NEMO

Novel high-performance enzymes and micro-organisms for conversion of lignocellulosic biomass to bioethanol

(EC project no 222699)
1.5.2009 – 30.4.2013

Final summary report

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4.1. Final publishable summary report

4.1.1. Executive summary

NEMO: Novel high-performance enzymes and micro-organisms for conversion of lignocellulosic biomass to bioethanol (EC project no 222699)

The NEMO project was carried out by 20 European partners during the years 2009-2013. The project focused especially on finding novel efficient enzymes capable for hydrolysing lignocellulose based raw materials and development of robust fermenting yeasts that can co-ferment pentose and hexose sugars. The NEMO project generated a great number of technological improvements that can be broadly exploited in production of 2nd generation bioethanol and in biorefineries in general.

Several strategies were used for identification of new efficient enzymes: screening of a large number of new fungal isolates from nature and culture collections, using metagenomic and cDNA library approaches, protein engineering and mining microbial genomes for yet unexplored enzyme encoding genes. These efforts provided cellulolytic and hemicellulolytic enzymes that are more thermostable and more efficient for hydrolysis of the resistant structures in lignocellulose, and enzymes with reduced affinity to lignin. Some enzymes with exceptional temperature optimum (90 - 100°C) were found from metagenomic libraries and enzyme engineering provided thermostable CBHI variants (75-79°C). The feasibility of production of many of the enzymes in filamentous fungal hosts in industrially interesting amounts was demonstrated. Thermo- and mesophilic enzyme mixtures were optimised for the chosen, relevant European raw materials, spruce, wheat straw and the energy crop *Arundo donax*.

The yeast strains used for bioethanol production need to be tolerant to process conditions and capable of converting all substrate sugars to high concentrations of ethanol. Screening of yeast strains from various environments and culture collections resulted in a NEMO yeast bank with more than 1000 strains. Novel natural *S. cerevisiae* strains that are clearly more temperature, ethanol, inhibitor and/or osmotolerant than current *S. cerevisiae* strains (e.g. Ethanol Red) were isolated, and genes responsible for the tolerances identified. Strains were further improved through evolutionary and genetic engineering. Several strains demonstrated excellent ethanol tolerance and growth at increased ethanol concentrations (up to 18-19%), and capability to grow at elevated temperatures (40-41°C) in the presence of lignocellulosic hydrolysates. Genes involved in improved pentose fermentation including novel sugar transporters were identified. Most importantly, using a robust industrial strain as a parent strain, new engineered strains were generated that have xylose fermentation rates that exceed current published results.

The new enzymes (together with some propriety enzymes from the NEMO industrial partners) and the new engineered yeast strains were tested in various process conditions (SSF and SHF) with lignocellulose hydrolysates as substrates, as well as in new regimes where high temperature prehydrolysis was applied as well as novel schemes for non-isothermal SSF. Clear improvements over reference enzymes and yeasts strains were obtained. Pilot scale experiments were carried out by combined efforts of the NEMO industrial partners using spruce (SHF) and *Arundo* (SSF) as raw materials confirming the improvements assessed in lab-scale. Process models were prepared and technoeconomic and LCA calculations carried out. The calculations indicated that the use of the NEMO enzymes and yeast strains in the process results in improved plant efficiency, decreased greenhouse gas emissions and lower estimated ethanol selling price when compared to the reference cases.

4.1.2. NEMO – Project context and objectives

The NEMO project was granted in the 7th Framework programme Call KBBE-2007-3-2-06: Bioethanol and beyond - Novel enzymes and microorganisms for biomass conversion to bioethanol. The project has developed new high performance enzymes and robust yeast strains and demonstrated their validity in process concepts considered to be the most relevant ones for bioethanol production (SHF, SSF) and in new process configurations addressed in NEMO. The overall goal has been to develop technologies allowing increased and more economical utilization of chosen European renewable lignocellulosic raw materials for the production of second generation bioethanol. The European raw materials chosen were the energy crop Arundo (giant reed), wheat straw, and softwood spruce.

The 2nd generation bioethanol processes that use lignocellulosic feedstock as raw material instead of starch or cane sugar were still in the development phase when the NEMO project was started. Meanwhile the field has developed and building of first commercial plants also by European companies has been initiated. Nevertheless, significant cost reductions and technological breakthroughs are still needed to make the processes truly economically feasible, particularly in Europe where raw material costs are high. Also other products than ethanol are produced or planned to be produced from renewable plant material, such as 1,3-propanediol, butanol, succinic and lactic acid. Lignocellulosic production of these products is yet to be realised. Ethanol is distilled while recovery of the other products from processes based on complex lignocellulosic materials is still a challenge reducing the overall production economy. The microbe used for production also suffers a combined stress caused by high titers of the product and inhibiting compounds present the lignocellulose hydrolysates. Consequently, how to improve production of cheap sugars and the robustness of the production microbes will remain key issues in biorefinery development also in continuation.

The two **main objectives** of the NEMO project were to generate:

- Novel thermostable and high performance enzymes and enzyme mixtures for efficient hydrolysis of the chosen lignocellulosic raw materials of European interest. Thermostability of the enzymes is important since it is expected to increase the rate of hydrolysis, general robustness of the enzymes and reduce contamination risk.

- Novel stress tolerant yeast strains for efficient fermentation of the biomass derived C6 and C5 sugars to ethanol with yields and productivity approaching that of the current 1st generation substrates. The yeast *Saccharomyces cerevisiae* is the most efficient industrial ethanol production organism. It is also favoured as a host for production of other biofuels or renewable chemicals.

More specifically, some main **enzyme development aims** of the NEMO project were to provide:

- Novel thermophilic cellulases and hemicellulases for high temperature hydrolysis at up to 70-85ºC
- Novel cellulases that have higher specific activity towards crystalline and amorphous cellulose than the existing enzymes and that are less end-product inhibited
- Cellulases that show optimal binding to substrate with minimal adsorption to lignin
- Novel thermo- and mesophilic raw material specific hemicellulases (e.g. side-chain cleaving esterases) that improve solubilisation of lignin-hemicellulose complexes and hydrolysis of hemicellulose

- Enzymes that can be produced in industrial fungal production hosts in bulk amounts

More specifically, the main **yeast development aims** of the NEMO project were to provide:

- Novel engineered *S. cerevisiae* hosts that convert the biomass C5 sugars xylose and arabinose with highest possible yields to ethanol and with preferably rates approaching that of glucose
- Novel *S. cerevisiae* strains that efficiently co-ferment the C6 and C5 sugars
- Novel robust *S. cerevisiae* hosts that are more stress tolerant than the currently existing strains, i.e. with combined ethanol, acid and inhibitor tolerance, e.g. capable of producing ethanol in the presence of inhibitors and 16% ethanol at 38°C or higher at similar rates than the current strains used in ethanol production
- Knowledge on mechanisms behind stress and inhibitor tolerance that can be adapted to other potential micro-organisms of interest

The work to reach these goals was divided into seven work packages (WP) as shown in Fig. 1 below. The Coordinator of the project was Merja Penttilä (VTT). The work package leader for WP1 was Frank Kose (Green Sugar GmbH), WP2 Ronald de Vries (University of Utrecht), WP3 Liisa Viikari (University of Helsinki) and Anu Koivula (VTT), WP4 Johan Thevelein (VIB), WP5 Laura Ruohonen (VTT), WP6 Lisbeth Olsson (Chalmers University of Technology), and WP7 Tommaso Di Felice (Chemtex Italia srl).

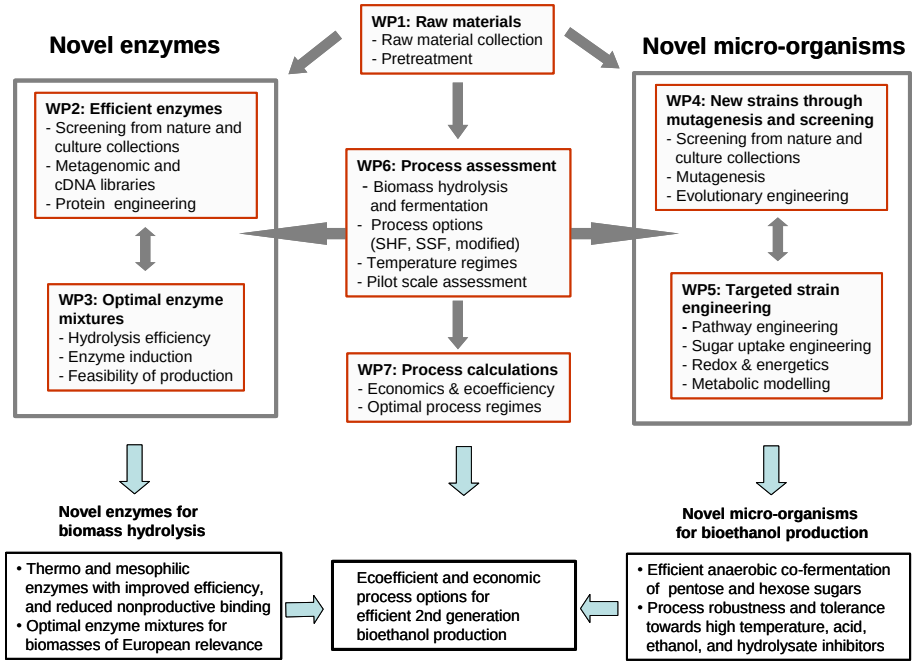

Figure 1: Outline of the NEMO project with WPs and expected outcome.

Basically, NEMO has successfully addressed all these aims mentioned above. It can now be envisioned that the **expected impacts,** which were outlined for NEMO if the project would succeed in its RTD goals, will be realised in industrial applications. These include:

- Reduced enzyme cost due to higher hydrolysis potency of the novel mesophilic and thermophilic enzyme preparations
- Decreased hydrolysis time due to improved activity, substrate binding and thermostability of the novel enzymes
- Decreased ethanol production price resulting from higher yields of ethanol from feedstock carbohydrates due to C5 sugar fermentation
- Reduced process running costs resulting from higher rates of C6 and C5 sugar co-fermentation
- Reduced bacterial contamination risk and better general reliability of the process due to higher stress tolerance of the fermenting microbe
- Reduced process costs due to the suitability of the novel enzymes and yeasts for high biomass consistency processes that result in high ethanol concentrations and reduced volumes of liquid streams in the fermentation residues and in the production plant
- Overall benefits in different types of bioethanol processes (SSF, SHF and novel modifications) through the use of the novel improved enzymes and fermentation organisms developed
- Improved ecoefficiency through development of more efficient 2nd generation biomass conversion and fermentation processes based of the most relevant biomasses for Europe (e.g. straw, wood residues, energy crops)

Table 1 shows the 20 Beneficiaries that took part in the NEMO project.

Table 1: List of NEMO Beneficiaries

Beneficiary Number	Beneficiary name*	Beneficiary short name	Country	Date enter project	Date exit project
1 (Coord.)	Teknologian tutkimuskeskus VTT *Prof. Merja Penttilä*	VTT	Finland	1	48
2	Lunds Universitet *Prof. Marie-Francoise Gorwa-Grauslund*	ULUND	Sweden	1	48
3	Helsingin yliopisto *Prof. Liisa Viikari*	UH	Finland	1	48
4	Universiteit Utrecht *Dr. Ronald de Vries*	UU	Netherlands	1	48
5	VIB *Prof. Johan Thevelein*	VIB	Belgium	1	48
6	Chalmers Tekniska Hoegskola Ab *Prof. Lisbeth Olsson*	CTH	Sweden	1	48
7	Johann Wolfgang Goethe Universitaet Frankfurt am Main *Prof. Eckhard Boles*	GUF	Germany	1	48
8	Ecole Polytechnique Federale de Lausanne *Prof. Vassily Hatzimanikatis*	EPFL	Switzerland	1	48
9	Universite de la Mediterranee D'aix-Marseille II *Prof. Pedro Coutinho*	UNIVMED	France	1	48
10	Universita Degli Studi di Milano – Bicocca *Prof. Danilo Porro*	UNIMIB	Italy	1	48

11	Koninklijke Nederlandse Akademie van Wetenschappen – KNAW *Dr. Teun Boekhout/Dr. Ronald de Vries*	KNAW	Netherlands	1	48
12	Univerza V Ljubljani *Prof. Ana Plemenitas*	UL	Slovenia	1	48
13	Chemtex Italia srl *Dr. Alessandra Frattini/Tommaso Di Felice*	CTXI	Italy	1	48
14	Dyadic Nederland BV *Dr. Jan Wery /Dr. Sandra Hinz*	DNL	Netherlands	1	48
15	Green Sugar GmbH *Dipl-ing. Frank Kose*	GS	Germany	1	48
16	Sekab E-Technology *Dir.Dr. Sune Wännström/Karin Hägglund and Marlene Mörtsell*	SE-T	Sweden	1	48
17	Syngenta Crop Protection AG *Dr. Arthur Steiger*	SCPAG	Switzerland	1	6
18	Roal Ou *Dr. Terhi Puranen/Dr Jari Vehmaanperä/*	Roal	Finland	1	48
19	c-LEcta GmbH *Dr. Klaus Pellengahr/Dr. Mathias Salomo*	c-LEcta	Germany	17	48
20	Wirtschaft und Infrastruktur GmbH & Co Planungs KG *Dr. Rainer Janssen*	WIP	Germany	25	48

*The original and latest names of the scientific contact persons and institutes are shown.

4.1.3. NEMO – Main scientific and technological results

4.1.3.1. Raw materials (WP1)

The main objectives of this WP were to collect and pretreat the selected raw materials of Southern and Northern European interest, an energy crop, straw from agriculture and wood, and to ensure a constant supply of these to the Consortium members from the very beginning of the project. This ensured that all enzyme and yeast work was targeted to suit industrially relevant and appropriately pretreated raw materials.

Raw materials

Before the start of the project the raw materials of choice were discussed and a safe supply of 9 different raw materials could be foreseen, including soft- and hardwood, residue streams from agriculture (straw) and energy crops (*Arundo donax, Miscanthus gigantheus*). The final choice of the raw materials was based on the expertise and insight of the NEMO industrial partners: Arundo (giant reed) (CTXI), softwood spruce (SE-T), and wheat straw (SCPAG, GS).

The NEMO industrial partners (CTXI, SE-T, GS, SCPAG) grow themselves or had good access to these European feed stocks. CTXI (Chemtex) provided throughout the project sufficient amounts of Arundo and SE-T (SEKAB) provided spruce, and SCPAG (Syngenta), a NEMO Beneficiary in the very beginning of the project, provided a large amount of wheat straw.

Arundo and wheat straw are similar in composition having a significant amount of pentose-rich hemicellulose (about 28-32% of dry weight), which consists of about 84-89% xylose and about 8-11% arabinose. The hemicellulose in spruce consists mainly of C6 sugars (glucose, mannose, galactose), which are easily naturally fermented by yeast, while xylan content is low (< 9%).

Pretreatment

Steam explosion

Since NEMO's goal was not to develop new pretreatment methods, the well-established and generally used pretreatment methods applying state-of-the-art steam explosion combined with acid treatment (mild or stronger) was used. Several NEMO partners had lab or pilot scale equipment for this method (e.g. ULUND, VTT, GUF, CTXI, GS). Steam explosion combined with stronger acid hydrolysis was used for spruce, routinely carried out by SE-T in large scale. In practise, the industrial partner CTXI provided most of pretreated Arundo and SE-T pretreated spruce, using their optimal in-house pretreatment protocols.

Chemical pretreatment and hydrolysis as a reference

GS (Green Sugar) worked on pretreatment of all the three raw materials. As a reference to steam explosion and enzyme treatment, GS evaluated in NEMO a new chemical method, which is based upon the "Bergius-Rheinau-Verfahren" technology for wood hydrolysis (Riehm, T. (1955) Verfahren und Einrichtung zur Hydrolyse von zellulosehaltigem Material mit hochkonzentrierter Salzsäure. DE927139B). This method generates a pure xylose fraction and sugar streams devoid of inhibitors and having sugar concentrations > 40%. Additionally, separate streams of carbohydrates with different degrees of hydrolysis such as dimers and tetramers can be produced. This approach allows testing in WP6 process options with a minimal amount of enzymes and yeast performance assessment in a completely different environment than the more standard hydrolysates.

The possibility to use a combination of HCl acid and enzymatic treatment in order to reduce the amount of acid and enzymes needed was evaluated but was not found feasible. Thus full acid hydrolysis method was a focus and was shown to produce after optimisation sugar streams that suited for yeast fermentations (GS: **Exploitable result 6**, p. 47; GS: **Patent appl.**, p. 48). Improvements were made to reduce the amount of acid needed and to modify the recycling method to suit industrial scale. Furthermore, the straw loading to the system could be significantly increased through a simple innovation of using a mixture of pellets and chaff instead of chaff only. All three NEMO raw materials are suited for this pretreatment/hydrolysis method. Thus, new combination of pretreatments is possible, which widen the variety of possible unit operations that can be economically compared.

Provision of pretreated materials

The main objective was to provide pretreated raw materials of consistent quality for all relevant partners to enable appropriate screening of enzymes and yeasts trains, and to enable comparable assessment conditions. A code system to trace each individual sample was generated and GS coordinated the provision of raw materials to partners. In total, 111 raw material samples have been distributed to a number of partners, which shows the integrated approach of activities targeted to the relevant raw materials from early on in the NEMO project.

