EUDP Final report

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Project title: **Yeast strains designed for 2. generation bioethanol production**
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Introduction

First generation bioethanol is produced from agricultural feedstock, such as sugar cane, sugar beet, and corn, whereas 2. generation bioethanol is produced from agricultural wastes, such as wheat straw, sugar cane bagasse, corn stover and wood chips.

The manufacturing process for 2G bioethanol involves pretreatment of biomass, enzymatic hydrolysis, fermentation, and distillation. Biomass contains hexoses, primarily from cellulose, and pentoses from the hemicellulose part of the plant material. After enzymatic hydrolysis yeasts ferment hexoses to ethanol whereas pentoses are not fermented to ethanol by any natural microorganism in sufficiently high concentrations.

The Bakers´ yeast *Saccharomyces cerevisiae* has a long tradition in alcohol production from glucose of e.g. starch, as this production process is simple and robust. *S. cerevisiae* has a stable and high ethanol productivity as well as high tolerance towards inhibitors and ethanol. The physiology of *S. cerevisiae* is very well known and a large toolbox is available for genetic engineering purposes.

Aim & goal

The aim of the project was to develop a suitable fermentation organism for 2G bioethanol production that would efficiently ferment all of the sugars in lignocellulosic biomass into ethanol at a commercially viable rate (comparable to yeast based 1G ethanol production).

More specifically, a yeast strain would be developed with the ability to ferment also the pentoses in lignocellulosic biomass and thereby increase the ethanol yield of the process by 30-45 % with a profound positive effect on the total process economy.

Strategy

The development strategies were to i) identify a *S. cerevisiae* entry strain that has optimal basic characteristics for industrial fermentations, ii) identify or develop an effective proprietary xylose isomerase, iii) obtain an enhanced xylose metabolism rate by relieving an unidentified bottleneck in *S. cerevisiae* and iv) combine the above points with further enhancement of the pentose phosphate pathway.

Yeast development

A small set of yeast hexose fermenting strains was chosen based on good transformability applying standard laboratory methods and which could be induced to sporulate allowing genetic analysis. Also, high resistance towards inhibitors from lignocellulosic biomass, in this case generated by the Inbicon process, was a prerequisite. These strains were tested and compared with state-of-the-art hexose fermenting Thermosacc from Lallemand under simulated industrial fermentation conditions at the Inbicon facility in Skærbæk. It could be demonstrated that specifically one strain met the requirement in the fermentation environment in the 2G ethanol industry by having good productivity in a cellulose simultaneous saccharification fermentation process. This specific strain served as the entry strain for the development work.

