NEMO Newsletter – April 2013

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The NEMO project

In the EU-funded project NEMO (Novel high performance enzymes and micro-organisms for conversion of lignocellulosic biomass to bioethanol), 19 partners from R&D and industry have been working on the development of technological improvements for the production of ethanol from lignocellulosic feedstocks such as wheat straw, giant reed (*Arundo donax*), and softwood spruce.

The three major project objectives are the discovery and optimization of new cellulases and hemicellulases, the development of robust, C6 and C5 sugar co-fermenting yeast strains, and the integration of novel enzymes and microbes into optimized production process configurations.

For more information on the NEMO project, please visit http://nemo.vtt.fi.

NEMO has been funded by the European Commission in the 7th Framework Programme (Project No. FP7-222699). Project duration was four years and activities started in May 2009.

In this Newsletter - NEMO Exploitable Results

In this NEMO newsletter, we would like to present several flyers elaborated to summarize Exploitable Results developed by partners of the NEMO Consortium, namely on 'Industrial yeast strains fermenting C6 and C5 sugars', 'Specific xylose and arabinose transporters', 'Novel hemicellulases', 'Overexpression of the *S. cerevisiae* gene *MCR1* in cell factory(ies)', 'Improved processes for the hydrolysis of pelletized biomass', and 'Engineered thermostable cellobiohydrolases'.

For more information on NEMO exploitable results, please contact the respective NEMO partner indicated in the flyers, the NEMO coordinator Prof. Merja Penttilä from VTT (merja.penttila@vtt.fi), or Dr. Rainer Janssen from WIP (rainer.janssen@wip-munich.de).

Result 1: Industrial yeast strain fermenting C6 and C5 sugars

An industrial *Saccharomyces cerevisiae* strain for fermentation of mixed-sugar lignocellulosic hydrolysates has been constructed which has expression cassettes for overexpression of an optimised pentose phosphate pathway stably integrated in its genome. Moreover, it carries overexpression constructs for the *Clostridium phytofermentans* xylose isomerase and a xylulokinase, as well as an optimised bacterial arabinose utilisation pathway. The recombinant yeast is further optimised for xylose and arabinose fermentation by evolutionary engineering. It will ferment mixtures of glucose, xylose and arabinose efficiently according to industrial requirements.

Flyer download: http://nemo.vtt.fi/Flyer_1_NEMO-ExploitableResult-1-GUF-120601.pdf

Result 2: A specific xylose transporter evolved by using a newly developed yeast-based screening system

The NEMO partner GUF has developed a new screening system for improved xylose utilisation of yeast strains in the presence of glucose. GUF has constructed a hexokinase/hexose transporter-deficient recombinant yeast strain, which is no longer able to utilise glucose, but expresses a xylose utilisation pathway. In this strain single sugar transporters with limited specificity for xylose have been expressed. However, in the presence of glucose, xylose cannot be transported by the sugar transporter as glucose inhibits nearly all sugar transporters. Suppressor mutants/mutations have been selected which enable the strain to utilize or ferment xylose even in the presence of increasing concentrations of glucose. The work has led to engineering of a specific xylose transporter which is no longer inhibited by glucose.

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Result 3: A specific arabinose transporter for improved fermentations of lignocellulosic hydrolysates with recombinant yeasts

Lignocellulosic hydrolysates used for the production of 2nd generation biofuels typically contain sugar mixtures consisting of glucose and xylose, and minor amounts of arabinose. The yeast *Saccharomyces cerevisiae* is the preferred microorganism for the fermentative production of ethanol but is only able to ferment the pentose sugars xylose and arabinose after genetic engineering. Although pentose fermenting *S. cerevisiae* strains have been constructed recently, pentose uptake is still a limiting step in mixed-sugar fermentations. The NEMO partner GUF has identified an arabinose transporter that can be expressed in *S.cerevisisae* in functional form.

Flyer download:

Result 4: Novel hemicellulases derived from *Myceliophthora thermophila* C1 for the degradation and modification of plant materials

Five novel hemicellulases have been found and produced in *Myceliophthora thermophila* C1. These hemicellulases include two different xylanases, one arabinofuranosidase and two acetyl xylan esterases, which are involved in the degradation of the hemicellulosic structure (arabino)xylan. Xylans are found in all plant materials and occur in high amounts in crops that are in use for the production of biofuels (up to 25%). These enzymes degrade the hemicellulose and therewith improve the complete enzymatic hydrolysis of the feedstocks. Besides the production of biofuels such enzymes are also used in other industries, such as the food/feed or the pulp & paper industry.