In particular at the beginning of the project pretreatment conditions were modified based on the feedback from partners. Also special samples were prepared to suit identification of novel enzymes (WP2) or preparation of optimised enzyme mixtures (WP3), or for yeast screening (WP 4-5). These were e.g. samples with higher amounts of unhydrolysed xylan or fractions with oligomers, or less or more inhibitor containing samples, respectively.

Analyses of the hydrolysed and/or fermented materials were carried out in appropriate task contexts with methods that were harmonised between partners as relevant. Due to the partly confidential pretreatment methods of the companies providing the raw materials, figures on the pretreatment conditions and sample characteristics cannot be made public.

4.1.3.2. Identification of novel efficient enzymes (WP2)

The first major objective of the NEMO project was to identify novel enzymes for more efficient hydrolysis of the chosen raw materials. The cost of enzymes for hydrolysis of the feed stocks to fermentable sugars is still a major factor of the overall production costs of bioethanol and other biofuels. It is therefore essential to improve not only the production of the enzymes, but also to design more efficient enzyme cocktails to either enable lower enzyme loading or to reach a higher yield of released sugars using the same enzyme loading.

The targets set for this WP were to identify individual novel enzymes or engineer enzymes that are more thermotolerant, have higher specific activity, reduced end-product inhibition and/or show less non-productive binding to the substrate than the commercially available enzymes, or which are lacking or limiting in the commercial enzyme preparations. Screening was targeted to the expected most important enzyme classes (cellobiohydrolase, hemicellulases) and from the very beginning of the project on enzymes that would be active on the chosen pre-treated raw materials.

The NEMO partners had access to various enzymes and unique microbial culture collections including large collections of mesophilic and thermophilic wood and litter decomposing basidiomycetous and ascomycetous fungi and bacteria, and unique metagenomic libraries. Furthermore, discovery of enzymes and substrate-binding modules were carried out through comparative approaches based on analysis of the public and in-house genomic data against the

CAZy database (www.cazy.org), and interesting individual enzyme sequences were identified or selected species chosen for construction of expression libraries. Individual genes or gene libraries were screened and expressed either in prokaryotic (*Escherichia coli*) or in eukaryotic hosts, such as *Trichoderma*, *Aspergillus*, and/or *Myceliophthora*. Furthermore, protein engineered versions of the enzymes were initially expressed in *S. cerevisiae*.

Screening from culture collections

In total 125 fungal strains from the culture collections were screened for relevant enzyme activities by KNAW, UL, UH and VTT. Fungal strains were grown on the pretreated substrates to select strains that produced efficient enzymes for hydrolysis of these substrates. Enzymes were identified in these strains by protein purification (UU, UH, VTT) or analysis of gene expression using RNA sequencing technology (DNL). Screening of the thermophilic ascomycetes for hemicellulolytic activities resulted in *Myceliophthora heterothallica* as the most promising species. This strain showed very good growth on the NEMO substrates that were rich in hemicellulose, while growth on substrates with low hemicellulose content was poor. For cellulolytic enzymes 86 basidiomycete wood rotting fungi were screened for their ability to degrade cellulose (UH). The most promising strain was *Dichomitus squalens* as this showed the highest cellobiohydrolase (CBH) activity during growth on Avicel medium. These strains were used for identification of genes and enzymes.

Screening from genomic databases

Fungi with available in-house or public genome sequences were grown and analysed on the NEMO substrates (UU). Their genome sequences were mined for putative genes encoding these activities as well as activities that are not present in the basic enzyme mixtures.

For screening from public genomic databases the first step was to compare the CAZy content of fungi with sequenced genomes for the total number of (hemi)cellulases (UU, UNIVMED). This resulted in a selection of 14 species with enriched genomes for these functions (**Table 2**).

Table 2: Species for which the genome is enriched in (hemi)cellulases.

Species	Class	Phylum
Phanerochaete chrysosporium	Agaricomycetes	Basidiomycetes
Coprinopsis cinerea	Agaricomycetes	Basidiomycetes
Schizophyllum commune	Agaricomycetes	Basidiomycetes
Aspergillus nidulans	Eurotiomycetes	Ascomycetes
Aspergillus terreus	Eurotiomycetes	Ascomycetes
Aspergillus niger	Eurotiomycetes	Ascomycetes
Fusarium oxysporum	Sordariomycetes	Ascomycetes
Nectria haematococca	Sordariomycetes	Ascomycetes
Trichoderma virens	Sordariomycetes	Ascomycetes
Chaetomium globosum	Sordariomycetes	Ascomycetes
Podospora anserine	Sordariomycetes	Ascomycetes
Magnaporthe oryzae	Sordariomycetes	Ascomycetes
Verticillium dahlia	Sordariomycetes	Ascomycetes
Phaeosphaeria nodorum	Dothideomycetes	Ascomycetes

Amino acid sequences encoded by the genes from these species were used for a phylogenetic analysis together with the sequence from characterised enzymes to identify candidate targets likely to differ in their enzymatic properties from previously known enzymes. This yielded a very promising set of endoxylanase targets from *P. anserina*, while a promising α-L-arabinofuranosidase

was identified in *F. oxysporum* (UU). Most promising cellobiohydrolase targets were identified in *C. cinerea* and four GH7 genes were selected for expression in *T. reesei* (Roal).

In parallel to the above mentioned CAZy analysis, analysis of VTT's in-house fungal genomic database for candidate GH7 cellulases was carried out. Three genes were originally chosen based on sequence similarity to known CBHI type of cellobiohydrolases for expression studies in *T. reesei* (VTT, Roal). Additionally, growth screening of thermophilic fungal strains was performed resulting in selection of one thermophilic strain, for which in-house partial genomic data was available (VTT). Cloning of three putative GH7 cellulase genes from this strain was carried out, but only one of these GH7 cellulases was readily produced in *T. reesei*, whereas poor production was obtained with the other two cellulase genes (VTT).

Furthermore, the genome of *Myceliophthora thermophila* (previously known as *Chrysosporium lucknowense*) C1 was analysed for candidate hemicellulases and 14 genes were identified for expression (DNL) (DNL: **Exploitable result 4**, p. 47).

Screening of cDNA and metagenomic libraries

Screening of bacterial metagenomic libraries expressed in *E. coli* on substrate plates resulted in the identification of four candidate endoglucanases and two xylanases that had good activities at higher temperatures (VTT).

Based on the above mentioned growth screening *M. heterothallica* was selected as the most promising thermophilic ascomycete (KNAW). Rather than generating a cDNA expression library, it was decided to grow two strains of this species on hemicellulose-rich NEMO substrates and perform RNA sequencing on samples from these cultures. This resulted in the identification of several new enzymes from which a CE3 xylan acetyl esterase has been selected to be produced (DNL).

Summary of the cloned and expressed enzyme encoding genes

As a conclusion, the different screening and protein engineering approaches mentioned above provided a number of candidate proteins and gene sequences with possibly unique and desired properties for further study. To be able to assess even at the preliminary level the enzyme properties, the genes needed to be cloned, codon optimised and expressed first at smaller scale in appropriate expression hosts for protein purification.

The expression hosts used in NEMO were *E. coli*, *B. subtilis*, *T. reesei*, *Pichia pastoris*, *S.cerevisiae*, *M. thermophila* and *Aspergillus vadensis*. In total 35 genes were expressed successfully covering 6 different activities. In some cases, more than one expression host was also tested (**Table 3**).

Table 3: Overview of the selected cloned and expressed new genes originating from different screening approaches (DNL, UU, VTT, UH, ROAL).

Activity	No. of genes	CAZy family	Expression host	Screening approach
Cellobiohydrolase	11	GH7	*T. reesei*	Genome mining, Culture collection screening
Cellobiohydrolase	5	GH6/GH7	*P. pastoris*	Culture collection screening
Endoglucanase	4	GH5	*E. coli/B. subtilis/T. reesei*	Metagenomic library screening
Endoxylanase	2	GH10/GH11	*M. thermophila*	Genome mining

Endoxylanase	2	GH8/GH11	*E. coli/T. reesei*	Metagenomic library screening
Endoxylanase	2	GH10/GH11	*A. vadensis*	Culture collection screening
Arabinofuranosidase	1	GH43	*M. thermophila*	Genome mining
Arabinofuranosidase	1	GH51	*A. vadensis*	Culture collection screening
Arabinofuranosidase	1	Nd	*E. coli*	Genome mining
Acetyl xylan esterase	3	CE1/CE3/CE5	*M. thermophila*	Genome mining, RNA sequencing
Acetyl xylan esterase	1	Nd	*E. coli*	Genome mining
β-xylosidase	1	Nd	*E. coli*	Genome mining
Feruloyl esterase	1	n/a	*M. thermophila*	Genome mining

Improvement of cellobiohydrolase properties

Seven different GH7 cellobiohydrolase variants with improved thermostability and activity were made using site-directed mutagenesis and shuffling of different carbohydrate-binding modules (CBMs). The cellulase variants were produced either in *S.cerevisiae* or in *T.reesei* (VTT, Roal) and purified. All variants were clearly more thermostable than the reference enzyme, *T.reesei* CBHI (TrCel7A) (**Fig. 2**) (VTT: **Exploitable result 7**, p. 47). The best GH7 variant was further tested in the novel enzyme cocktail, called Thermomix, under WP3 and WP6 and used also in the pilot runs.

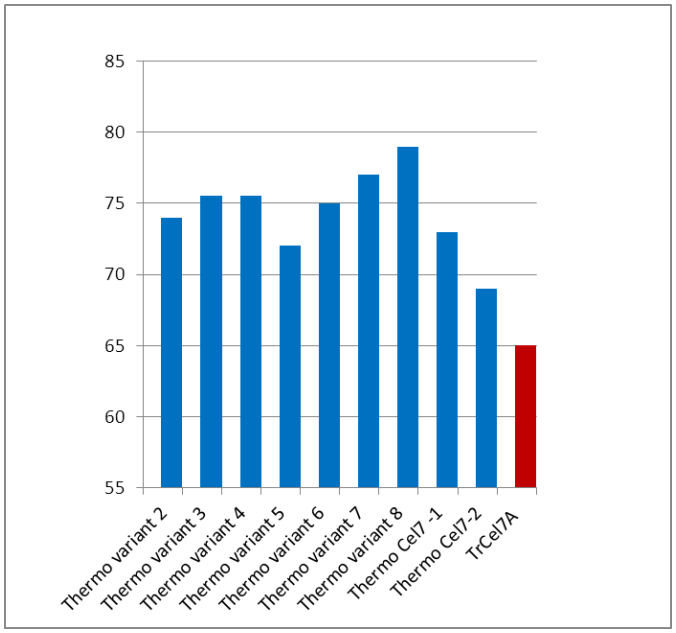

Figure 2: Thermostabilities of the novel GH7 cellobiohydrolases engineered (Thermo variants 2-8), or discovered (Thermo Cel7-1 and Cel7-2) within NEMO project. The *T.reesei* CBHI (TrCel7A) was used as a reference. The unfolding temperatures (Tm) were measured with CD spectroscopy and the error in the Tm values is ± 1 °C.

4.1.3.3. Optimised enzyme mixtures (WP3)

The objective of WP3 was to analyse selected novel enzymes originating from the screening approaches (WP2) more thoroughly, and to generate optimal enzyme mixtures for the selected NEMO raw materials and process concepts (WP6). The goal was to enable efficient hydrolysis of the pretreated raw materials and to reduce the enzyme amount needed.

More specific objectives were

- Identification of limiting enzyme activities
- Induction of enzyme profiles for tailoring enzyme mixtures
- Production, purification and characterisation of most relevant enzymes
- Design of optimal mixtures for the process concept(s)

Limiting components in lignocellulosic materials and bottleneck enzymes

Solubilisation and hydrolysis of chosen lignocellulosic substrates was studied under different conditions using the new identified enzymes separately, and in different combinations (UH, VTT, DNL). The results clearly show that several identified factors limit the hydrolysis of the substrates. A systematic approach was taken to study the individual roles of various components and develop methods to overcome these limitations. Improvement of the specific activity and performance of cellulases, particularly cellobiohydrolases, was thus one of the main aims in the NEMO project, and this was successfully achieved. Characterisation of the seven engineered GH7 cellobiohydrolases (Thermo variants 2-8) demonstrated that they all had higher thermostability (**Fig. 1**, WP2) and were also less inhibited by cellobiose than the reference enzyme, *Trichoderma reesei* Cel7A (*Tr*Cel7A). Two of these variant were also shown to be less inhibited by lignin (**Fig. 2**). In addition, four of the variants were shown to hydrolyse pretreated *Arundo donax* at 71 °C more efficiently than that of the reference enzyme, TrCel7A, at 60 °C, thus fulfilling one of the targets set for NEMO (**Fig. 3**). The two best CBHI variants were selected for process assessment (WP6) and one of them (Thermo variant 4) was eventually chosen for the pilot testing on spruce. Additionally, two novel GH7 family cellobiohydrolases discovered from thermophilic fungi (Thermo Cel7-1 and Cel7-2) demonstrated that both cellulases are more thermostable and have higher hydrolytic activity at 60 °C than the reference enzyme TrCel7A (**Fig. 3**).

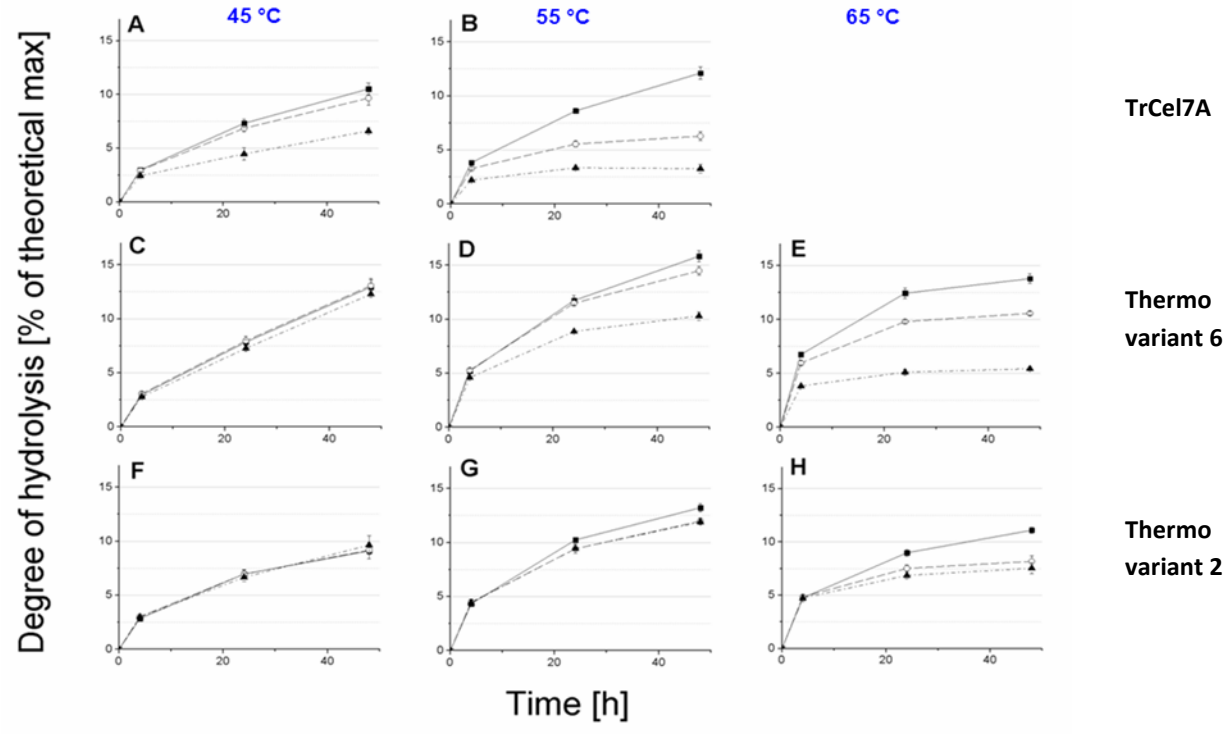

Figure 3: Hydrolysis of Avicel (10 mg/ml) in the presence of lignin by the two novel GH7 Thermo enzyme variants and *T.reesei* Cel7A, a reference. Hydrolysis was performed at three different temperatures (45, 55, and 65 °C) at pH 5 with and without added lignin. Reactions without added lignin (■), with 10 mg/ml of wheat straw EnzHR lignin added (○) and with 10 mg/ml of spruce EnzHR lignin added (▲). Hydrolysis reactions were supplemented with 500 nkat/g cellulose with a thermostable β-glucosidase (Roal), and assays performed in triplicates. Solubilized reducing sugars were measured with the PAHBAH reagent. Results are presented as percentage of the theoretical maximum glucose yield from 10 mg/ml of Avicel.