In lignocellulosic biomass roughly 1/3 of the sugars are pentoses, whereas 2/3 of the sugars are hexoses. Hexoses are natural substrates for yeasts, whereas pentoses are not. However, 80-90% available pentose from lignocellulosic biomass is xylose, therefore the main focus has been on the xylose pathway. *S. cerevisiae* is capable of metabolizing the 5-carbon sugar D-xylulose through the nonoxidative part of the pentose phosphate pathway (PPP), from which the carbon enters the central glycolytic pathway as fructose-6-phosphate and glyceraldehyde-3-phosphate (Fig. 1). To enable *S. cervisiae* to grow on D-xylose it is sufficient to introduce the ability to convert D-xylose into D-xylulose.
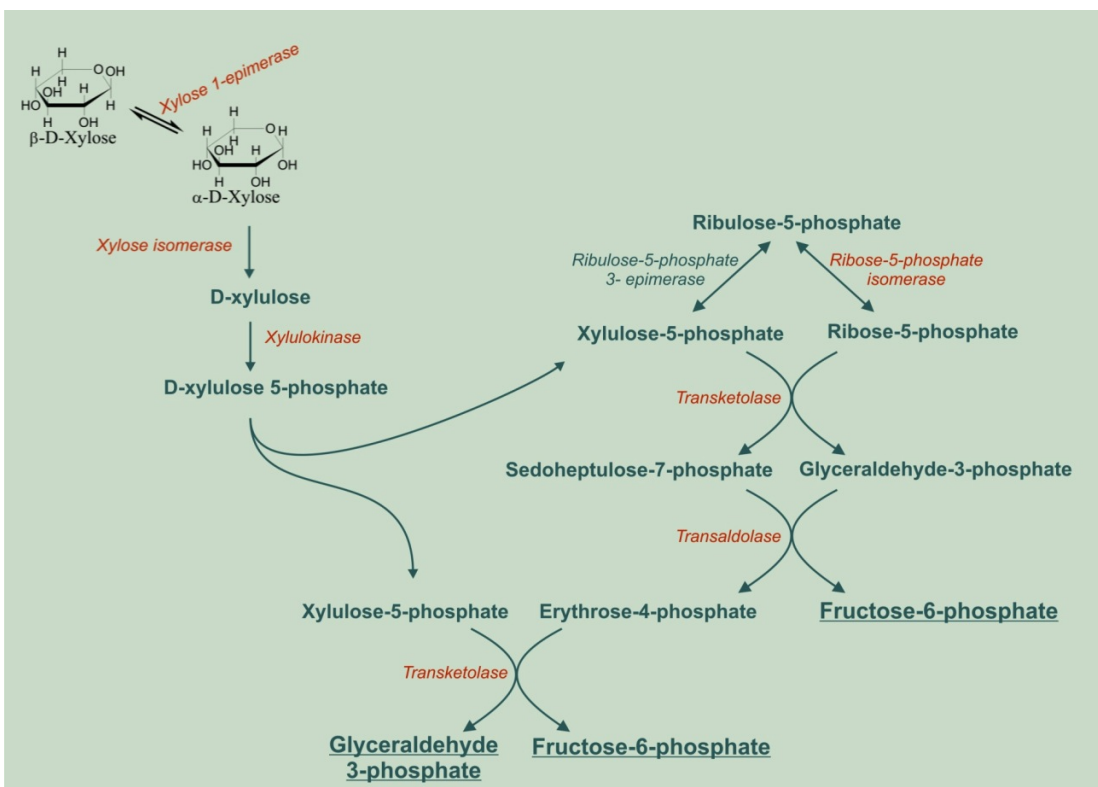

Fig. 1. Pentose phosphate pathway including xylose isomerase pathway

For D-xylose conversion to D-xylulose there are two pathways either of fungal or bacterial origin. In most bacteria, D-xylose conversion proceeds via direct isomerization to D-xylulose using xylose isomerase (XI). In yeasts and most fungi, D-xylose conversion is carried out by xylose reductase (XR) and xylitol dehydrogenase (XDH). XR is NADPH cofactor specific whereas XDH is NAD+ cofactor specific. The difference in cofactor preference of XR and XDH leads to the

formation of an unwanted byproduct xylitol under anaerobic conditions. We have preferred the bacterial XI pathway as it is biochemically simpler, giving higher efficiency, and also the patent situation is simpler, which allows for freedom to operate.

Hence, a XI from *Lactococcus lactis* was identified, cloned and expressed in the chosen entry strain (Fig. 1). The effect was measured in terms of activity in yeast and ability to confer growth on xylose as carbon source. Our experiments using the XI gene derived from *Lactococcus* demonstrate similar performance to that of the *Piromyces sp. E2* state-of-the–art XI gene with respect to growth pattern.