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Result 5: Overexpression of the *S. cerevisiae* gene *MCR1* in cell factory(ies)

In industrial processes the organism used for production is exposed to several stresses that can lead to lower production titers, lower production rates and lower yield of the product. Studying how to improve the cell factory robustness as well as gaining deep understanding of its innate potentiality can help in overcoming this challenging bottleneck. One way to obtain or improve strain robustness is the manipulation of the antioxidant capacity of the cell, since many stressor agents lead to accumulation of reactive species, mainly oxygen species (ROS). A yeast strain overexpressing the *MCR1* gene (encoding for a mitochondrial NADH-cytochrome b(5) reductase) demonstrated higher resistance to stresses such as oxidative stress. These cells performed better and had higher ethanol productivity than the reference strain.

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Result 6: Procedure and apparatus for hydrolysis of pelletized biomass using hydrogen halides

When hydrochloric acid was used in the past as an agent for saccharification it was almost exclusively applied to wood. This was for two reasons. Firstly, the dry matter of wood exceeds in density that of other biomasses such as straw or grass two to three-fold when filled into the hydrolysis unit. Accordingly an equal throughput corresponds with a two to threefold increase in terms of the installed hydrolysis capacity. Secondly, biomass with a lower level of lignification such as straw showed a tendency to clog the reactors when acid is replaced by water, leading to an interruption of the procedure applied. Simple solutions were invented leading to a five-fold increase in material load when straw is used. This was reached by using a mixture of chaff and pellets instead of only pellets. In comparison to wood an increase of 30-40% could be reached. Further, by simple modification of the reactors the clogging can be avoided.

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Result 7: Engineered thermostable cellobiohydrolases for improved lignocellulose hydrolysis

Despite extensive screening and protein engineering efforts, there is still a need for better enzymes for the bioprocessing of lignocellulosic biomass. At present, the *Trichoderma* cellulases are the most commonly used industrial enzymes in biomass hydrolysis and other applications. The industrial processes are normally carried out at 45-55°C due to the instability of these fungal enzymes to tolerate higher temperature. For applications it would, however, be desirable to have enzymes that are more active on crystalline substrates and/or work at higher temperatures. Thermostable enzymes could additionally render the enzymes stable against other harsh application conditions i.e. inhibitors, solvents, etc. VTT has improved the thermostability and activity of a fungal GH7 family cellobiohydrolase by different protein engineering approaches. Several cellobiohydrolase variants having improved activity in biomass hydrolysis have been created. Superior performance at elevated temperatures has also been demonstrated.

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Result 8: Industrial yeast strain combining inhibitor and temperature tolerance

An industrial *Saccharomyces cerevisiae* strain named ISO12, derived from the industrial strain Ethanol Red, has been isolated from a long-term adaptation experiment using spruce hydrolysate and increasing temperatures. In contrast with Ethanol Red, ISO12 is able to grow and ferment undetoxified spruce hydrolysate at 39°C without prior strain adaptation. ISO12 represents a good platform for strain engineering as well as for the identification of tolerance factors.

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Result 9: Robust xylose fermenting industrial yeast strain for bioethanol production

A robust industrial yeast strain has been developed that efficiently converts xylose into ethanol. The strain was developed by mutagenesis and multiple rounds of evolutionary engineering, including genome shuffling from an Ethanol Red strain expressing a bacterial xylose isomerase. Further improvements were introduced by targeted genetic engineering using superior allele tools for improvement of complex traits. The new strain efficiently produces ethanol close to theoretical maximum yield from both hexoses and xylose in lignocellulose hydrolysates.

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Result 10: **Optimized temperature profile in simultaneous saccharification and fermentation**

The process option simultaneous saccharification and fermentation (SSF) is an interesting process option since end-product inhibition on hydrolysis can be minimized by the removal of sugars by fermentation. Also the capital costs may be decreased due to the integration of enzymatic hydrolysis and fermentation.
However, the process temperature needs to be a compromise between the optimum temperature for enzymatic hydrolysis and that for fermentation. A procedure has therefore been designed to find the optimum temperature for a non-isothermal SSF process. Depending on the feedstock used, process advantages in terms of in particular a shorter process time can be accomplished.