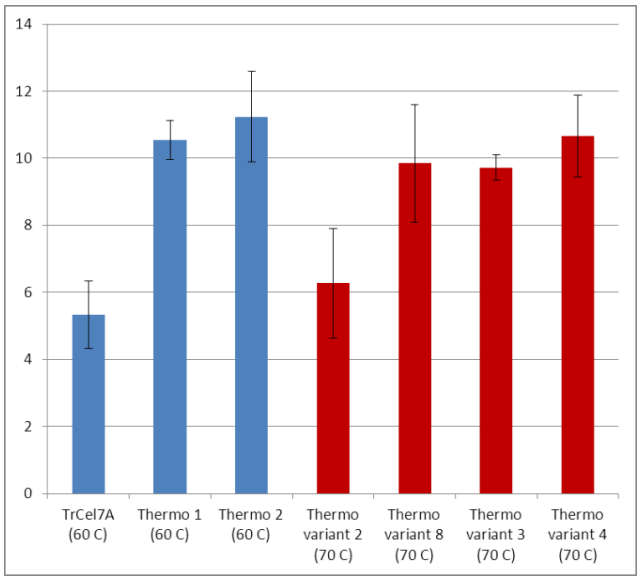

Figure 4: Hydrolysis of 1% pretreated *Arundo donax* (from Chemtex) at 60°C or 70°C (71 °C), pH 5, with novel thermostable GH7 family cellobiohydrolases discovered (2 different) or engineered (4 different) within the NEMO project. The reaction was followed for 48h and the reducing sugars were measured with PAHBAH reagent against cellobiose standards. Solubilization (w/w) was calculated from the total carbohydrate amount (68 %) of the pretreated *Arundo donax*. The time point shown here is after 24h hydrolysis. The *T. reesei* enzyme (TrCel7A) was used as a reference.

Hemicelluloses are known to be structurally interlinked with cellulose and lignin, and thus may form together with lignin layers hindering the access of cellulases to the cellulose substrate. The amount and structures of hemicelluloses varied in the substrates (pretreated straw, spruce and *Arundo donax*). The hydrolysis of the remaining hemicelluloses was shown to clearly restrict the hydrolysis of cellulose by using pure enzymes. It was shown that xylanases (along with non-specific endoglucanases) have the most significant impact, and addition of acetyl xylan esterase and to minor extent by arabinosidase further improved the effect (UH). The new NEMO enzymes (such as *Myceliophthora thermophila* C1 endo-xylanases) tested were shown to be superior as compared to the commercial reference enzymes in removing these hindering structures (DNL). On spruce substrates, the solubilization of galactoglucomannans, even though present in only small amounts, enhanced the glucose yield.

Lignin affects the hydrolysis negatively due to its location and structure in the pretreated lignocellulosic materials. Lignin prevents the access of cellulases to cellulose and binds hydrolytic enzymes unspecifically and thus decreases the hydrolysis rate. As explained above, novel thermostable cellulases were engineered in the NEMO project and shown to bind less to lignin than the reference enzyme (**Fig. 3**). In addition, enzymatic modification of lignin by laccases was studied as a means to improve the hydrolysis (UH). The effects of laccase catalyzed modification of lignin were adverse in case of the two raw materials tested; the hydrolysis of pretreated spruce was improved whereas the hydrolysis of *Arundo donax* was impaired. The effect was concluded to be partially due to modification of lignin leading to changed binding properties of enzymes to lignin. Spruce lignin was also found to have a more inhibiting role in the hydrolysis than the *Arundo donax* lignin. The enzymatic hydrolysis of spruce was improved by 47% with laccase when electron transferring mediator molecules were added to the treatments. All laccase and mediator treatments improved the enzymatic hydrolysis of spruce.
(UH: **Exploitable result 12,** p. 47**)**

Induction of enzyme profiles for tailoring enzyme mixtures

New information about regulation of fungal enzyme expression and strains with improved enzyme production profiles was obtained during the project. Induction of enzymes was studied in fungal expression hosts *T.reesei*, *Aspergillus vadensis* and *Myceliophthora thermophila* (previously known as *Chrysosporium lucknowense*) C1. *T. reesei* cpc1 gene has been isolated earlier (in another project at VTT) as a putative cellulase gene repressor. During the NEMO project, the gene was deleted from *T. reesei* QM9414 strain and the deletion strain was shown to produce 2-3 fold more cellulase activity (MULac activity) on lactose, when compared to the parental strain (VTT). Concerning the xylanolytic regulator XlnR, overexpression in *A. vadensis* was achieved (UU). However, adding multiple copies of the target enzyme encoding genes resulted in a higher increase in enzyme production than adding extra copies of the regulator. Comparison of *M. thermophila* C1 strain (producing the enzyme mixture Alternafuel Cmax1) grown on mildly pretreated wheat straw (from GS) and *Arundo donax* (from Chemtex) demonstrated that an improved enzyme mixture for saccharification can be obtained when the fungal strain is grown on the same substrates as the saccharification is performed (DNL).

Larger scale production and characterisation of the novel enzymes

Several partners (c-LEcta, UU, VTT, DNL and Roal) were participating in larger scale production of the novel enzymes. Various production hosts were tested and used, and bulk scale production of several novel enzymes was achieved. **Table 4** summarises the enzymes that were successfully expressed in larger scale for detailed characterisation.

One of c-LEcta's major tasks in the NEMO project was to express different hemicellulases and endoglucanases in *Pichia pastoris* or *Bacillus subtilis*. The enzymes were derived from literature, and partners VTT and UU. Partner UU expressed 10 novel hemicellulases in *A. vadensis* and these are currently being evaluated for their enzymatic characteristics and their relevance to biofuel pre-treatments. VTT expressed one thermostable cellulase in *T.reesei*, and Roal expressed altogether 13 (hemi)cellulases, derived from public genome screenings or from partner laboratories, in the *T. reesei* strain lacking the genes coding for the four major cellulases.

The enzyme production at Roal was carried out in laboratory scale fermentation and at least 500 mg protein/litre production level was obtained. This fulfils one of the goals set for the NEMO project, the initial demonstration of the feasibility of production in relevant industrial fungal hosts as an indication of possibility for production improvement to commercially relevant levels in the future. Moreover, this is a good productivity in a heterologous host especially for the Basidiomycete enzymes, as well for the metagenomic bacterial (hemi)cellulases. Furthermore, DNL expressed altogether eight hemicellulases, derived from DNL and UU, in a C1 strain having a low cellulase background. These were also expressed in level of at least 500mg/L.

Table 4: Enzymes produced in larger scale and characterised in NEMO. Enzymes with unconfirmed data of adequate production levels are listed below the dotted line.

Enzyme abbr.	Details (Partner)	Activity	Production host (Partner)
GH10 Xyl	Genomic (DNL)	Endo-xylanase GH10	C1 (DNL)
GH11 Xyl	Genomic (DNL)	Endo-xylanase GH11	C1 (DNL)
CE5 Axe	Genomic (DNL)	Acetyl xylan esterase, CE5	C1 (DNL)
CE1 Axe	Genomic (DNL)	Acetyl xylan esterase, CE1	C1 (DNL)
CE3 Axe	Screening (DNL)	Acetyl xylan esterase, CE3	C1 (DNL)
GH43 Abf	Genomic (DNL)	Arabinofuranosidase, GH43	C1 (DNL)
Fae	Genomic (DNL)	Ferulic acid esterase	C1 (DNL)

ABF-FOX	Genomic (UU)	Arabinofuranosidase, GH51	C1 (DNL)
Thermo variant 3	Engineered (VTT)	Cellobiohydrolase GH7	*T. reesei* (Roal)
Thermo variant 4	Engineered (VTT)	Cellobiohydrolase GH7	*T. reesei* (Roal)
Thermo variant 5	Engineered (VTT)	Cellobiohydrolase GH7	*T. reesei* (Roal)
Thermo variant 6	Engineered (VTT)	Cellobiohydrolase GH7	*T. reesei* (Roal)
Thermo Cel7-2	Screened (VTT)	Cellobiohydrolase GH7	*T. reesei* (Roal)
Thermo Cel7-3	Screened (VTT)	Cellobiohydrolase GH7	*T. reesei* (Roal)
Basidio Cel7-1	Genomic (UNIVMED)	Cellobiohydrolase GH7	*T. reesei* (Roal)
Basidio Cel7-2	Genomic (UNIVMED)	Cellobiohydrolase GH7	*T. reesei* (Roal)
Basidio Cel7-3	Genomic (UNIVMED)	Cellobiohydrolase GH7	*T. reesei* (Roal)
Basidio Cel7-4	Genomic (UNIVMED)	Cellobiohydrolase GH7	*T. reesei* (Roal)
CBHII	Screened (UH)	Cellobiohydrolase GH6	*T. reesei* (Roal)
Tr10S22-CBM1	Metagenomic (VTT)	Endoglucanase GH5	*T. reesei* (Roal)
TrKP3	Metagenomic (VTT)	Xylanase, GH11	*T. reesei* (Roal)
Thermo Cel7-1	Screened (VTT)	Cellobiohydrolase GH7	T.reesei (VTT)
10S22-CBM1	Metagenomic (VTT)	Endoglucanase GH5	*Bacillus* (c-LEcta)
10S37-CBM1	Metagenomic (VTT)	Endoglucanase GH5	*Bacillus* (c-LEcta)

Optimal enzyme mixtures

In the beginning of the project reference conditions and enzymes (baseline assay) were chosen based on the experience of the partners. *Trichoderma reesei* monocomponent enzymes or enzymes mixtures, e.g. commercial Celluclast provided with the β-glucosidase preparate (Novozym 188), were used as baseline enzymes. In addition, for the thermostable enzyme mixtures, proprietary thermostable enzyme components from Roal were used. The substrates used for the assays were model compounds, i.e. Avicel for development of cellulases and birch xylan for hemicellulases. In addition, pre-treated biomass materials from partners from WP1 were used. A number of enzymes were tested in the baseline assays and further in optimized conditions for the improved hydrolysis.

One of the chosen process concepts to be tested comprised a 6 h prehydrolysis at higher temperature (60°C) followed by SSF at a lower temperature of 35 °C. The substrates were pretreated *Arundo donax* (from Chemtex) and spruce (from SE-T). The final enzyme mixture optimization for the spruce hydrolysis was performed using thermostable enzymes (UH). All enzymes in the mixture need to be thermostable and for this reason the NEMO enzyme(s) were combined with propriety enzymes from the NEMO partner Roal. The final mixture was called Thermomix and contained cellobiohydrolase II (CBHII), endoglucanase II (EGII) and β-glucosidase from Roal, and the new thermostable NEMO CBHI (Thermo variant 4). To optimize the enzyme concentrations in the Thermomix for most efficient hydrolysis result in the scale-up tests (WP6), response surface modeling was used and a statistical D-optimal design was chosen (UH). The optimization was carried out with an enzyme load of 10 mg/g DW. Celluclast 1.5L (10 mg/g DW) and Novozym 188 (500 nkat/g DW) was used as the reference. The target, *i.e.* to improve the hydrolysis yield by 30% when replacing Celluclast + Novozym188 with the new Thermomix, was clearly exceeded **(Fig. 5).**

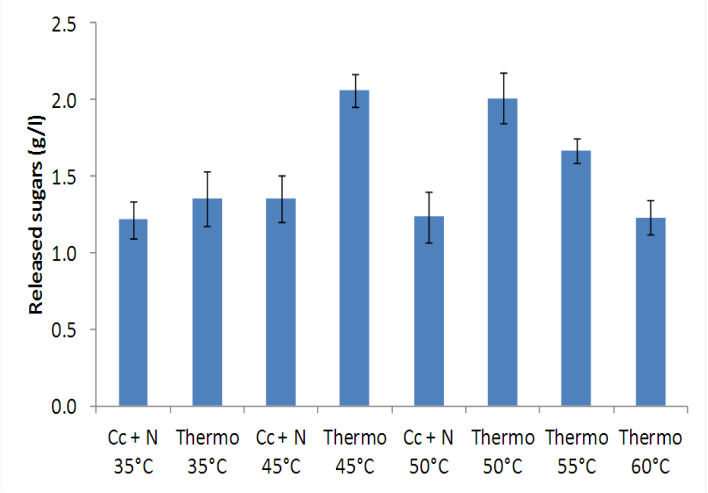

Figure 5: Spruce hydrolysis at different temperatures with the reference enzyme mixture, Cc+N (10 mg/g DW Celluclast + 500 nakt/g DW Novozymes), compared with the optimized Thermomix, Thermo (10 mg/g DW: 36% CBHI, 49% CBHII, 15% EGII + 1.5 g/DW β-GLU). The hydrolysis was performed for 24 h.

For the *Arundo* hydrolysis, the separate hydrolysis and fermentation (SHF) process concept was chosen. The enzyme optimisation was carried out at 55 °C, pH 5 using the Celluclast + Novozym 188 as a basic enzyme mixture in combination with novel (hemi)cellulases (DNL). Replacing Celluclast (10mg/g DW) partly with the novel GH10 xylanase, derived from *M. thermophila* C1, resulted in an improved enzyme mixture showing similar release of xylose and glucose with a 30% lower enzyme dosage. In addition, replacing Celluclast partly with the GH10 xylanase and the thermostable CBHI (Thermo variant 4) also showed similar release of xylose and glucose with a 30% lower enzyme dosage (**Fig. 6**). The GH10 xylanase was delivered to WP6 for pilot scale saccharification experiments.

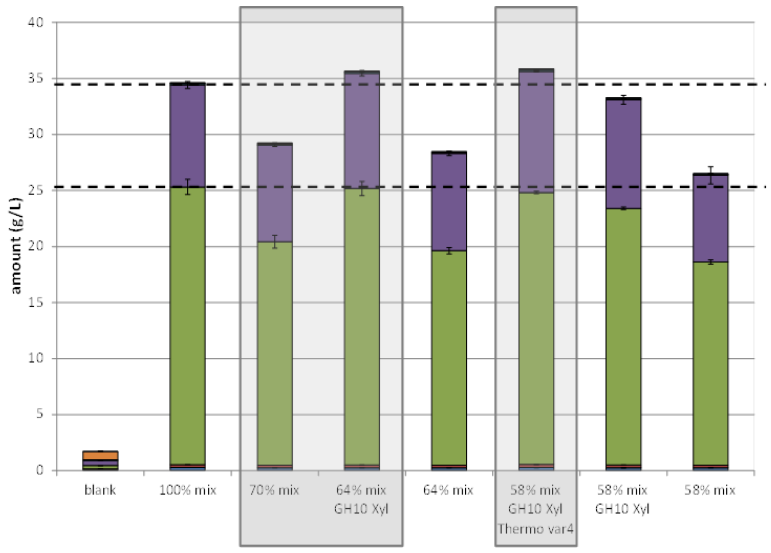

Figure 6: Saccharification of mildly pretreated *Arundo donax* (Chemtex) with baseline Celluclast + Novozyme188 in combination with the novel GH10 xylanase derived from *Myceliophthora thermophila* C1 alone, or in combination with novel CBHI Thermo variant 4 (engineered by VTT). Experiments were performed at a DM content of 10%, using a baseline enzyme loading of 1%, 0.64% and 0.58% total protein of baseline and a mono component enzyme loading of 0.1% target protein, at 55°C, pH5 during 24 hours. Green bars: glucose; violet bars: xylose; purple bars: galactose; orange bars: arabinose. The columns in grey boxes show the hydrolysis yields with the enzyme mixtures when a 30% reduction in enzyme loading was used.

4.1.3.4. New strains through mutagenesis and screening (WP4)

The second main objective of the NEMO project was to develop robust industrial yeast strains with high pentose sugar fermentation capacity and high tolerance to the inhibitors present in lignocellulose hydrolysates. The aim of WP4 was in particular to screen for robust yeast strains, identify the genes responsible for process tolerances and to engineer pentose fermenting strains.

Selection and screening of novel robust *S. cerevisiae* strains

A large collection consisting of 1018 *S. cerevisiae* strains was compiled through combined efforts of NEMO partners (KNAW, VIB, UL) and screened for tolerance to different stress conditions. These traits included ethanol tolerance, thermotolerance, acetic acid tolerance and tolerance towards lignocellulose hydrolysates, which are all of crucial importance for optimal fermentation performance of industrial yeast in second-generation bioethanol production. The work resulted in seven thermotolerant strains (up to 41°C), eight highly acetate tolerant strains, and fourteen strains with higher ethanol tolerance on YP or YPD medium than that of the industrial reference strain selected for the project, Ethanol Red. Ethanol Red showed the highest capacity of the *S. cerevisiae* isolates to tolerate spruce hydrolysate at different temperatures (GUF). Evolutionary engineering of Ethanol Red by increasing temperature from 35°C to 39°C in the presence of 50% spruce hydrolysate (from SEKAB) further improved its tolerance resulting in a new isolate named ISO12 that showed the fastest growth and fermentation performance (ULUND). By means of evolutionary engineering, a highly thermotolerant strain (41 °C) named T1 was also isolated, this strain showed different colony morphology and growth pattern (aggregation) in liquid medium suggesting significant changes in the cell wall. Moreover, Fermipan (a baker's yeast) was selected by VIB among 600 strains as being the best strain for high fermentation rate (shorter lag phase) in spruce hydrolysate.

As a conclusion, a number of robust and improved industrial strains were identified in the NEMO project that have unusually high tolerance to the stress conditions experienced by the yeast in bioethanol production on lignocellulos hydrolysates. These serve as excellent parent strains for further strain engineering work.