It is questionable whether the XI pathway is optimal. It is known that D-xylose in aqueous solution is a mixture of 5 configurations. However, in practice 99.5% is present in two forms: β-D-xylopyranose and α-D-xylopyranose (Fig. 2). Equilibrium exists between these two forms, of which 2/3 is in the β-form and 1/3 is in the α-form. Both forms are rather stabile and a spontaneous mutarotation occurs between the two forms, but it is only the α-form, which is a substrate for XI.
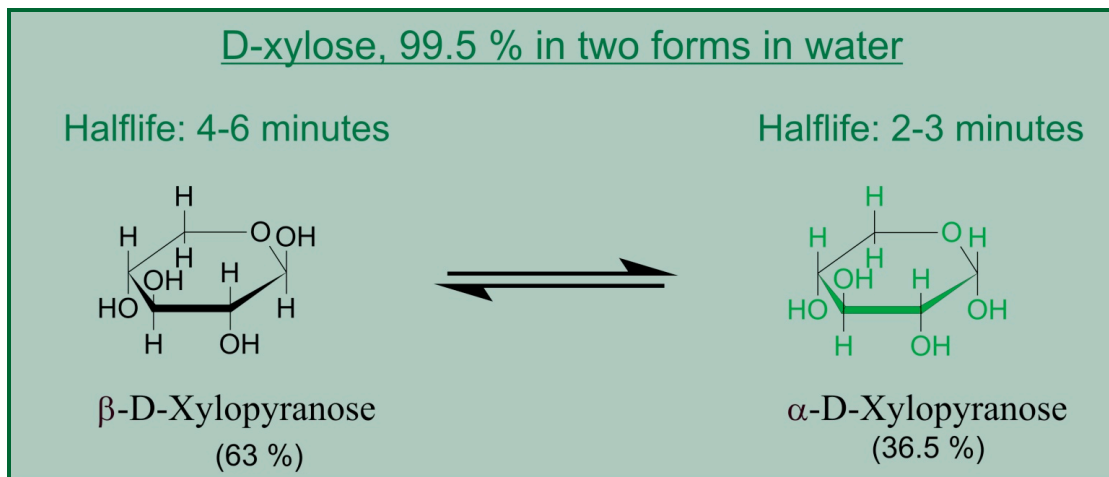

Fig. 2. Epimerization of Xylopyranose

The spontaneous conversion between the two forms is potentially rate limiting. However, there are enzymes (mutarotases or epimerases) catalyzing the conversion between the β and α forms. In literature there are examples where genes encoding epimerases are located in the same operon as xylose degrading genes. We have identified a gene from *Lactococcus lactis* encoding a aldose 1-epimerase acting on xylose and this gene has been expressed (Fig. 1) in varying amounts together with plasmid based constant expression of XI in standard laboratory yeast and a growth effect on xylose can be demonstrated.

When yeast ferment glucose less than 2% passes through the PPP, the rest passes through glycolysis. Thus, to ensure efficiency of the PPP it is essential to upregulate key PPP enzymes (shown in red in Fig. 1). We have made genetic constructs by combining genes known from literature to upregulate the PPP together with genes encoding XI and epimerase with a number of chosen strong constitutive glycolytic promoters. These constructs have been inserted in

the genome of our chosen basic entry strain and the various combinations were then subjected to long-term adaptation under selective pressure i.e. evolutionary engineering, where variants of a cell population with a selective advantage exponentially take over the initially dominating cells. The evolutionary engineering programme was conducted in repetitive batch cultures in shake flasks by screening for reduced generation time under aerobic conditions and improved fermentation efficiency on xylose.

Yeast testing, strain Q10

Early tests were performed in small flasks sealed with rubber stoppers into which cannulas were inserted for sampling and release of exhaust gas (Fig. 3). Fermentations were started with 1g/l yeast biomass and 50-60 g/l xylose in rich medium at pH 5.5 and 30°C. It could be shown that fermentation time of yeast sampled at different time points during the process of evolutionary engineering was step-by-step reduced from almost infinite down to two days.
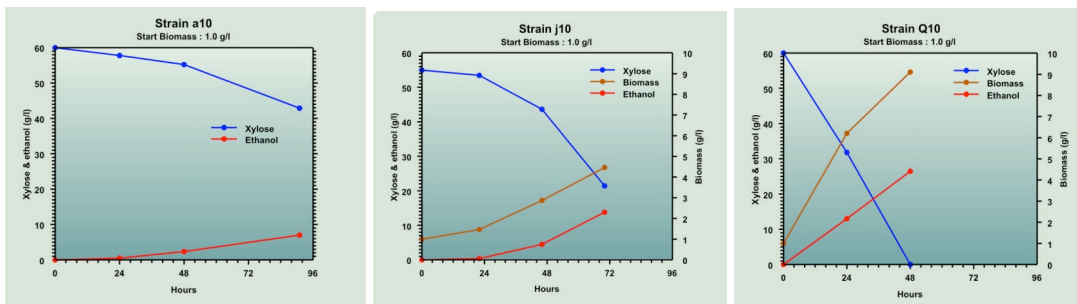

