 rpm. By following the weight loss of the culture, a consequence of the production and escape of CO_2 gas from the culture, a relative measure for the fermentation capacity can be obtained – the faster the weight loss the higher the fermentation capacity and concludingly more active i.e. viable yeast was present in the dried yeast. A reference measurement is made upon making of the dry yeast, viability at time zero, which sets the 100% viable yeast capacity.

The industry standard for yeast viability is six months. The viability of the ADY product in this project has so far been recorded to 75% after more than twenty months of storage in the fridge. Also, the product is genetically stable as demonstrated in propagation of yeast in synthetic medium with 15% trehalose content followed by long-term continuous fermentation experiments.

WP8: Combining and optimizing complete dry yeast process parameter & yeast viability

Viability, believed to be due to the high intracellular contents of trehalose (15 (g/g)), proved satisfying, An emulator such as sorbitan monostearate (E491) has been described in literature to make the yeast, or microbe, more resistant to desiccation. It is currently being used by industrial yeast producers such as Lesaffre, France. In the trials that have been performed at Terranol, the challenging part appears to be the mixing of sorbitan and the yeast: sorbitan being a white waxy substance not readily dissolvable in water, and only in ethanol at temperatures above 50°C. Since liquid yeast does not tolerate these conditions very well, it makes the homogenization and mixing of sorbitan challenging. A possible approach could be to spray the liquid sorbitan directly onto the yeast during the fluid bed

stage thus coating the yeast. However, since we were able to produce a stable ADY with a trehalose content as high as 15% it was finally decided not to include an emulator in the drying process.

WP9: Development of onsite 10 x propagation strategy, 24/48 h

The propagation strategy described in WP1 was scaled up by a factor of hundred. Thus, 100 gDW of yeast would be pitched in 90 L of defined media with a sugar concentration of 50 g/l. A feed of 30 L and 300 g/L sugar would be fed over 72 hours.

WP10: Optimization and validation of 10x inoculate quality in biomass process, small and large scale

In addition to the viability assay as described in WP2, functional properties have also been tested and access to 2G sugar streams is required so that these can be performed under realistic conditions. Access to sugar streams have been gained from Finland, Sweden, Estonia, Austria and England (manufactured in industrial scale pilot using either enzymatical or chemical methods). The functionality of Terranol's dry yeast has been tested on all these sugar streams as a minimum in small scale. Likewise, the functionality has been tested in laboratory fermentor scale for selected enzymatically and chemically produced streams. In all cases, the dry yeast has proved functional to the same degree as we have previously achieved with freshly made yeast. In addition, laboratory-scale fermentor experiments have also required 10x amplification of the yeast, which has been developed in parallel. The yeast is rehydrated in tap water and then fed directly to a propagation medium which allows the necessary growth. The propagation medium is designed so that no control of media feeding is required as fermentative growth does not occur. Thus, it is also possible to achieve more than 10x propagation simply by using a larger propagation tank (and more time).

WP11: Demonstration of 10 x propagation protocol in pilot/demo scale

The 100x propagation strategy described in WP9 was evaluated on a 120L scale at the facility of Sekab E-technology AB, Örnsköldsvik, Sweden. It proved successful as it provided the same result in the laboratory scale at Terranol.

All in all, the project objectives to develop a scalable propagation and formulation of Terranol's C5-yeast into a stable Active Dry Yeast product and to document maintained production quality after 10 x propagation of the dry yeast in 10 m3 demonstration scale was accomplished.

Dissemination of results

Results have been disseminated to the scientific community e.g. in the article "Optimising fermentation" in Biofuels International Magazine, issue 3, volume 13.

In addition, the availability of the ADY formulation of strain cV-110 has been communicated in conjunction with presentation of Terranol technology e.g. at "40th Symposium on Biotechnology for Fuels and Chemicals", Florida, USA, May 2018,

"35th Annual International Fuel Ethanol Workshop & Expo" in Indianapolis, USA June 2019, and "BESTF2 Final Forum" in Brussels October 2019.

Furthermore, Terranol's website has been redesigned and updated in order also to reflect the availability of dry yeast samples and protocol for efficient yeast propagation.

Special emphasis has been made towards directing the communication to potential end users of the demonstrated technology and samples have been shipped to a number of customers/partners.

1.6 Utilization of project results

A scalable propagation and formulation of Terranol's proprietary pentose and hexose fermenting yeast into a stable Active Dry Yeast product was developed.

Initially, it was agreed by letters of intent by three end users to test the ADY formulation by the use of their respective proprietary biomass pretreatment and hydrolysis technology followed by ethanolic fermentation and evaluate the industrial suitability of the ADY sample in collaboration with Terranol.

In one instance has the propagation strategy been scaled up by a factor of one hundred and evaluated on a 120 L scale by the partner Sekab E-technology AB, Örnsköldsvik, Sweden. Thus, 100 gDW of yeast was added to 90 L of defined medium with a sugar concentration of 50 g/L and within 72 hours 30 L were fed with a sugar concentration of 300 g/L of sugar. The demonstration experiment proved successful and gave the same result as was obtained in laboratory scale.

With respect to the remaining two intended end users we did not manage to obtain results either due to bankruptcy or unwillingness to share results.

However, in connection with the sale of a license to an English company, tests have been conducted in laboratory scale and agreed in 600L pilot scale for a subsequent demonstration scale experiment in the UK. The results of these trials were not yet available in the course of the project period.

In another partnership with a Scandinavian energy company, who is planning a 150 million Euro investment in a commercial 65.000 MT per year 2G bioethanol facility to be operational in 2021, Terranol will provide ADY for the precommercial trials and the ADY is considered for their facility.

Furthermore, several potential customers have by now received ADY samples and marketing of the ADY has thus already been initiated and search for further potential customers is ongoing.

It was not the intention that the project should take out patents or include educational activities.

1.7 Project conclusion and perspective

In this project an efficient aerobic yeast propagation process with a high yield of biomass, followed by a robust preservation process that results in a stable active dry yeast strain product has been developed.

The propagation strategy has been scaled up by a factor of one hundred and evaluated on a 120 L scale by the partner Sekab E-technology AB, Örnsköldsvik, Sweden. The demonstration experiment proved successful and gave the same result as was obtained in laboratory scale.

In another partnership with a Scandinavian energy company, who is planning to invest in a commercial 65.000 MT per year 2G bioethanol, Terranol will provide ADY for the precommercial trials whilst the ADY is considered for their facility.

Terranol is in contact with potential end users of the demonstrated technology and samples have already been shipped to a number of customers/partners.

After the project period Terranol will maintain the equipment for producing ADY to be used for current and future projects. Also, ADY samples will continue to be shipped to partners/customers. Finally, and if needed, the developed technology will be demonstrated for potential 2G producers and contract manufacturers of yeast.