Identification of mutations causing superior traits

VIB developed a novel genomic technology, pooled-segregant whole-genome sequence analysis (AMTEM). This technology was applied to elucidate the genetic basis of several complex traits determining the different stress tolerance characteristics of the identified yeast strains. At the beginning of the NEMO project, most information on the genetic basis of tolerance traits had been obtained with laboratory yeast strains, which in general display much lower stress tolerance than industrial and natural yeast strains. Little was known about the genetic determinants of the extreme stress tolerance that is required for second generation bioethanol production.

Ethanol tolerance
Using both the AMTEM technology and pooled-segregant whole genome sequence analysis (**Fig. 7**), several causative genes involved in this trait were identified. Deletion of *APJ1* contributes positively to ethanol tolerance. This finding has been confirmed with a range of industrial strains (Ethanol Red and other industrial strains VR1, ES2 and PE2). *SWS2* and *VPS70* mutant alleles, which confer higher ethanol tolerance, are currently being tested in industrial strains. *URA3* and *MKT1* cannot be used for industrial strain improvement since it was noticed that Ethanol Red already contains similar alleles. (VIB: **Patent appl.** Swinnen et al, p. 48)

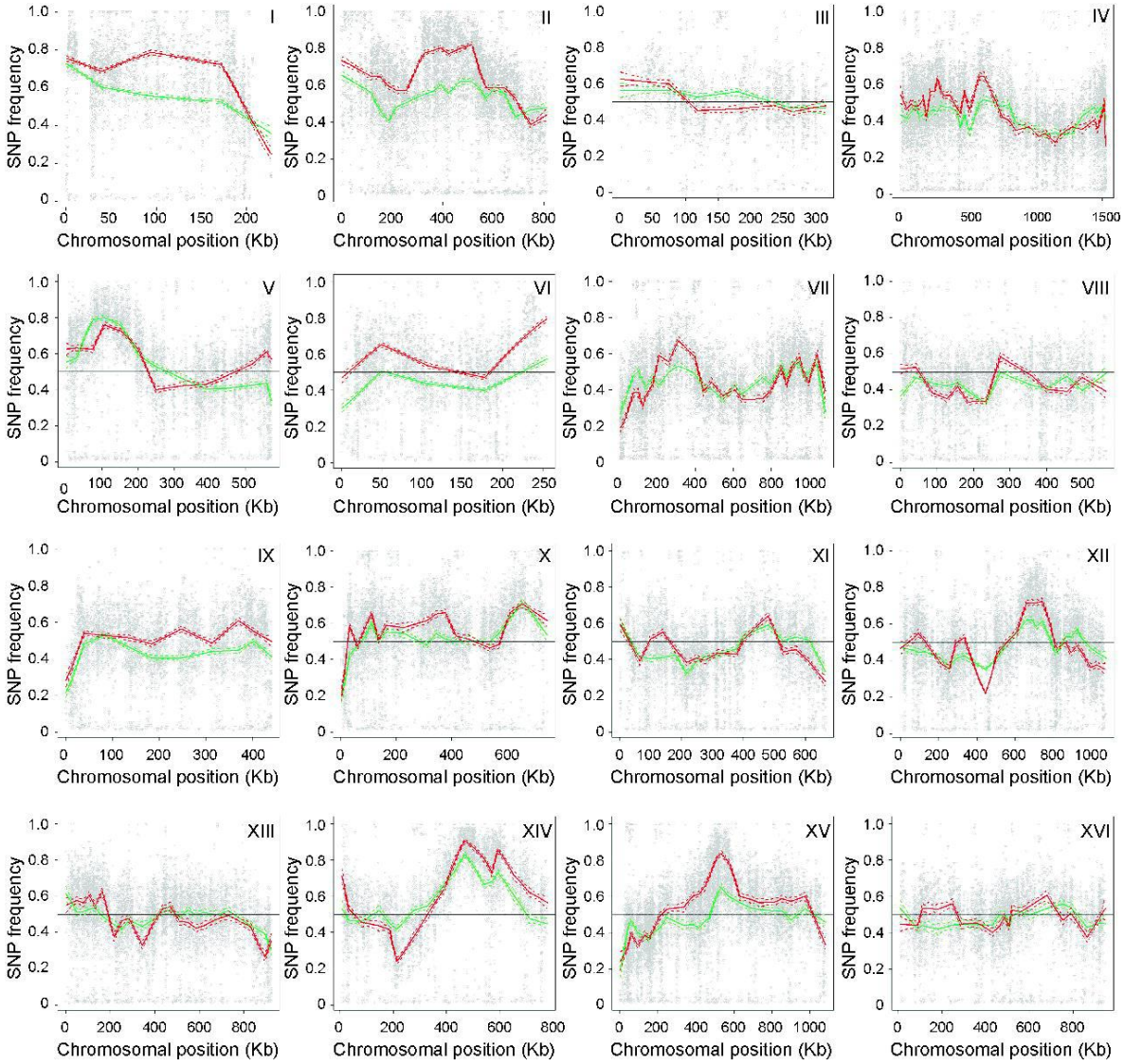

Figure 7: QTL were mapped by whole-genome sequence analysis of DNA extracted from a pool of 136 segregants tolerant to at least 16% ethanol (pool 1; green line) and from a pool of 31 segregants tolerant to at least 17% ethanol (pool 2; red line). The genomic DNA of the parents, VR1-5B and BY4741, and of the two pools was sequenced and aligned to identify SNPs. The nucleotide frequency of quality-selected SNPs in the sequence of the pool was plotted against the chromosomal position. Significant deviations from the average of 0.5 indicate candidate QTL linked to high ethanol tolerance. Upward deviations indicate linkage to QTL in the ethanol tolerant parent VR1-5B. The three major QTL on chromosomes V, X and XIV are not significantly different between the two pools. However, in several instances, e.g. on chromosomes II, XII and XV, minor loci can be identified showing a significant difference between the two pools. These candidate QTL are more distinctive in pool 2 (17% ethanol) compared to pool 1 (16% ethanol). The difference in SNP frequency between the two pools is certainly significant when the simultaneous confidence bands do not overlap.

Thermotolerance

To identify the genetic basis of high thermotolerance, VIB first mapped multiple QTLs using a natural yeast strain, MUCL28177, compared to the laboratory strain, BY4742. To identify minor loci, downgraded parental strains, in which inferior alleles replaced the superior alleles, were used in a second QTL mapping experiment. Mutant alleles of *MKT1*, *END3* and *NCS2* genes were identified, however, those were previously pointed out as causative genes in QTLs with an allele-specific influence on thermotolerance. *PRP42* and *SMD2*, which have related functions and are novel alleles, suggest an important role for RNA processing in thermotolerance. Interestingly, the

PRP42 superior allele was identified in the inferior parent strain. (VIB: **Patent appl.** Yang et al., p. 48**)**

Acetic acid tolerance
The loci identified by pooled-segregant whole genome sequence analysis are rather broad and therefore it is a laborious task to downscale the loci to single gene resolution. VIB introduced a new approach to narrow down the loci by internally crossing segregants from the hybrid diploid strain. This new methodology was applied in crossing segregants from the acetic acid susceptible Ethanol Red strain and the acetic acid tolerant strain JT22689. Using segregants from the first and the 7th generation (F1 and F7), several genes responsible for high acetic acid tolerance were identified. The *HAA1* gene has a major contribution to acetic acid tolerance. VIB found a mutant allele that confers higher tolerance and this finding has been confirmed in different *S. cerevisiae* strains. Mutant alleles of the *ERG6* and *YJR030C*, and *MVP1* genes were also identified and confirmed by reciprocal hemizygosity analysis.

Osmotolerance
Osmotolerance was assessed in an industrially relevant manner by measuring maximal ethanol accumulation capacity in high-density fermentations containing about 33% glucose. In such fermentations nearly all glucose is converted to ethanol and about 17-18% ethanol is accumulated.This analysis identified *ADE1*, *URA3*, and *KIN3* encoding a protein kinase involved in DNA damage repair, as specific causative genes for maximal ethanol accumulation capacity. These genes, as well as the previously identified *MKT1* gene, were not linked in this genetic background to tolerance of cell proliferation to high ethanol levels. The newly discovered superior alleles will be used in the future to improve osmotolerance/maximal ethanol accumulation in high-density fermentations.

Low glycerol yield/high ethanol yield
VIB also performed polygenic analysis of this metabolic trait and successfully identified several mutant alleles for *SSK1*, *GPD1*, *HOT1* and *SMP1* genes that are responsible for lower glycerol production and higher ethanol production. These alleles do not seem to affect the growth rate and stress tolerance properties of the yeast in a negative way. This shows that also metabolic pathways can be improved by applying polygenic analysis of complex traits to a metabolic process. (VIB: **2 Patent appls.** Thevelein et al., Goovaerts et al., p. 48)

Development of a robust industrial yeast strain with high xylose fermentation capacity

At the beginning of the NEMO project there were only laboratory yeast strains published and available with efficient anaerobic pentose fermentation. These strains are not suitable for use in industrial fermentations because they are not robust enough and because their fermentation capacity is too low. Hence, it was a major objective of the NEMO project to develop an inhibitor tolerant industrial *S. cerevisiae* strain with high pentose fermenting activity.

For the construction of a good NEMO strain, GUF first constructed the recombinant yeast strain HDY-GUF5, which was based on the industrial yeast strain Ethanol Red as parent, in which a cassette was inserted in two copies that contained the xylose isomerase (XI) encoding gene from *Clostridium phytofermentans* as well as a xylulokinase gene, several genes of the pentose phosphate pathway and genes for arabinose fermentation. This strain is still very poor in xylose fermentation (see **Fig. 10** later). VIB exploited then further the methods of strain selection, mutagenesis, genome shuffling and subsequent evolutionary engineering using this strain constructed by GUF. Two robust xylose fermenting yeast strain isolates, GS1.11-26 and GS1.11-14, were obtained and selected because of their good fermentation performance on wheat straw (provided by GS) and

Arundo (provided by Chemtex) hydrolysates.

The xylose utilizing strain GS1.11-26 was evaluated for growth and fermentation in both artificial medium and two different lignocellulosic hydrolysates (**Figs. 8 and 9**). The mutagenesis step appeared to have generated some negative effect on the aerobic growth rate in glucose and loss of tolerance towards osmotic stress in high gravity fermentation at 35 % glucose concentration. To cure this, the strain was crossed with the industrial strains Ethanol Red and Fermipan, which generated the strains GSE16, and GSF335 and GSF767, respectively. In complex media, the D-xylose utilization rate by the hybrid strains ranged from 0.36 to 0.67 g/gDW/h, which was reduced compared to that of GS1.11-26 (1.10 g/gDW/h). However, in batch fermentation of undetoxified acid pretreated spruce hydrolysate, the hybrid strains showed comparable D-xylose utilization rates as GS1.11-26, and produced up to 23 % more ethanol compared to Ethanol Red due to efficient D-xylose utilization and inhibitor tolerance. GSF335 and GSF767 demonstrated significantly better tolerance to inhibitors, however, with slower xylose fermentation rate than the GSE16 strain. GSF335 presented lower tolerance to ethanol. GSE16, that has only Ethanol Red genetic background, was identified as the best xylose fermenting yeast strain in lignocellulose hydrolysates. This strain demonstrated rapid aerobic growth on glucose. Therefore, the NEMO strain GSE16 has been selected for further improvement and is being evaluated in pilot scale fermentations at different companies. (VIB: **Exploitable result 9**, p. 47).

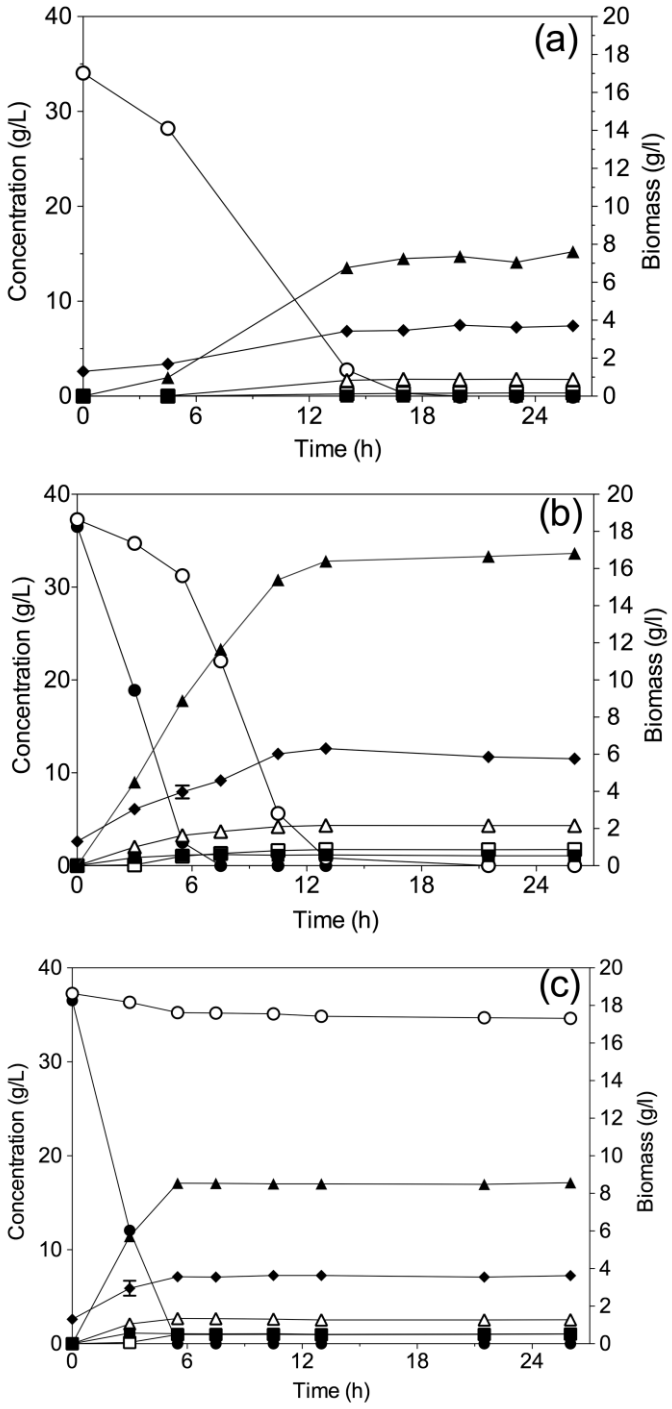

Figure 8: Performance of strain GS1.11-26 in semi-anaerobic batch fermentations with D-xylose and a glucose/D-xylose mixture. (a) Strain GS1.11-26 in synthetic medium with D-xylose. (b) Strain GS1.11-26 in rich YP medium containing 36 g/L glucose and 37g/L D-xylose. (c) Parent strain HDY.GUF5 in rich YP medium containing 36 g/L glucose and 37g/L D-xylose. (●) Glucose, ((○) D-xylose, (▲) ethanol, (△) glycerol, (■) acetate, (□) D-xylitol and (◊) biomass. Each experiment was performed in duplicate, and error bars represent standard deviation from the average of duplicate values.

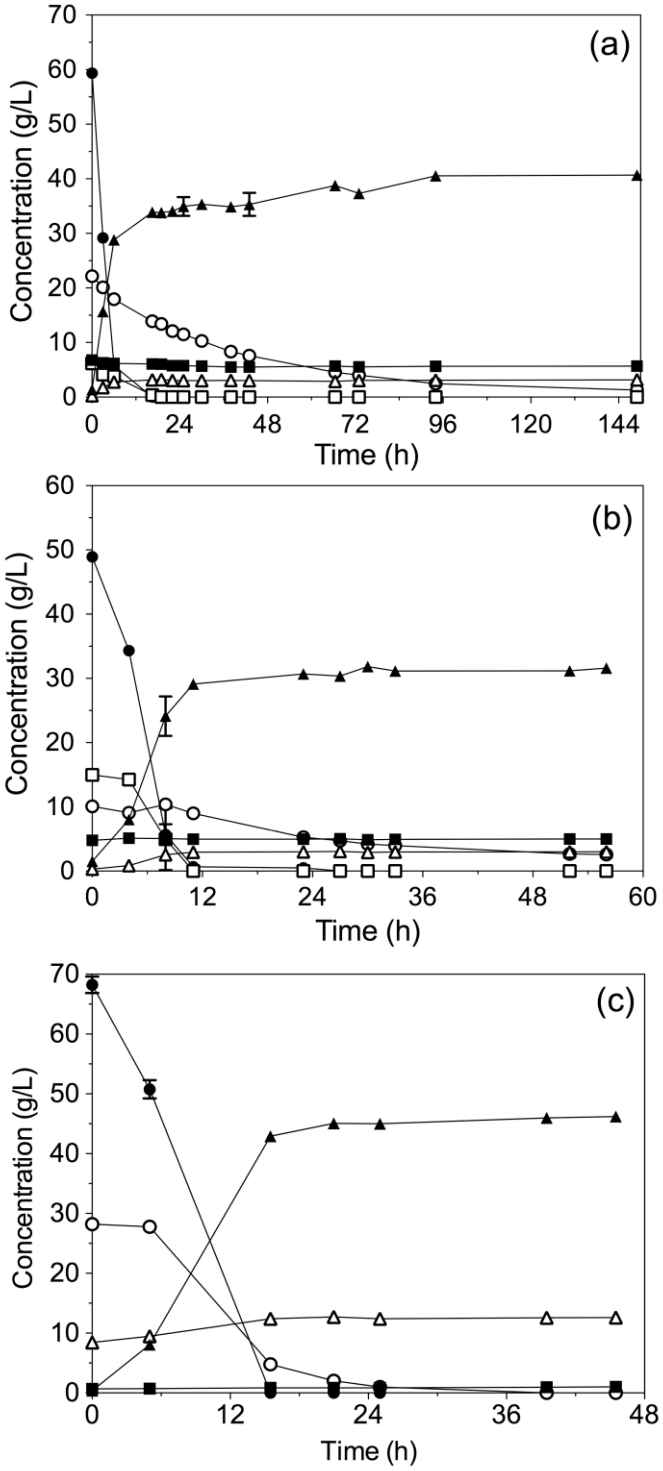

Figure 9: Performance of strain GS1.11-26 in semi-anaerobic batch fermentations with three different lignocellulose hydrolysates. (a) *Arundo donax*, (b) spruce and (c) mixture of wheat straw/hay. (●) Glucose, ((○) D-xylose, (▲) ethanol, (△) glycerol, (■) acetate, and (□) D-xylitol.