Fig. 3. Early testing of yeasts in temperature controlled flasks

A fermentation performed under similar conditions (Fig. 4) in a mixture of 100 g/l glucose and 50 g/l xylose showed that our first yeast prototype, Q10, could ferment both sugars in a one pot reaction resulting in an ethanol yield of 83% of theoretical maximum.
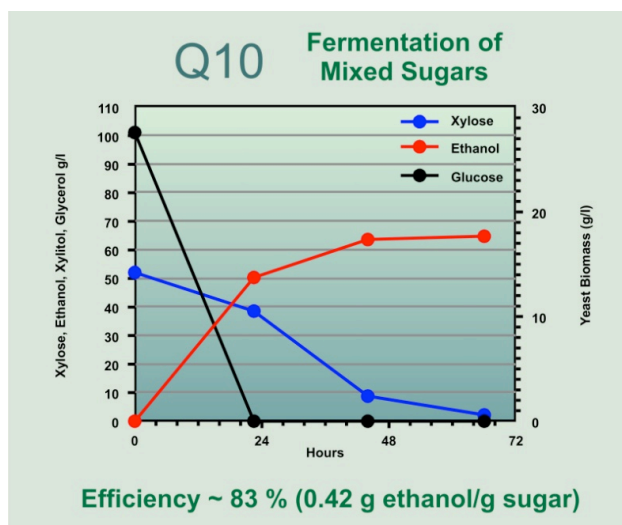

Fig. 4. Mixed sugar fermentation of strain Q10

Continued test of strain Q10 in hydrolyzed fiber fraction from Inbicon with added xylose was performed in a 2L fermentor (Fig. 5), which also allowed for more frequent samples. Results from small-scale experiments could be confirmed under controlled conditions in larger scale and in lignocellulosic biomass. An ethanol yield of 87% of theoretical maximum could be obtained starting with 125 g/L glucose, 50 g/L xylose and 0.25 g/L yeast inoculum.
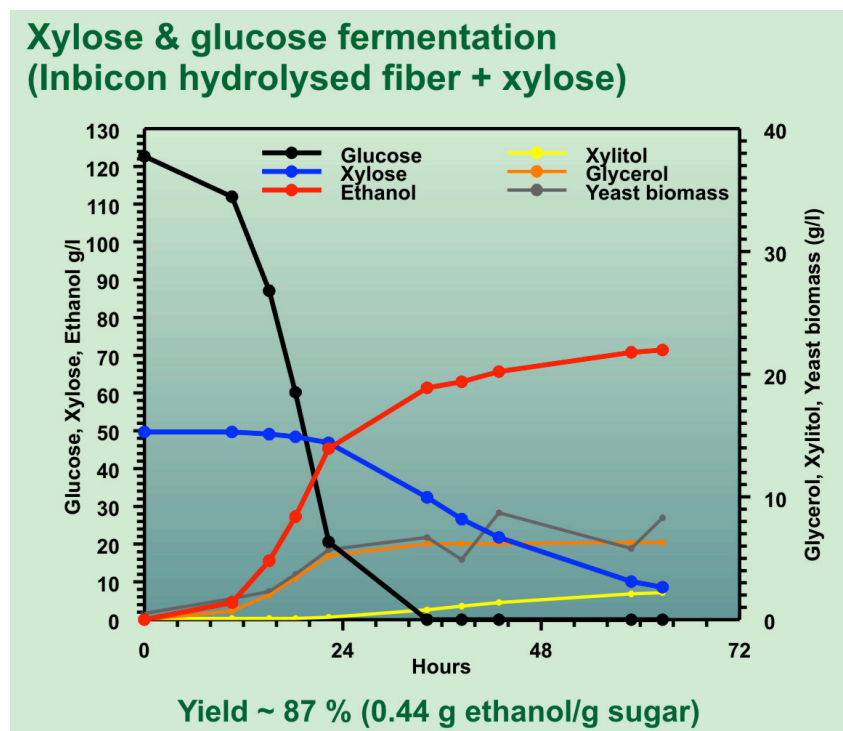

Fig. 5. Q10 controlled fermentation in hydrolyzed Inbicon fiber fraction with added xylose

Further testing of strain Q10 was performed in real hydrolysed lignocellulosic biomass with a total solid content of 20%, yeast inoculum of 1 g/L at pH 5.5 and 30°C with addition of 2-3 g/L urea as nitrogen source (Fig. 6). Tests were performed both with corn stover hydrolysate and the first version of complete wheat straw slurry from Inbicon.
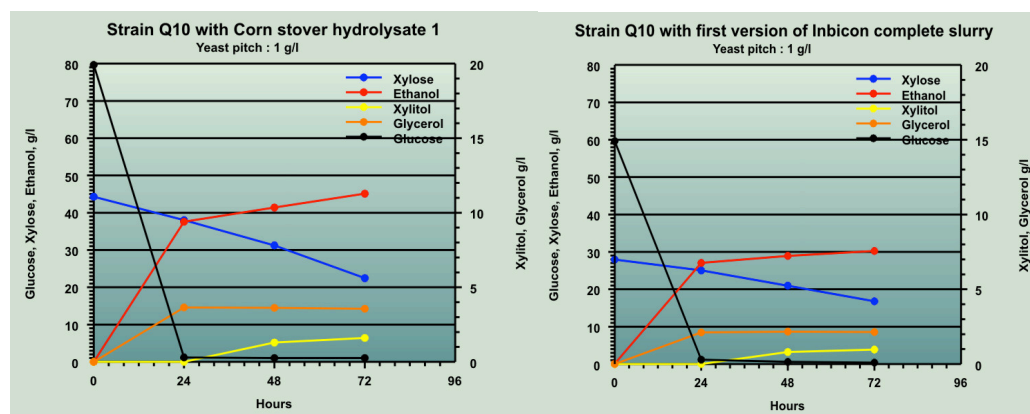

Fig. 6. Q10 fermentation in corn stover and wheat straw hydrolysates

From these fermentation tests it could be concluded that the fermentation times of Q10 in lignocellulosic biomass were prolonged compared to the previous tests in rich medium. During pretreatment of lignocelulosic biomass the release of sugar monomers is often accompanied by release of compounds that inhibit growth and metabolism of the yeast. Some of the inhibitory compounds are present in the biomass and others are formed from degradation of sugar and lignin molecules during the pretreatment process. Thus, the prolonged fermentation times were ascribed to the presence of such inhibitors in the lignocellulosic biomass. Further development of strain Q10 employing evolutionary engineering was desirable to enhance the resistance towards biomass-derived inhibitors and also to increase the fermentation rate of xylose. The evolutionary engineering programme was conducted in repetitive batch cultures in shake flasks using a cocktail of inhibitors that is present in biomass hydrolysate and led to the development of strain V1.

Yeast testing, strain V1
Initial comparison of strain Q10 and V1 in rich medium (Fig. 7); yeast extract with the addition of 80 g/L glucose and 60 g/L xylose, could demonstrate that strain V1 is considerable faster giving a higher ethanol yield.
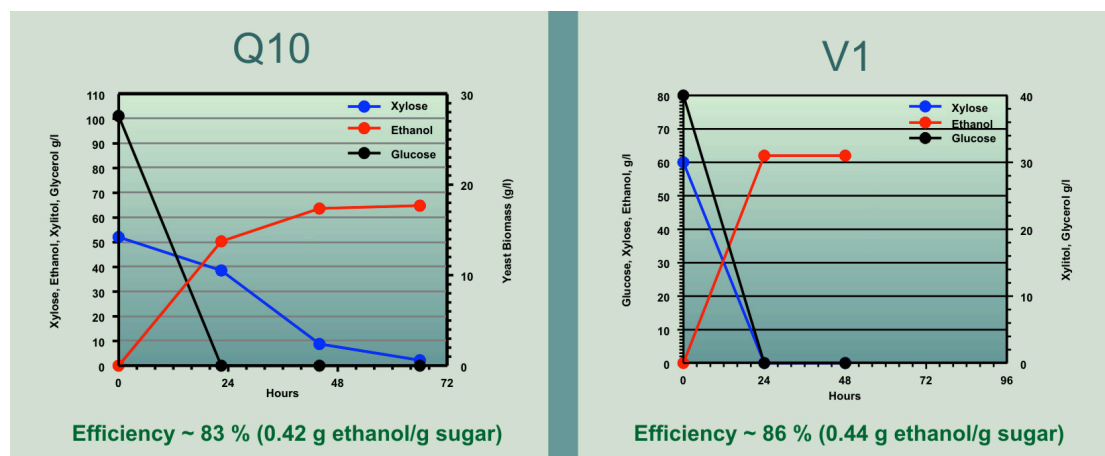