In an alternative approach, GUF developed the efficient strain HDY.GUF12, in which all genes needed for xylose and arabinose utilization were overexpressed from genomic locations in order to construct a stable Ethanol Red based industrial yeast strain. Minimal evolutionary engineering was used to avoid loss of any beneficial properties. The ethanol yield from a total straw hydrolysate (obtained from GS) could be increased by more than 20% by fermentation with a further improved strain HDY.GUF9. GUF also introduced mutated transporters for pentose sugars (see Chapter 4.1.3.5.) into HDY.GUF9 and the fermentation rate of xylose in the presence of glucose could be increased. The further improved HDY.GUF12 strain fermented xylose completely with 55% of the maximal specific consumption rate of glucose. In mixed sugar fermentations (glucose-arabinose) in mineral medium HDY.GUF12 fermented arabinose with 8.5% of the maximal specific consumption rate of glucose. **Fig. 10** shows strain examples from the development of the industrial pentose utilizing yeast strains by GUF, and demonstrates the significant engineering and evolution efforts needed to produce superior strains for pentose fermentation. (GUF: **Exploitable result 1**, p. 47; **Patent appl.** Boles & Subtil, p.48).

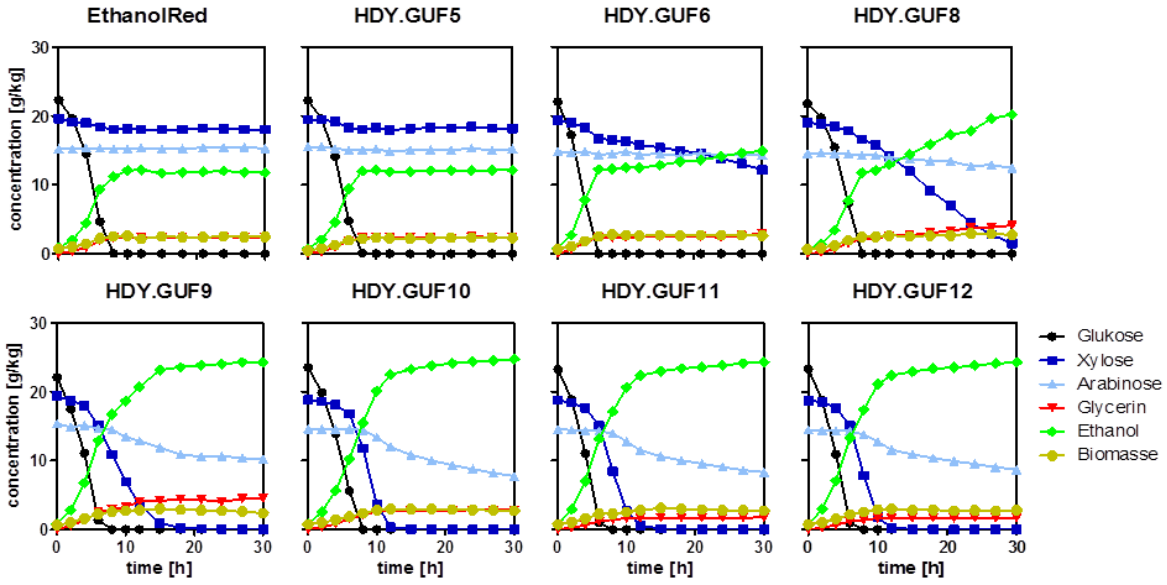

Figure 10. Industrial pentose utilizing yeast strains developed by GUF studied for anaerobic fermentation capacity on medium containing pure sugars. Ethanol Red is the industrial parent strain, where the XI gene and pentose phosphate pathway genes were introduced to generate strain HDY.GUF5. Further evolution and strain engineering resulted in increased improvements on pentose fermentation. Finally, incorporation of mutant pentose transporters in the strain HDY.GUF9 led to further improved strains. See text for explanations.

4.1.3.5. Targeted strain engineering (WP5)

The objective of WP5 was to use rational metabolic engineering approaches by combining several genetic alterations into one strain to improve stress tolerance and yields and rates of C6 and C5 co-fermentation, aided by physiological systems biology analyses and metabolic modelling.

More specifically the objectives were

- Physiological and systems biology studies of recombinant, xylose-fermenting *S. cerevisiae* strains
- Modelling of xylose fermentation to identify novel targets for metabolic engineering to enhance xylose fermentation and improve tolerance to industrially relevant raw material hydrolysates
- Targeted engineering of xylose-fermenting *S. cerevisiae* to enhance strain robustness and tolerance
- Enhancement of pentose uptake by identification and engineering of novel pentose transporters

Advanced modelling and systems biology analyses for identification of targets for strain improvement

Laboratory and industrial *S. cerevisiae* strains, engineered to ferment xylose either via the isomerisation pathway by xylose isomerase (XI) or the oxido-reductive pathway by xylose reductase (XR) and xylitol dehydrogenase (XDH) were studied under controlled bioreactor cultivations to obtain understanding on the physiology of xylose fermentation. Additionally, *Kluyveromyces marxianus* was studied to obtain comparative insight to naturally xylose-utilising yeast species.

The xylose utilising laboratory strain VTT-C-10883 used as a reference strain in the NEMO project, containing the XR/XDH pathway and xylulokinase overexpressed, was constructed by VTT (and CTH). This was compared to the industrial evolved NEMO strains containing the XI pathway, constructed by GUF and VIB (see chapter 4.1.3.4.). The most informative culture conditions were anaerobic bioreactor batch cultures on a mixture of glucose (20 g/L) and xylose (50 g/L). As expected, the XI strains showed the highest ethanol yields and productivities; with the HDY.GUF9 strain the volumetric productivity was 0.52 g/L/h, the overall yield on glucose and xylose was 0.43 g/g and the *average* specific productivity was 0.23 g/g cell dry weight/h in cultures carried out by VTT. When the HDY.GUF9 strain was cultivated on 30 g/L glucose and 10 g/L xylose, the *maximal* specific productivity observed was 1.65 g/g cell dry weight/h (GUF).

Physiological characterisation of *K. marxianus* (strain CBS712) in micro-aerobic batch cultures on a mixture of glucose and xylose confirmed that this species is strongly oxygen dependent, xylose consumption and growth on xylose was not observed under anaerobic conditions (UNIMIB). This relates to the cofactor imbalance of the *K. marxianus* XR enzyme (NADPH-dependent), but the data also indicated post-translational control of the xylose pathway enzymes since the XR and XDH activities decreased as the oxygen supply decreased, which did not directly correlate with the transcript levels of the corresponding genes.

In order to provide means to analyse the intracellular fluxes VTT modified a genome-wide flux model for *S. cerevisiae*, and developed a dynamic model fitted to the extracellular metabolite

measurements from the bioreactor cultures. This allowed accurate estimation of specific accumulation rates for the flux and metabolic control analysis. Approaches for solving intracellular fluxes in batch cultivations were established, enabling the use of the thermodynamically curated flux model developed by EPFL and the modified genome-wide flux model.

EPFL developed the first ever thermodynamically curated genome-scale model of the yeast *S. cerevisiae*. The *S. cerevisiae iMM904* model was curated to account for the thermodynamic properties of metabolites and reactions and for proper modelling of transport reaction thermodynamics between the cell compartments. EPFL further developed a method for consistent reduction of genome-scale metabolic models to central core subsystems while avoiding loss of useful information with respect to the whole genome-scale model. This included large-scale kinetic models of the yeast metabolism with enzymatic mechanisms with sigmoidal kinetics and capability to include allosteric effects as modifiers. These models ensure reliable prediction of responses of metabolism to the variation in environmental and cellular conditions in spite of scarce and uncertain kinetic data.

EPFL invested considerable efforts in the development for methodologies for building these thermodynamically and physico-chemically consistent kinetic models for the integration and analysis of experimental flux and systems biology (e.g. array) data provided by partners (VTT, CTH). EPFL developed further the ORACLE (Optimization and Risk Assessment of Complex Living Entities) framework that relies on formalism from process control and metabolic control analysis to identify the rate-limiting steps in metabolic pathways. Motivated by the challenges encountered within NEMO, EPFL developed a methodology based on the Principal Component Analysis (PCA) for characterisation of feasible flux profiles in the absence of ^{13}C-labelling data. In addition, EPFL developed methodology that allows constructing dynamical, non-linear models of metabolic networks to investigate temporal responses of metabolic networks under a wide range of perturbations and the stability of the metabolic networks.

EPFL performed array of analyses of the cultivation (and transcriptional profiling) data of both XI and XR/XDH strains provided by VTT. Metabolic control analyses of the interplay between the upper glycolysis and xylose uptake pathway in the VTT-C-10883 strain (XR/XDH) revealed that the hexokinase (HXK) reaction has a negative impact on xylose uptake. Moreover, the studies pinpointed the probable cause of this effect as a biochemical coupling through ATP between HXK and xylulokinase (XK). VTT verified experimentally the improved effect of reduced hexokinase activity on specific xylose consumption rate. The utilisation rates of glucose and xylose became more similar due to lowered hexokinase activity, resulting in a longer period of glucose and xylose co-consumption (**Fig. 11**). However, overall ethanol productivity was not improved. Other bottle neck targets revealed by the modelling were also studied: The increase of phosphate availability in the medium, and increase in phosphofructokinase (PFK) activity by expressing the *E. coli PfkA* gene in the XI strains from VIB and GUF. However, no clear enhancement on xylose fermentation was observed under the conditions studied.

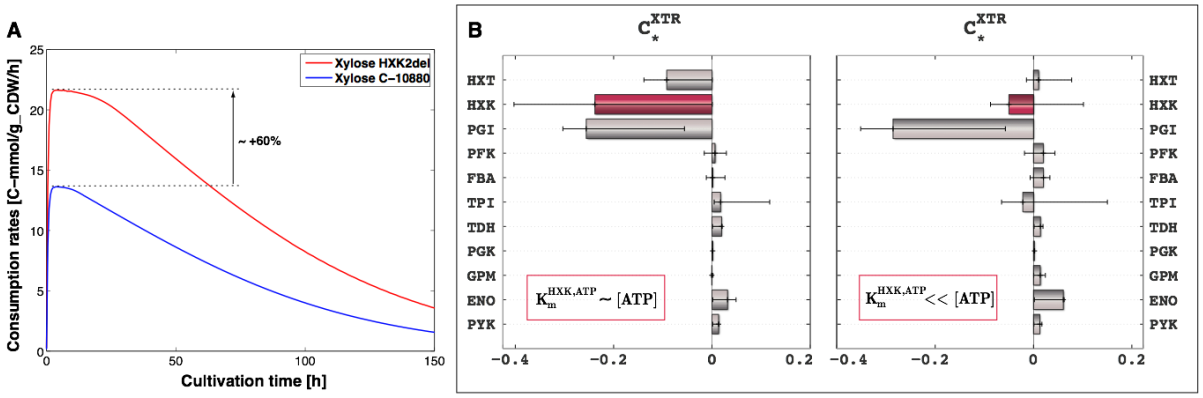

Figure 11: Panel A: Consumption rates of xylose in the engineered *HXK2*-negative strain (red) versus the ones in the parent VTT-C-10880 strain (blue); Panel B: distribution of the flux control coefficients of the xylose uptake during glucose-xylose co-utilisation. The bars are the mean values of the control coefficients through xylose transporters (XTR). The error bars denote a range containing 50% of the samples closest to the mean. The HXK (hexokinase) reaction has a negative impact on XTR (Panel B, left). When ATP saturates the HXK enzyme, the negative effect of HXK on XTR diminishes significantly (Panel B, right).

Targeted genetic modifications for improvement of strain robustness

Tolerance to industrially relevant raw material hydrolysates is an important attribute for efficient bioethanol production hosts. ULUND constructed an industrial strain with improved growth in the presence of non-detoxified spruce hydrolysate, through the overexpression of the transcription factor *YAP1* in the industrial Ethanol Red based strain. The use of this strain may be advantageous for reducing the length of batch processes during biomass generation for ethanol production. (ULUND: **Exploitable result 8**, p. 47).

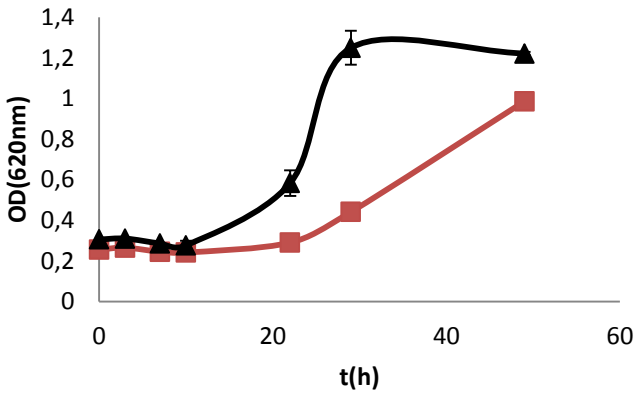

Figure 12: Aerobic growth on 50% (v/v) non-detoxified spruce hydrolysate (30°C) for the strain overexpressing YAP1 (black) and the control industrial strain (red). Cells were pre-grown on 30% hydrolysate. Figure shows one of the profiles for each strain.

Previously developed yeast strains by UNIMIB producing vitamin C, shown to be more tolerant to different stresses, were tested in conditions relevant for the process of ethanol production from pre-treated lignocellulose. Growth on pre-treated spruce was comparable to the control strain, while tolerance to acetic acid stress was enhanced. A new strain was developed, with addition of a plant gene involved in ascorbic acid recycling, resulting in more robust strain than its parental strain. Said strain was tested on pre-treated spruce, and at low pH a growth advantage together with a higher ethanol production was observed. Furthermore, the *ScMCR*1 gene encoding an activity involved in

erythro ascorbic acid recycling was overexpressed in the industrial xylose-fermenting strain. The work by UNIMIB has resulted in several laboratory and industrial strains and modifications of the best pentose fermenting NEMO strain that are presently being tested by the Consortium under relevant process conditions.(UNIMIB: **Exploitable result 5**, p. 47).

Due to its high intracellular concentration and low redox potential, glutathione has been regarded as the main redox buffer in living cells. Based on some of the known effects of lignocellulosic inhibitors on microbial metabolism, and data on the effect of furaldehydes on redox metabolism, CTH overexpressed two genes involved in glutathione metabolism, *GSH1* and *CYS3* in yeast. To increase the capacity of the cells to recycle oxidised glutathione to reduced glutathione, *GLR1* was additionally overexpressed. These strains were evaluated in process-like conditions in SSF of pretreated spruce. All strains carrying the *GSH1* overexpression construct stayed viable for a longer time than the wild-type and consequently produced 50 – 69% more ethanol. Thus, engineering of the redox metabolism by increasing the intracellular glutathione level was a successful strategy to increase strain robustness in the context of lignocellulosic bioethanol production. (CTH: **Exploitable result 11**, p. 47)

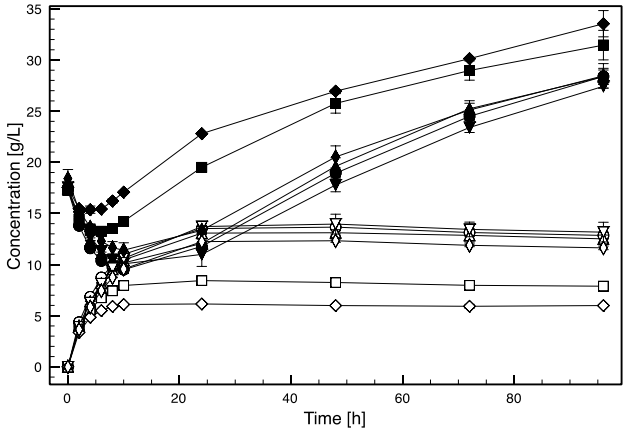

Figure 13: Time course of glucose and ethanol concentrations in SSF of spruce with the strains generated by CTH. Squares: CEN.PK 113-7D, circles: *GSH1*, triangles: *GSH1/CYS3*, diamonds: *GLR1*, reversed triangles: *GSH1/GLR1*, stretched diamonds: *GSH1/CYS3/GLR1*. The filled and open symbols represent glucose and ethanol, respectively. The error bars represent the standard deviation (n=2).

CTH also studied the effect of 5-hydroxymethylfurfural (HMF) and furfural on redox and energy state of yeast in a chemostat setup in the VTT-C-10883 strain. The data indicated that the presence of furfural and HMF in the medium is affecting the cytosolic redox state. This effect appeared only after the lag-phase, capturing the experimental observations that HMF is degraded upon complete degradation of furfural. The catabolic and anabolic reduction charges were significantly decreased in stressed cells indicating that HMF and furfural are draining the cells of reducing power. Genome-wide transcriptome profiling identified several key genes that were differentially expressed upon inhibitor challenge. The cultivation data was also used for thermodynamic modelling (see above), which revealed that during stress the metabolism of *S. cerevisiae* was less flexible, i.e. the ranges of reaction Gibbs energies and the concentrations of the associated metabolites had more constrained feasibility ranges (**Fig 14**). Data from this experimental set-up is valuable material for further kinetic modelling studies, aiming at revealing strain engineering targets within the yeast metabolic network.

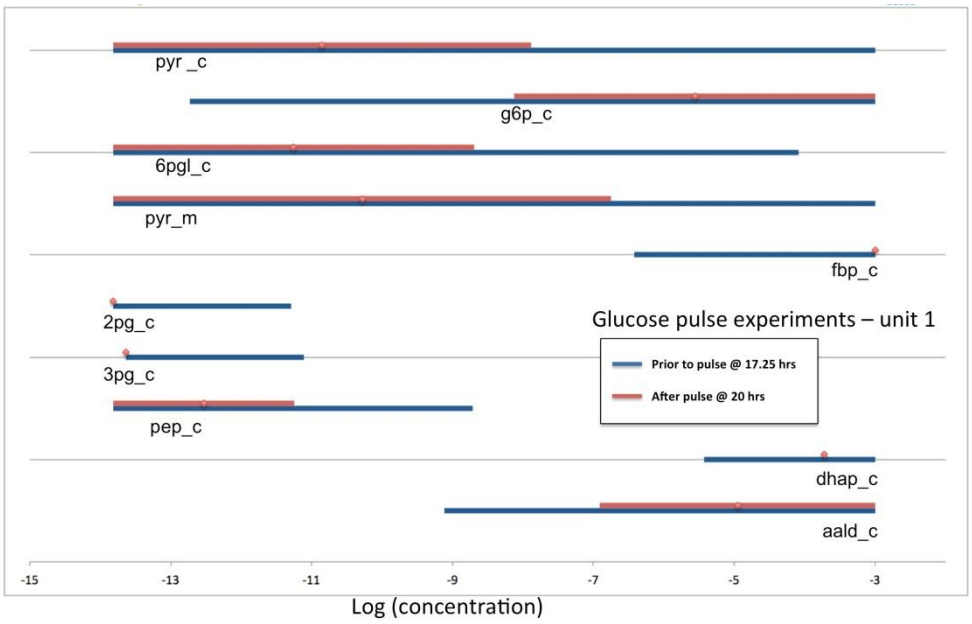

Figure 14: Thermodynamically allowable ranges of 10 representative metabolites in the metabolic network for two cases: (i) prior to pulse of furfural and HMF (blue) and (ii) after the pulse (red).

Improvement of sugar uptake for efficient co-fermentation of hexoses and pentoses

In mixed sugar fermentations with recombinant *S. cerevisiae* strains able to ferment xylose and arabinose the pentose sugars are normally only utilised after depletion of glucose which significantly increases the fermentation time. GUF showed that glucose impairs the simultaneous utilisation of pentoses mainly due to inhibition of pentose uptake. A screening system for xylose and arabinose transporters was developed by GUF. A new high-affinity arabinose transporter, Stp2 from the plant *Arabidopsis thaliana*, was identified (GUF: **Exploitable result 3**, p. 47; GUF: **Patent appl.** Boles & Subtil, p. 48). This transporter mediated the uptake of arabinose into *S. cerevisiae* cells especially at low concentrations but not of glucose. Several attempts to find specific xylose transporters for heterologous expression in *S. cerevisiae* were not successful. Although GUF tested several putative xylose transporter genes from *S. stipitis*, *A. thaliana* and *Neurospora crassa*, and screened cDNA libraries from *Aureobasidium pullulans*, *Magnaporthe grisea* and *Trichoderma reesei*, no specific xylose transporter being not inhibited by glucose was found.

The screening system was further developed to rule out the possibility of maltose transporters (Mal31, Mal21) to be responsible for glucose transport in the *hxt*^{0}-strain background (EBY.VW5000). The system was used for evolutionary engineering of yeast hexose transporters and to screen for improved xylose-transporters after epPCR-mutagenesis. This way two important amino acid positions in yeast hexose transporters were identified to render pentose transport resistant to inhibition by glucose. These two amino acid positions in the *S.cerevisiae GAL2* and *HXT7* transporters were subjected to mutagenesis to identify the best amino acid substitution at each position, and the mutant clones were expressed in the industrial pentose fermenting strain HDY.GUF9 (see Chapter 4.1.3.4.). Simultaneous co-fermentation of xylose and glucose could be significantly improved, the fermentation of xylose in the presence of glucose was increased up to 1.9-fold (**Table 5**). (GUF: **2 Exploitable results, 1** and **2**, p. 47).

Table 5: The ratio of xylose consumption rate to glucose consumption rate was calculated to 'normalize' for overall metabolism. Considering the ratio r_{xyl}/r_{glc} as a measure for co-fermentation capability, the fermentations show that overexpression of the mutant transporters Gal2-N376F and Hxt7-T213 have a clearly beneficial effect on co-fermentation, increasing the fermentation of xylose in the presence of glucose up to 1.9-fold (88%). Overexpression of the wild type Gal2 has only marginal effects.

	consumption rates		ratio	
	glucose (r_{glc})	xylose (r_{xyl})	r_{xyl}/r_{glc}	
HDY.GUF9	[$g_{glc}*g_{dm}^{-1}*h^{-1}$]	[$g_{xyl}*g_{dm}^{-1}*h^{-1}$]	absolute	relative
empty vector	3.45	0.22	0.065	1
Gal2-wt	2.22	0.15	0.069	1.07 (7.1 %)
Gal2-N376F	2.02	0.25	0.122	1.88 (88 %)
Hxt7-T213G	2.76	0.27	0.099	1.54 (54 %)

4.1.3.6. Process assessment of the novel enzymes and yeast strains (WP6)

The main objective of WP6 was to assess the novel enzymes and engineered yeast strains in process relevant conditions. Standardised SHF (separate hydrolysis and fermentation) and SSF (simultaneous hydrolysis and fermentation) conditions were assessed and also new process options where prehydrolysis at elevated temperatures was used to evaluate the benefit of the new thermostable enzymes and non-isothermal SSF were designed to best utilise the capacity of the enzymes and the yeast during the different phases of SSF. Finally the aim was to carry out selected process options at pilot scale.

Base case comparison of pretreated Arundo and spruce

The three different pretreated NEMO raw materials were used in the evaluations: wheat straw, Arundo and spruce. Arundo was physically pretreated by an steam explosion pretreatment step by CTXI. Spruce was chemically pretreated by an acid pretreatment step (SE-T), whereas wheat straw was totally hydrolysed using concentrated acid (GS). The conditions for pretreatment were also modified during the course of the project according to best in-house know-how of the companies and based on the feed-back obtained from NEMO partners. To be able to perform comparable experiments, a significant effort was made to develop standard operating procedures (SOPs) to ensure that assessment protocols allowed a proper comparison basis.

In the beginning of the project reference (base case) conditions were decided based on the state-of-the-art at that time, available reference materials and best experiences of the partners. In the base case assessments, as a reference enzyme a mixture of Celluclast 1.5L (10mg/g raw material WIS (water insoluble solids)) and Novozyme 188 (500 nkat/g WIS) (supplying extra β-glucosidase) was used on 10% (w/w) WIS substrate. These industrial enzyme preparates were a gift of Novozymes (also provided in amounts sufficient for piloting). As reference yeast strains the industrial strain Ethanol Red (non-xylose fermenting, for fermenting pretreated spruce) was used, and the recombinant xylose fermenting strain VTT-C-10883 (XR/XDH/XK pathway introduced into the

laboratory strain CEN.PK) for fermentations of xylose containing wheat straw and Arundo. Fermentations were carried out at 32 °C.

In the base case assessment, spruce exhibited the highest ethanol yield when fermented in the SSF mode compared to SHF (0.35 g/g compared to 0.32 g/g), whereas the opposite was found for Arundo (0.21 g/g in SSF compared to 0.25 g/g in SHF). The relative low ethanol yield in the Arundo case was due to a low hydrolysis yield partly caused by xylo-oligomers present in the material, which inhibited the action of the cellulolytic enzymes. Furthermore, the ethanol yield on xylose was suboptimal, due to significant xylitol formation and low xylose consumption rate with the reference yeast strain (XR/XDH pathway).

In general, there will be a compromise between the temperature selected for the hydrolysis and the fermentation. Since higher temperature in general relates to faster processes, there is a significant interest in finding thermotolerant enzymes and yeast strains. If the temperature optimum is different for the hydrolysis and the fermentation, different alternative process schemes should be designed for evaluating and combining the potential benefits of the new enzymes and yeast strains as efficiently as possible. These new process options were compared (see below) with the base line SHF and SSF cases.

Improved enzyme cocktails

A number of novel enzymes were already evaluated in WPs 2-3 and a few selected ones were tested more extensively in complete process conditions. For the process assessments in particular novel xylanases were selected for pretreated Arundo, and for the spruce case, novel thermostable enzyme mixtures – including the new thermostable mutant GH7 CBHI enzyme.

Replacing part of the reference enzyme preparate with the novel NEMO xylanase, xyl11, supplied by DNL (see 4.1.3.3.), an improved hydrolysis of pretreated Arundo was reached, primarily due its action on xylo-oligomers present in the pretreated material. This decreased the inhibition of the cellulose hydrolysis resulting also in higher yields of monomeric glucose. Furthermore, in SSF experiments using pentose fermenting *S. cerevisiae* strain GS1.11-26 (see chapter 4.1.3.4.) an increased ethanol yield was obtained, 0.26 g ethanol/g total sugar, compared to 0.22 g ethanol/g total sugar obtained with the reference enzymes with similar loading. However, more residual xylose was observed when using xyl11 in the enzyme cocktail, indicating a limiting rate of fermentation. Time course data during SSF indicates that the new xylanase improves the initial rates of xylose release from the oligomers present in the liquid fraction of the pretreated material.

The NEMO Thermomix (see 4.1.3.3.) was designed to be used for spruce hydrolysis. It consisted of a mixture of propriety enzymes from Roal and improved mutant G7 CBHI generated by VTT) (CBHI thermo variant 4 at 3.6, CBHII 4.9, EGII 1.5, and β-glucosidase 1.5 mg/g WIS, respectively) (see also chapter 4.1.3.3.). When testing the Thermomix enzymes, SSF experiments were carried out at 37 °C. This temperature was chosen as a compromise between the temperature suitable for both enzymatic hydrolysis and fermentation, and this was also the highest temperature to be used in the pilot SSF experiments. During fermentation of pretreated spruce using *S. cerevisiase* Ethanol Red an increased ethanol yield from 0.22 g ethanol/g total sugar (base case) to 0.27 g ethanol/g total sugar was obtained using Thermomix compared to the reference enzymes.

Improved NEMO yeast strains

As with the enzymes, the new yeast strains and effects of identified individual genes were evaluated in the WPs 4-5 also using pretreated materials. In this WP6 selected strains were tested in process assessment conditions for efficiency of pentose fermentation and process robustness.

Early on in the project, an already robust industrial strain (Ethanol Red) was used as a background for the strain development, in order to obtain a superior process strain. This strain has been further evolved and modifed (see chapters 4.1.3.2. and 4.1.3.3.) to tolerate for instance higher temperatures and spruce hydrolysates, and verified in process relevant conditions. Since spruce does not contain relevant amounts of xylose such strains are useful even without xylose fermenting capability.

The pentose fermenting strains developed in WP4 (see chapter 4.1.3.4.), GS1.11-26, GSE16 and HDY.GUF6 were tested. In SSF experiments with pretreated Arundo conducted at 39 °C all three xylose fermenting strains were able to convert xylose efficiently into ethanol resulting in a final ethanol yield over total sugars of 0.28 g/g (HDY.GUF6), 0.29 g/g (GS1.11-26), and 0.26 g/g (GSE16). This is significantly more than with the reference parental strain Ethanol Red, which had a yield of 0.22 g ethanol/g total sugars. The residual xylose levels were below 1 g/liter and minimal xylitol formation resulted. A common problem with xylose utilizing *S. cerevisiae* (in particular with XR/XDH pathway) is that they produce xylitol, yields in the range of 0.1-0.2 g/g are commonly reported. It should therefore be emphasized that the NEMO strains were shown to be superior not only due to their good xylose fermentation capability and robustness but also due to their low by-product formation as assessed in process relevant conditions.

Novel process options at elevated temperatures

The basic process options SHF and SSF can be modified in various ways, creating hybrid processes or novel process configurations. Within the NEMO project, two main approaches were developed and evaluated namely (*i) pre-hydrolysis*, i.e. the combination of initial high temperature enzymatic hydrolysis, and *(ii) non-isothermal SSF*, i.e. an SSF process during which the temperature is changed.

Cellulase enzyme mixtures typically have a temperature optimum higher than the maximum allowable temperature for yeasts. The main benefit of SHF is to be able to select the temperatures optimally for hydrolysis and fermentation. However, in case the end-product inhibition of the enzymes becomes significant, a benefit may be obtained through removal of the monosaccharides by yeast fermentation. In order to improve the process we have elaborated different prehydrolysis options and their influence on the overall process performance. Thermophilic enzymes may show a benefit even at standard conditions. Indeed, when the hydrolysis efficiency of Thermomix on pretreated spruce was compared to the reference enzymes Celluclast and Novozym 188 at 45 ˚C, the ethanol yield was slightly better (0.34 g/g) using the Thermomix enzymes compared to the reference enzymes (0.33 g/g). The behaviour of Thermomix was tested also at elevated temperatures. Significantly more glucose (27.6 g/l) was released after 6 hours prehydrolysis at 55 ˚C compared with the lower temperature 45 °C (18.6 g/l). Ethanol production using *S. cerevisiae* GS11.1-26 strain at 32 °C was more rapid at the beginning due to higher sugar amount but, however, the total final ethanol yield calculated both from the C6 and C5 sugar content was similar than when the hydrolysis was carried out at 45°C.

Another process option evaluated was the application of a temperature profile during the process, which could allow a benefit for the fermentation at controlled lower temperatures and hydrolysis at higher temperatures. In contrast to the pre-hydrolysis approach, the principle of non-isothermal SSF is to design a temperature profile that takes best advantage of the enzymatic hydrolysis throughout

the SSF and the capability of the yeast. The non-isothermal SSF can be carried out with a predefined temperature profile based on intuitive reasoning, or an optimal temperature profile can be obtained based on modeling of the SSF process, including temperature effects. The best design found allowed temperature increase towards the end of the process, resulting in higher glucose release in hydrolysis at the point when the hydrolysis rate normally decreases. It also allowed better yeast viability than if higher temperature was applied at the beginning of the process.

In case of pretreated spruce, two different slopes, 0.073 °C/h and 0.135 °C/h giving final temperatures after 96 h of 39 °C and 45 °C, respectively, using a starting temperature of 32 °C were evaluated. There was no significant difference in the final ethanol concentrations between the tested conditions. In the case of pretreated Arundo, with lower inhibitor levels compared to spruce, more severe temperature profiles could be applied (**Fig. 15**). An increase in the final ethanol concentration (16.5g/l) was observed in comparison to baseline experiment (15.3g/l), and improvement in production rate could also be observed. (ULUND: **Exploitable result 10**, p. 47)

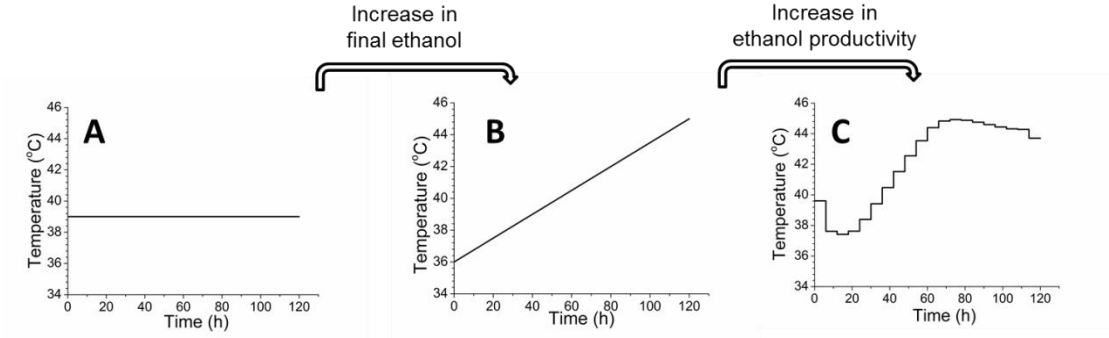

Figure 15: Progression of temperature based process improvements in SSF carried out on pretreated Arundo.