Fig. 7. Comparison of Q10 and V1 in rich medium

Further testing of strain V1 (Fig. 8) in 2L controlled bioreactor demonstrates that V1 is capable of completely fermenting glucose and xylose in several different pretreated lignocellulosic biomasses, i.e. wheat straw hexose/pentose solution from Inbicon.
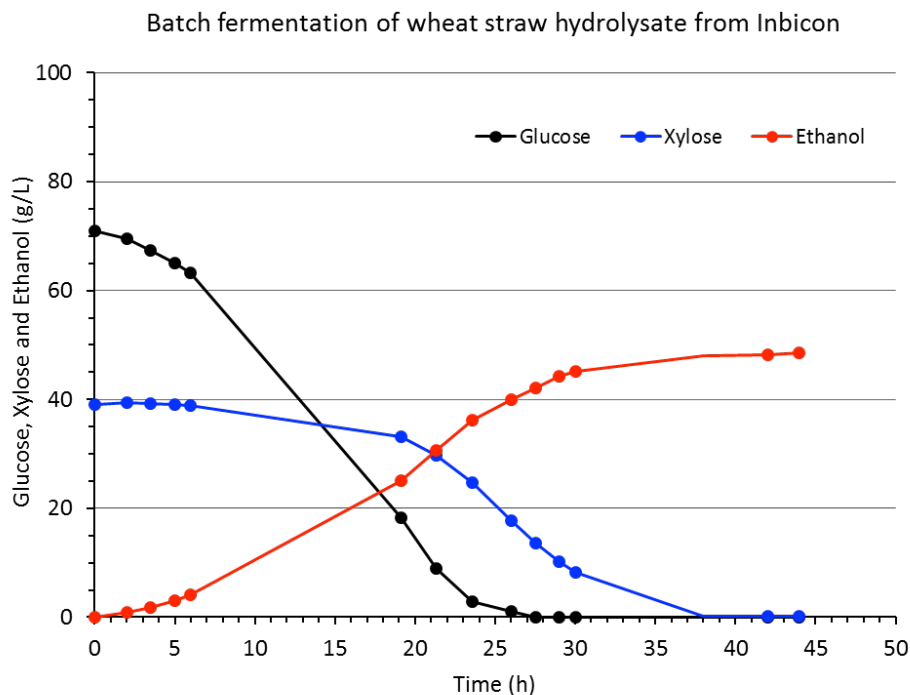
Fig. 8. V1 batch fermentation of wheat straw hydrolysate from Inbicon.

This material contains more than 7 g/L acetic acid and fermentation conditions were 1 g/L yeast inoculum at pH 5.5. Complete fermentation was obtained within 48 hrs giving an ethanol yield of 91% and a final ethanol concentration of 50 g/L. Also, co-fermentation of glucose and xylose is observed.

To conclude batch fermentation testing of strain V1 in the frame of this project Terranol, Inbicon and Novozymes entered into a joint exploratory research agreement. Pilot scale batch fermentations (500L) of wheat straw hydrolysate prepared by Inbicon using Cellic CTec2 enzymes from Novozymes were conducted. Fermentations were run in a hydrolysate containing app. 60 g/L glucose and app. 30 g/L xylose at 30°C, pH of 5.2, 3 g/kg of urea and 1 g/l of yeast inoculum (Fig. 9).

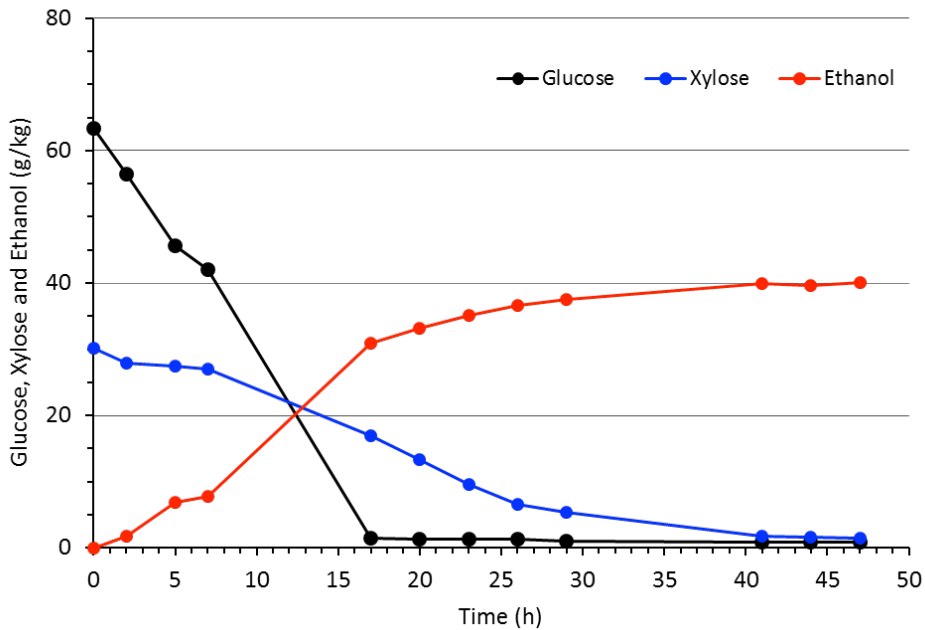Pilot scale batch fermentation of wheat straw hydrolysate from Inbicon

Fig. 9. Pilot scale batch fermentation of V1 employing wheat straw hydrolysate

This subproject demonstrated that no problems were encountered in the upscaling process and that the same results can be achieved in larger scale as an overall ethanol yield of 90% of the theoretically possible was achieved. Glucose was digested within the first 18 hrs and the fermentation was completed within 42 hrs reaching an ethanol concentration of more than 4 g/kg.