Fermentation of HCl hydrolysed raw materials

As a reference to the main pretreatment methods in NEMO relying on enzymatic hydrolysis, GS hydrolysed wheat straw by the total HCl acid method. The hydrolysis resulted in a sugar solution mainly consisting of monosaccharides, with only a minor fraction of oligomers. While the first wheat straw materials were poorly fermentable, process optimization led to material that could be fermented well. Raw materials in the range of 10-30 % of consistency were tested, and in general the fermentability of the hydrolysates decreased with increasing consistency. In the best cases an ethanol yield of 0.45 g ethanol/g monosaccharides was obtained at a consistency of 10% wheat straw. Under most conditions xylose and some glucose remained unfermented. Finally, also spruce and Arundo were hydrolysed with HCl and the three raw materials fermented with the NEMO yeast HDY.GUF12 (**Fig. 16**). In comparison to fermentations on pure sugars (chapter 4.1.3.4.), co-fermentation of xylose is reduced on the hydrolysates.

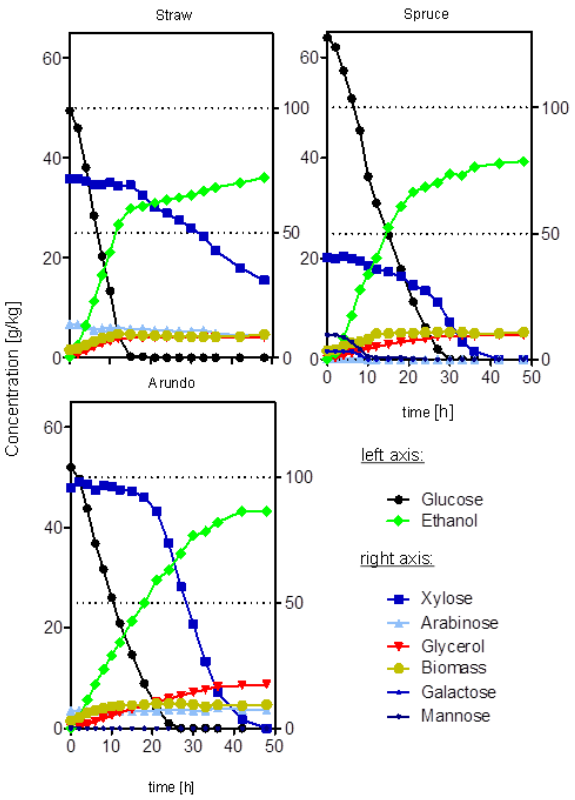

Figure 16: Fermentation of HCl hydrolysates from GS with the *S.cerevisiae* strain HDY.GUF12

Pilot plant testing

The pilot scale design needed to be decided 1.5 years before the project end to be able to produce sufficient amounts of NEMO enzymes, to carry out the piloting and be able to calculate (in WP7) the possible benefits of the novel enzymes and yeasts strains in terms of technoeconomy and LCA. The most feasible enzymes, yeast strains and advanced process concepts that were possible to implement in pilot scale were tested. SE-T (SEKAB) carried out piloting on steam exploded/acid treated spruce at the Örnsköldsvik demo plant (scale 3 m^3) and CTXI (Chemtex) on steam exploded Arundo at their pilot (scale 100kg biomass inlet). The enzyme companies Roal and DNL were each producing over 20-30kg of enzymes for the pilot tests, and the commercial Novozymes preparates Celluclast and Novozym 188 were used as a reference. Basically it was not possible to carry out any repetitions of the pilot experiments and below is given the results which are considered most representative.

The pilot plant testing of pretreated spruce was performed by SE-T in several SHF modes using different temperatures in the hydrolysis. The results confirmed that the NEMO Thermomix is more efficient than the reference enzyme mix at elevated temperatures. Thermomix resulted in a more efficient hydrolysis at 55 °C compared to the reference enzyme mixture during the first 72 hours of the SHF process. The reference enzyme mixture was able to hydrolyse the cellulose at both 45°C and 32°C, but at a lower rate than Thermomix at 55°C. It should be noted that due to less than intended severity during pre-treatment, the enzymatic degradability was very low for both enzyme mixtures since the cellulose was not well susceptible to enzymatic attack. This caused low overall hydrolysis and fermentation yields, but the results still clearly showed the improvements of the new Thermomix enzyme mixture.

Thermomix was also tested by SE-T on spruce in the pre-SSF concept where prehydrolysis at 55 °C was carried out prior to SSF at 32°C. This resulted in higher overall ethanol yield compared to the same process concept with the reference enzyme mixture.

During pilot trials at CTXI on pretreated Arundo, it was demonstrated that the replacement of part of the reference mixture by the xylanase Xyl11 improved the hydrolysis yield compared to the reference enzymes (~20% increase). The NEMO *S. cerevisiae* strain GSE16 was successfully used in fermentation of pretreated Arundo, with a consumption of more than half of the xylose during the first 48 h of fermentation.

4.1.3.7. Process calculations (WP7)

WP7 activities focused on the evaluation of NEMO process options and the benefits when using the novel enzymes and yeast strains developed in the NEMO project. The base case process schemes were first laid out. Process calculations and techno-economic analyses were performed as well as impact on energy efficiency and GHG emissions of the process regimes. Finally, the reference chemical pretreatment method based on total acid hydrolysis was evaluated using a particular MC (Monte Carlo) simulation.

The main feedstocks considered were spruce and Arundo. The outline of the scheme logic on which the calculations were based on is shown in **Fig. 17**. Similar schemes were used for spruce and Arundo. The reference base cases (BC) were calculated based on the lab scale SSF and SHF processes of WP6, and the processes where NEMO enzymes or yeast were used were based on the optimal lab scale results, also pilot scale results were used for the Arundo processes.

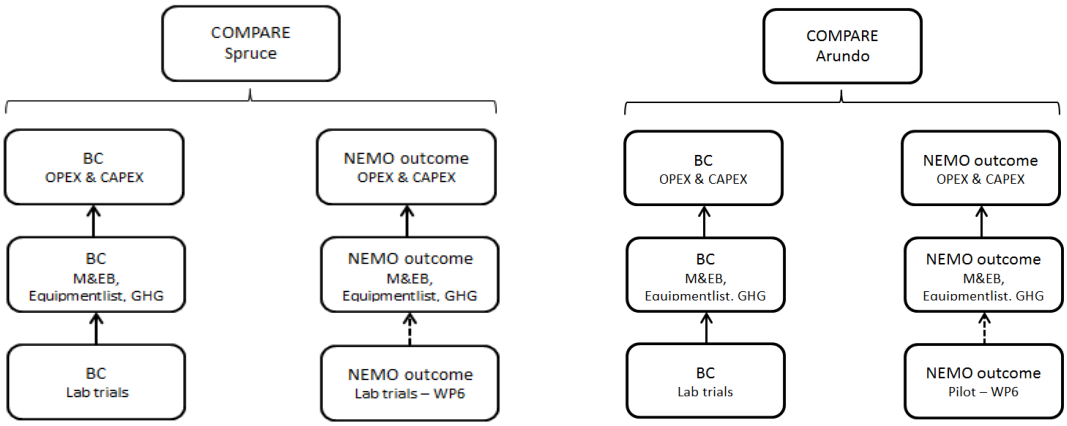

Figure 17: Scheme for process calculations for spruce (left) and Arundo (right). See text for explanations.

First the reference base cases (BC) were calculated. For spruce the chemical pretreatment method of SE-T was calculated, and for Arundo the steam explosion pretreatment of CTXI. Initial Mass & Energy Balances (M&EB) were calculated, equipment lists collected, and preliminary evaluations of GHG and OPEX & CAPEX were also carried out. Results so gathered were helpful for identifying the best experimental operative conditions for both spruce and Arundo, which were thus implemented in the last lab scale (spruce) or last pilot scale (Arundo) trials.

The process calculations were based on an annual production capacity of 40 000 tons ethanol/year. The minimum ethanol selling price (MESP) was then calculated for the improved cases, i.e. the use of NEMO enzymes or yeast, and compared to the MESP for the processes based on the base case results. For each NEMO case, a significant reduction in the MESP was obtained.

The description of the processes is presented in **Fig. 18**.

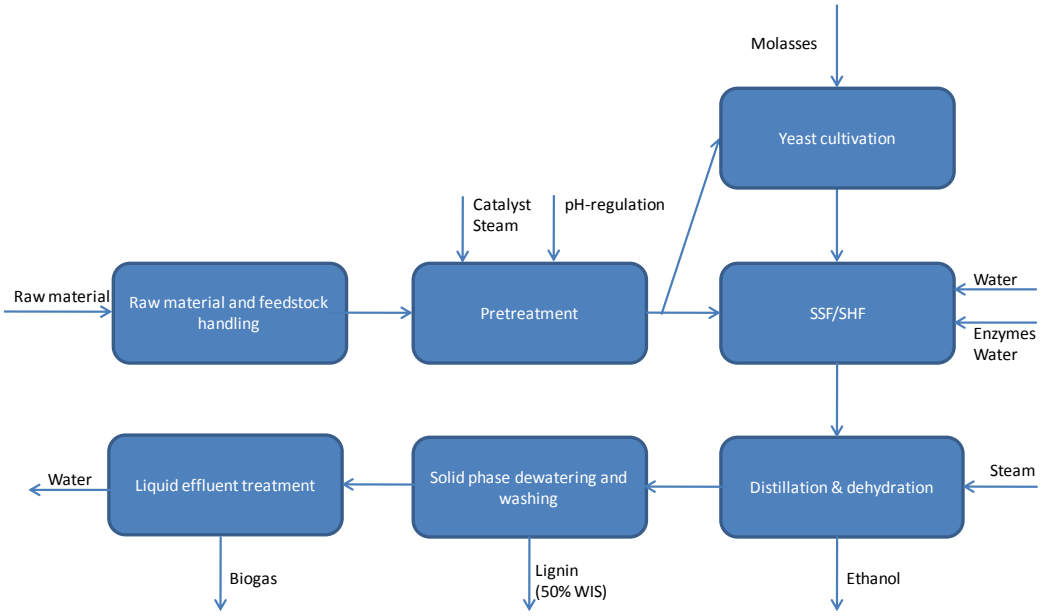

Figure 18: Principal description of the NEMO-processes used for OPEX and CAPEX calculations.

Spruce

Main results for spruce are gathered in **Table 6**.

Table 6: Main process calculations and minimum ethanol selling price (MESP) results for spruce feedstock.

Spruce	Overall ethanol yield (% kg DM/kg ethanol)	Investment (million €)	MESP (€/ L ethanol)
SSF Base case	5.1	105	0.77
pre hydrolysis-SSF NEMO	5.1	95	0.72
SHF Base case	7.6	142	1.00
SHF NEMO	6.8	118	0.80

Global main parameter values as well as the lifecycle GHG emissions for spruce are gathered in **Table 7**. GHG emissions reductions (%) with respect to gasoline are also shown.

Table 7: Main process calculation and GHG emission results for spruce feedstock.

SHF Spruce	Overall process yield	Net Plant Efficiency	LCA – GHG reduction	
Base case	6.3 kg DM/kg EtOH	24.9 %	33 gCO_2_eq/MJ	Red: 60 %
NEMO	6.0 kg DM/kg EtOH	24.6 %	31 gCO_2_eq/MJ	Red: 63 %

A reduction in terms of GHG emissions compared to the baseline case was obtained, with a final value of 31 gCO2_eq/MJ of ethanol, when using the NEMO Thermomix in an SHF process. The assumed conversion of 100% available mannose to EtOH during the fermentation step has a considerable impact on the global result, due to the positive impact on the overall process yield.

Arundo

Main results for Arundo are gathered in **Table 8.**

Table 8: Main process calculations and minimum ethanol selling price (MESP) results for Arundo feedstock.

SHF Arundo	Overall ethanol yield (% kg DM/kg ethanol)	Investment (million €)	MESP (€/ L ethanol)
Base case	7.6	142	1.00
NEMO	6.8	118	0.80

Global main parameter values as well as the lifecycle GHG emissions of 2nd generation bioethanol from Arundo are gathered in **Table 9.** GHG emissions reductions (%) with respect to gasoline are also shown.

Table 9*:* Main process calculations and GHG emission results for Arundo feedstock.

SHF Arundo	Overall process yield	Net Plant Efficiency	LCA – GHG	
Base case	7.6 kg DM/kg EtOH	21.8 %	41 gCO_2_eq/MJ	Red: 51%
NEMOed	6.8 kg DM/kg EtOH	27.5 %	36 gCO_2_eq/MJ	Red: 57.5%

In case of bioethanol from Arundo, global parameter outputs as well as LCA results have highlighted an improvement with respect to the baseline case scenario when a NEMO xylanase and pentose fermenting yeast are used. Particularly, the reduction of GHG emissions demonstrates the key role of process optimization through novel enzymes and yeast for improvement of the environmental parameters in biofuel production.

Reference process based on total acid hydrolysis

Finally, a MC simulation of a hypothetical ethanol production facility of 40 000 tons ethanol/year based on the total acid hydrolysis process of GS was performed, considering all three NEMO feedstocks: Arundo, spruce and wheat straw. The aim of this conclusive MC simulation study was to assess the feasibility of 2nd generation bioethanol technologies taking also specific uncertainties into consideration.

The techno-economic backbone of this MC simulation is based on an ethanol production facility (EPF) which in principle consists of a GS hydrolysis unit and a molasses based fermentation unit (since the NEMO hydrolysates are similar in their fermentation performance to molasses).

The minimum ethanol selling prices (MESP) were calculated as shown in **Fig. 19** based on the fermentation trials performed by GUF using their novel pentose fermenting yeast strain HDY.GUF12 on hydrolysates made from all three NEMO biomasses (chapter 4.1.3.6).

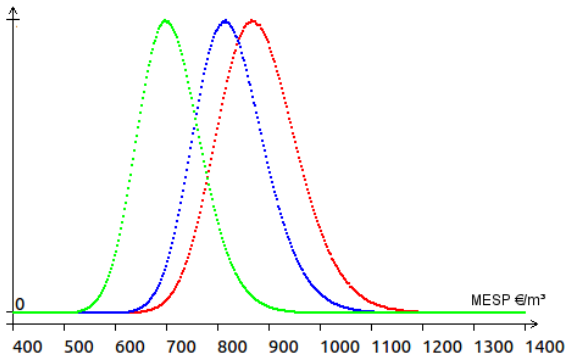

Figure 19: Result of MC-simulation (10 Mio trials). X-axis: MESP in €/ton of ethanol made from chosen biomasses. Red: wheat straw. Blue: spruce. Green: Arundo. Y-axis: Number of occurences a MESP has been returned by MC-simulation.

The most probable value of MESP for ethanol made from straw was 864€/ton. For spruce a MESP of 814 €/ton and for Arundo 695€/ton was obtained. The differences are mainly caused by the price of the biomass (Arundo has the lowest price of 48 €/ton) and the yield of carbohydrates (which was higher for spruce than for wheat straw). The **Table 10** below gives an overview on the Overall Process Yield (OPY) and Net Plant Efficiency (NPE) of this type of an ethanol production facility with the three biomasses used as raw material.

Table 10: OPY (kg dm/kg ethanol) and NPE for chosen biomasses.

Biomass	OPY	NPE %
Spruce	3.08	42.93
Wheat straw	3.68	48.18
Arundo	3.18	52.28

The results show that an economically competitive cost of around 500€/ton is still far from reality. Nevertheless, an economic opportunity is within reach if the ethanol production facilities containing GS's saccharification method are scaled up to a range of at least 80 000 ton ethanol output. Those facilities should use biomass sources, which are still more available such as rice straw or corn cobs. Under those constraints a MESP of ~500€/ton can become a reality.

4.1.4. NEMO – Potential impact

4.1.4.1. Scientific and technological impact

The main aim of the NEMO project was to identify and engineer novel enzymes and yeasts strains that would provide significant benefits in 2nd generation lignocellulosic ethanol production processes. Lignocellulosic bioethanol production is becoming a reality, and many commercial initiatives are being realised also by European companies, mainly in USA and Brazil. Another aim of NEMO was to develop the enzymes and yeasts to particular suit European biomasses covering broad geographic locations, namely the energy crop Arundo, wheat straw and the soft wood spruce, thus supporting near future initiatives that would be localised in Europe itself.

NEMO has achieved or exceeded all the goals set in the original research plan through the combined efforts of the key academic and industrial research groups in Europe. Some of the main achievements are:

- A large number of novel cellulases and hemicellulases that are useful in lignocellulose hydrolysis
- Cellobiohydrolases (GH7/CBHI) that have the highest thermostabilities reported
- Novel enzymes that have shown feasibility for production in industrial hosts
- New enzyme cocktails than reduce the enzyme amount needed for hydrolysis by 20-30%
- A large collection of yeast strains that are more robust in process conditions than the industrial strains currently used
- Robust pentose fermenting industrial yeast strains that are amongst the best, or the best, strains currently available
- Yeast strains that have xylose fermentation rates close to that of glucose, and can efficiently co-ferment the sugars
- Thermostable enzyme cocktails and yeast strains that are suited to new process concepts, e.g. those carried out at elevated temperatures
- Technoeconomic and LCA calculations that show benefits in respect to plant efficiency, greenhouse gas emissions and minimal selling price of ethanol when the NEMO enzymes and yeasts strains are used in the process

More than 50 technical Deliverable reports have been submitted to Commission describing the RTD results. The benefits of the new NEMO developments span much broader that could be realised within the NEMO project itself. Only part of the identified genes, enzymes and yeast strains could be examined in ethanol production processes during the project. The science and insight into enzyme and yeast requirements have expanded significantly. The partners are in a very good position to continue their work from a cutting-edge science base and apply the NEMO results and further developments in industrially relevant process configurations.