Through development of fed-batch strategies employing automatic feed control the ethanol yield and productivity can be further improved. In the example shown below (Fig. 10) (1 g/L total yeast inoculum, pH 5.5) an ethanol yield of 93% and final ethanol concentration of 55 g/l is obtained within 48 hrs.
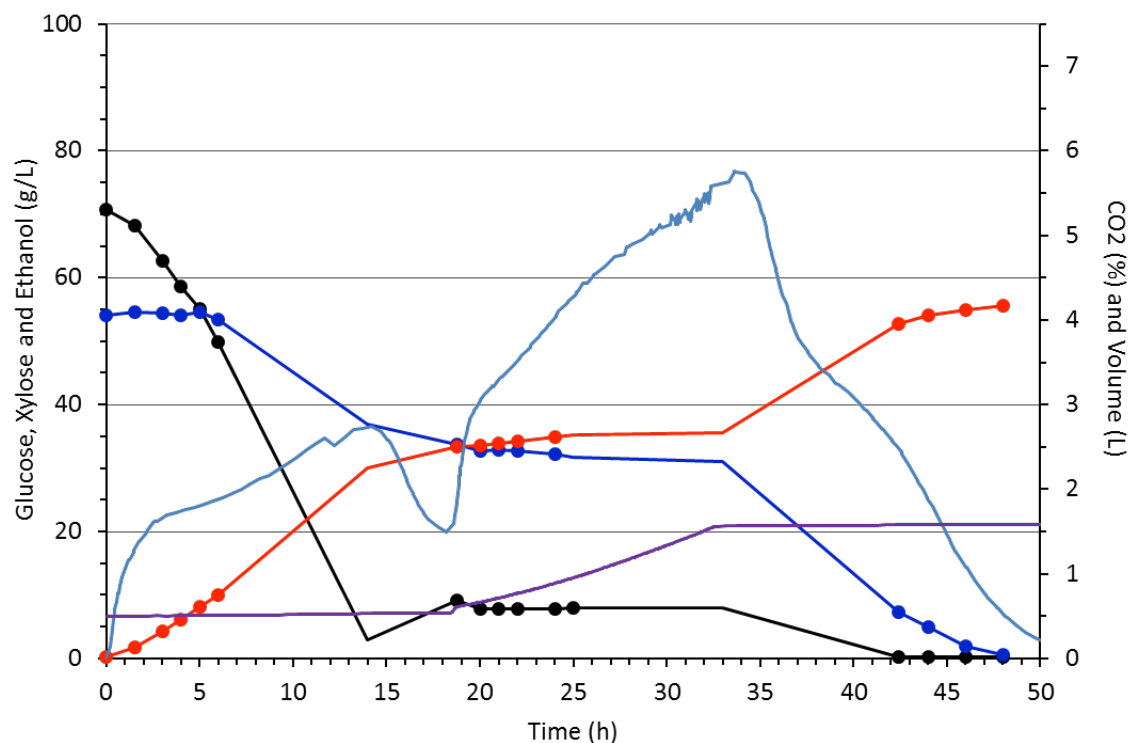
Fig. 10. Fed-batch fermentation of corn stover hydrolysate with automatic feed control. Glucose (•), xylose (•), ethanol (•), volume (-), CO2 (-)

Weak acid detoxification ability

Acetic acid is released from lignocellulosic biomass during the pretreatment process and is regarded as a growth inhibitor. A technique for conferring ability to reduce acetic acid to ethanol at the expense of ATP and NADH has been tested in a laboratory strain. It was originally planned to implement this technology in the entry strain, but the long-term adaptation process of the yeast, which was described previously, demonstrated that we could develop yeast that tolerated more than 100mM acetic acid simply by adaptation. Thus, this line of work was not pursued further.

Addition of arabinose fermentation ability

Like D-xylose, L-arabinose is metabolized through the pentose phosphate pathway after an initial conversion into D-xylulose-5-phosphate. This three-step conversion is well known from the literature, and patents concerning arabinose metabolizing *S. cerevisiae* strain generation are confined to the use of specific combinations of certain genes from certain bacteria thus, there is freedom to operate outside those combinations.

As mentioned earlier, 80-90% available pentose from biomass material is xylose and therefore the main focus has been on xylose. In this project work has been initiated to prepare the yeast for addition of arabinose fermenting ability by synthetizing the genes *AraA*, *AraB* og *AraD* relevant for conversion of L-arabinose to D-xylulose 5-phosphate: These

genes have been combined with various promoters resulting in expression cassettes which then at a later point will be inserted in the yeast.

Fermentation results with strain V1 can thus be summarized as follows:

- A low yeast inoculum (0.25-1 g/L) is viable for fermentation of lignocellulosic hydrolysates
- Ethanol titers up to 7 % (w/w) can be achieved
- Ethanol yields well above 90 % of theoretical (on glucose and xylose) can be achieved
- Complete fermentation of all sugars (glucose and xylose) available can be fermented within as little as 48 hrs
- The strain is inhibitor tolerant
- The strain can tolerate high acetic acid (e.g. above 7 g/L) and ethanol concentrations
- Low xylitol formation, below 0.5 g/L
- Cell mass and glycerol are the only major fermentation products – apart from ethanol

In conclusion, we have combined rational engineering for efficient xylose fermentation and evolutionary engineering, inserted an efficient proprietary XI, relieved an unidentified bottleneck by epimerization of α and β forms of D-xylose, enhanced PPP; all by stable chromosomal integration into an industrial strain which was previously demonstrated to perform well under industrial conditions.