The process concepts for the conversion of lignocellulosic substrates into ethanol are becoming more diverse, as compared to the traditional SSF or SHF processes. Thus, in the novel concepts, the improved properties of biocatalysts can be more efficiently benefited, resulting in decreased costs of the overall conversion processes of lignocellulose into ethanol. The NEMO results and process configurations can be applied in various current and emerging bioethanol processes but also directly in other biorefinery applications, e.g. in chemicals production. A key question common for all fermentation based processes is how to obtain cheap sugar through pretreatment and enzymatic hydrolysis. And robust yeasts strains, including pentose utilising, are of utmost importance as highly valuable hosts for further engineering towards also other fuels and chemicals than ethanol.

Novel enzymes

Presently, the key targets for enzymes are enhanced thermal stability and identification of improved enzyme components enhancing the hydrolysis yield. Cellulases still make up a significant cost factor in the production of cellulosic ethanol. Currently, most commercial enzymes are only able to act at a temperature range close to 40-50°C [Viikari,L., Vehmaanperä, J., Koivula, A. (2012) Lignocellulosic ethanol: from science to industry. Biomass and Bioenergy 46, 13-24]. Thermostable enzymes offer potential benefits in the hydrolysis of lignocellulosic substrates; higher specific activity decreasing the amount of enzymes needed, enhanced stability allowing improved

hydrolysis performance, better inhibitor tolerance and increased flexibility with respect to process configurations, all leading to improvement of the overall economy of the process.

Thus, in the NEMO project, novel CBHI enzymes could be engineered having some of the highest thermostabilities reported for any GH7 cellobiohydrolases. One of these engineered CBHI was used in the new NEMO Thermomix, which performed in a superior manner on pretreated spruce hydrolysis as compared to the classical mesophilic reference enzyme mixture, Celluclast + Novozym 188. The Thermomix used in the NEMO project is a novel base for further improvements. Complemented with other thermostable enzymes, these enzymes will form a base for next generation industrial enzymes.

In the search for reducing the biomass conversion costs, additional hemicellulase enzymes were found to further improve the hydrolysis yield of pretreated *Arundo donax*. The enzyme dosage of Celluclast + Novozyme188 could be lowered with 30% when replacing 10% of the baseline enzyme with the novel GH10 Xyl11 derived from *Myceliophtora thermophila* C1 strain. In addition, 30% lower enzyme dosage could also be used when replacing 20% with the GH10 xylanase and CBHI Thermo variant 4.

In most pretreatment processes, lignin is retained in the pretreated raw material and used as energy source for the process or potentially used for upgrading into value-added products. Lignin, however, poses problems by limiting the access and free movement of enzymes on the complex substrates. Thus, lignin is one reason for the unproductive binding of enzymes and limiting their efficiency. Various approaches have been taken to diminish this effect; by developing less lignin-binding enzymes or modifying the structure of lignin. In the NEMO project, cellobiohydrolases binding significantly less to lignin were used. In another approach the hydrolysis yield was improved by simultaneous oxidative enzyme treatments. Thus, improved enzymes and increased knowledge on enzyme mechanisms and their interactions with the substrate led to significant improvements of the hydrolysis yield. The enzyme dosage could be decreased by various alternative or complementary methods, evaluated individually. In future, when these improvements could be additive, even more significant achievements are to be expected.

Efficient production of industrial enzymes is a prerequisite for reducing the conversion costs. In the NEMO project, high production yields in the industrial production hosts, *T.reesei* and *Myceliophtora thermophila* C1, could be achieved for various Ascomycete enzymes, and also what si significant for Basidiomycete enzymes as well for the metagenomic (hemi)cellulases. Results obtained in the project will form a basis for further design of optimized enzyme mixtures in industrial production hosts and will be continued in the enzyme producing companies. The analysis of the fungal genomes also demonstrated that we have only scraped the surface of the enzymatic potential that is present in the fungal kingdom, indicating that approaches such as used in NEMO can identify novel enzymes that will significantly improve the efficiency and lower the costs of the hydrolysis step of biofuel production.

Novel yeasts

NEMO succeeded well in reaching its two major goals in terms of novel yeasts: development of industrial yeast strains with high pentose fermentation capacity and strains with high stress tolerance.

The strains developed in the NEMO project are among the best strains currently available for 2nd generation bioethanol production, if not the very best. It is to be noted that construction of pentose

fermenting strains was started from "zero" in the NEMO project since the aim was to use an industrial robust *S. cerevisiae* strain as a parent (Ethanol Red). The work also illustrated the significant efforts that are needed to engineer strains based on the xylose isomerase (XI) pathway for efficient anaerobic pentose fermentation. It is not sufficient to introduce XI (and genes for arabinose utilization) and several genes for upregulation of the pentose phosphate pathway enzymes into the yeast, but additionally for instance evolutionary engineering and selection is needed (Demeke et al. (2013). Biotechnology for Biofuels, in press). Furthermore, NEMO partners succeeded in isolation and engineering of xylose and arabinose transporters that allow pentose uptake in the presence of glucose, i.e. co-fermentation of C6 and C5 sugars, which is essential for good fermentation efficiencies. The strain HDY.GUF12 fermented xylose completely with 55% of the maximal specific consumption rate of glucose.

Selected pentose fermenting yeast strains (GUF and VIB series) have been tested on various NEMO hydrolysates and process relevant conditions in SHF and SSF mode and in new process regimes. Albeit xylose fermentation seems still affected by inhibitors present in some industrial hydrolysates, the strains perform well and are suited for industrial processes.

In addition, a large collection of *S.cerevsisiae* strains was established which show improved tolerance towards a variety of physical and chemical stresses that the yeast encounters in process conditions. These strains are superior to the current robust industrial strains such as Ethanol Red and Fermipan. Several strains demonstrated excellent ethanol tolerance and growth at increased ethanol concentrations (up to 18-19%), and capability to grow at elevated temperatures (40-41°C) in the presence of lignocellulosic hydrolysates. Evolutionary and metabolic engineering have been used to further improve the strains for superior robustness.

A very powerful technology for the genetic analysis of complex traits was developed and applied in NEMO, the AMTEM technology. This (and whole genome sequencing) was successfully applied in identification of genes behind stress tolerances, metabolic characteristics as well as high pentose fermentation capacity, that are required in an industrial yeast strain for 2nd generation bioethanol production. Results have been published already for ethanol tolerance (Swinnen et al. 2012, Genome Res. 22:975-984), low glycerol/high ethanol production (Hubmann et al. 2013, Met. Eng. 17:66-81 & Biotechnol. & Biofuels 6:87) and osmotolerance/maximal ethanol accumulation (Pais et al. 2013, PLoS Genetics 9(6):e10003548). They show that the NEMO group is the world leader in applying polygenic analysis to yeast traits of commercial importance. This polygenic analysis methodology for complex traits now provides a very efficient tool for further gradual improvement of the industrial yeast strains already available, in order to make them exceed the performance of any competing strain in this field.

As a conclusion, several interesting yeasts strains remain to be exploited and a number of stress tolerance traits to be combined in strain improvement strategies, which were not possible address within the framework of the NEMO project. These will provide valuable know-how and toolbox for various biorefinery applications. In addition, the advanced metabolic models and modelling tools that have been developed in NEMO are indispensable when physiology and metabolic insight need to be combined with future metabolic pathway engineering and synthetic biology approaches towards other chemicals and novel products.

Process considerations

It is a challenge within a 4 year long EU project to proceed from identification of previously unknown enzymes or yeasts and the relevant genes to demonstration of their industrial benefit at

pilot scale followed by technoeconomic and LCA calculations. This needs very good coordination on specifying the relevant initial screening and selection procedures and prioritization of the methods and materials that are worthwhile – or that are feasible time-wise – to take forward. **Fig. 20** shows the different steps that were needed for enzyme development in NEMO. A similar scheme could be outlined for yeast development.

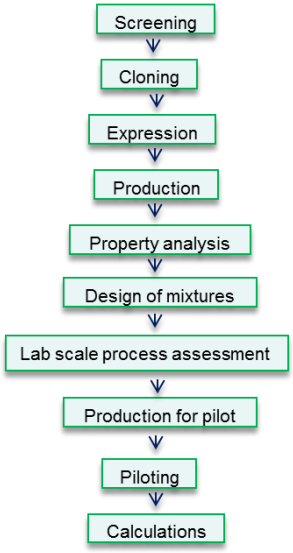

Figure. 20. Scheme of the different steps performed in NEMO to identify novel enzymes with shown benefits at pilot scale

Testing and screening was carried out on pretreated NEMO raw materials from the very beginning and enzymes and yeasts assessed in process relevant conditions. Combinations of selected enzymes and yeast strains were then evaluated in process regimes that are relevant for bioethanol (and biorefinery) production processes, i.e. SHF and SSF. Furthermore, different process conditions could be tested (e.g. biomass consistency, temperature). One of the aims was also to design new type of process concepts where the new enzymes and yeasts would show benefits. NEMO was successful in demonstrating that enzymatic pre-hydrolysis of spruce at elevated temperatures with a specific four-component thermophilic enzyme cocktail was superior to the commercial enzyme preparate (consisting of a multicomponent mixture of lignocellulosic enzymes). NEMO also demonstrated that having more thermostable enzymes and thermotolerant yeast strains at hand enables gradual temperature profiles to be implemented through hydrolysis and fermentation phases that bring benefits it terms of overall process yields in ethanol production. The NEMO process know-how can now be used in bioethanol process development but is equally relevant for other fermentation based biorefinery applications as well.

Only a selected pentose fermenting NEMO yeast and a couple of NEMO enzyme cocktails could be tested in pilot scale due to time and resource constraints. Proper pilot testing would demand a project more dedicated to larger scale evaluation of already existing strains and enzymes. Nevertheless, the benefits seen in laboratory scale could be seen also at pilot scale. Preliminary technoeconomic and LCA calculations carried out by the industrial partners of NEMO showed benefits for all NEMO cases in terms of plant efficiency, GHG emissions and minimal selling price for ethanol. Even though these results provide no absolute figures and are only indicative, they show that enzyme and yeast development are key issues in improving the sustainability of 2nd

generation bioethanol (and biorefinery) processes. The additive benefits of combination of more of the still non-evaluated NEMO achievements remain still to be examined and calculated.

4.1.4.2. Socio-economic and societal impact

The aims, activities and outcomes of the NEMO project are fully in line with the future key priorities of the of European programmes for research and innovation: Excellent science, Industrial leadership, and Societal challenges.

Established with a Communication from the Commission from 30.11.2011 [COM(2011)], the coming EU Framework Programme for Research and Innovation - **Horizon 2020** - is the financial instrument implementing the Innovation Union, a Europe 2020 flagship initiative aimed at securing Europe's global competitiveness. Running from 2014 to 2020 with a suggested €80 billion budget, this EU's new programme for research and innovation is part of the drive to create new growth and jobs in Europe. Horizon 2020 will focus resources on three key priorities, namely Excellent Science, Industrial Leadership, and Societal Challenges.

With respect to Excellent Science, Horizon 2020 aims to raise the level of excellence in Europe's science base and ensure a steady stream of world-class research to secure Europe's long-term competitiveness. With respect to Industrial Leadership, Horizon 2020 aims to make Europe a more attractive location to invest in research and innovation (including eco-innovation), by promoting activities where businesses set the agenda. The following main Societal Challenges addressed in Horizon 2020 reflect the policy priorities of the Europe 2020 strategy and address major concerns shared by citizens in Europe and elsewhere:

- Health, demographic change and wellbeing;
- Food security, sustainable agriculture, marine and maritime research and the bio-economy;
- Secure, clean and efficient energy;
- Smart, green and integrated transport;
- Climate action, resource efficiency and raw materials;
- Inclusive, innovative and secure societies.

NEMO has formed a good basis for the partners to be ready for the next programme Horizon 2020. NEMO has created excellent scientific achievements (described in previous sections). There are already close to 30 peer-reviewed scientific publications produced in NEMO and additional publications are under preparation. NEMO has significantly contributed to advancement in science in the fields of pre-treatment of biomass raw material, efficient enzymes and yeasts, as well as improved process concepts, all cornerstones for the establishment of the future global bio-based economy. Along with the applied targets also basic scientific discoveries and insight has been generated, e.g. identification of novel transportes, insight into microbial stress tolerance and enzymology.

Industrial leadership within NEMO has been guaranteed through the active involvement of several industrial partners as Beneficiaries, who have engaged in research and development as well as deployment and industrial validation activities of the research results. The industries have provided their in-house expertise in producing pretreated materials, enzyme production and pilot facilities, and dedicated efforts in testing NEMO materials. In order to facilitate the commercial exploitation of NEMO results, large efforts were further taken to contact and inform relevant

outside companies and organisations with potential interest in the transfer of NEMO results into industrial applications. *10 NEMO Exploitation Leaflets* have been elaborated, disseminated and promoted to inform industry stakeholders about concrete NEMO exploitable results and cooperation opportunities (see section 4.1.4.4).

Finally, within NEMO - where focus has been on 2nd generation lignocellulosic transport fuel bioethanol - significant achievements have been realised with respect to the following **Societal challenges**:

- Development of the bio-economy
- Secure, clean and efficient energy
- Smart, green and integrated transport
- Resource efficiency and raw materials.

Contributions to tackling these abovementioned Societal challenges (as detailed in NEMO reports, scientific publications, dissemination activities, patent applications, exploitation flyers, and industrial validation activities) have been achieved in the following main fields:

- Development of *improved yeast strains fermenting C6 and C5 sugars*
  → This result improves the fermentation of lignocellulosic raw material and leads to cost reductions in the production of second generation bioethanol and other bio-based products.
- Development of *industrial yeast strains with improved inhibitor tolerance*
  → This result improves the fermentation of lignocellulosic raw material.
- Development of *novel improved enzymes*
  → This result improves degradation of hemicellulose, thereby improving the complete enzymatic hydrolysis of lignocellulosic raw material.
- Development of *improved process concepts* such as optimized temperature profile in simultaneous saccharification and fermentation
  → This result improves the overall processing of lignocellulosic raw material, and leads to cost reductions in the production of second generation bioethanol and other bio-based products.

Results of the NEMO project thus contribute to the development of technologies allowing increased and more economical utilization of European renewable lignocellulosic raw materials for the production of second generation bioethanol as well as other bio-based products such as chemicals. Novel high-performing enzymes, efficient micro-organisms, as well as optimized process concepts provide the basis of future resource efficient use of lignocellulosic raw materials with the aim to contribute to secure, clean and efficient energy (mainly: fuel) supply, to a greener and more sustainable transport, as well as to the **overall development of the bio-economy in Europe and globally**.

Specifically, in the field of biofuels NEMO results are supporting the aims of the Commission as stated in their proposal for an amendment of the Renewable Energy Directive (RED) [COM(2012) 595 final] published on 17 October 2012. This proposal suggests to limit to 5% the amount of first generation biofuels (from agricultural feedstock) counting towards the RED targets, and to promote advanced non-land using biofuels, such as wastes and lignocellulosic material, in order to reduce the so-called "indirect land use change (iLUC)" impacts of biofuels.

Finally, activities and outcomes of the NEMO project are fully in line with the vision document put forward by the Horizon 2020 Bio-Based Industries Initiative.

This vision document sees bio-based industries as core part of the bio-economy holding the potential to decouple economic growth from resource use while leading the transition towards a post-petroleum society. The industry vision is to use renewable resources, bio-waste and side streams as the major input source for materials, food and feed ingredients, fuels and chemicals, maximizing the added value along the value chains. At the centre of this vision, bio-refineries will gradually replace oil-refineries, by sustainably processing biomass into a spectrum of marketable products and energy.

The NEMO technologies and developments can be directly applied in the Biobased PPP, and should be taken forward in this near Horizon 2020 program.

4.1.4.3. Dissemination

In order to increase the visibility of the NEMO project and to facilitate dissemination activities, a **NEMO project logo** (**Fig. 21**) was created upon project launch.

Figure 21: The NEMO logo

Furthermore, a **website** for the dissemination of NEMO project activities and results was created under *http://nemo.vtt.fi*. Basic project data such as project objectives and activities as well as consortium partner contacts are made publicly available. The website also serves as a communication platform for project partners. The password protected internal section is used for file sharing and document storage. The NEMO website has been continuously updated. The NEMO project website will be maintained and updated for at least 2 years after the project duration.

Three **NEMO leaflets** have been elaborated to provide interested stakeholders of the specific target audiences in the scientific and industrial community with an overview of the aims and objectives of the NEMO project (Fig. 3). The first NEMO leaflet provides background information on second generation bioethanol production as well as aims of NEMO activities. The second NEMO leaflet describes midterm results obtained in the field of “novel enzymes for hydrolysis of biomass” and “efficient yeasts for fermentation of biomass sugars”. The third NEMO leaflet launched at the end of the project describes 10 exploitable results from the NEMO project (see chapter 4.1.4.4.). These leaflets can be downloaded from the NEMO web site.

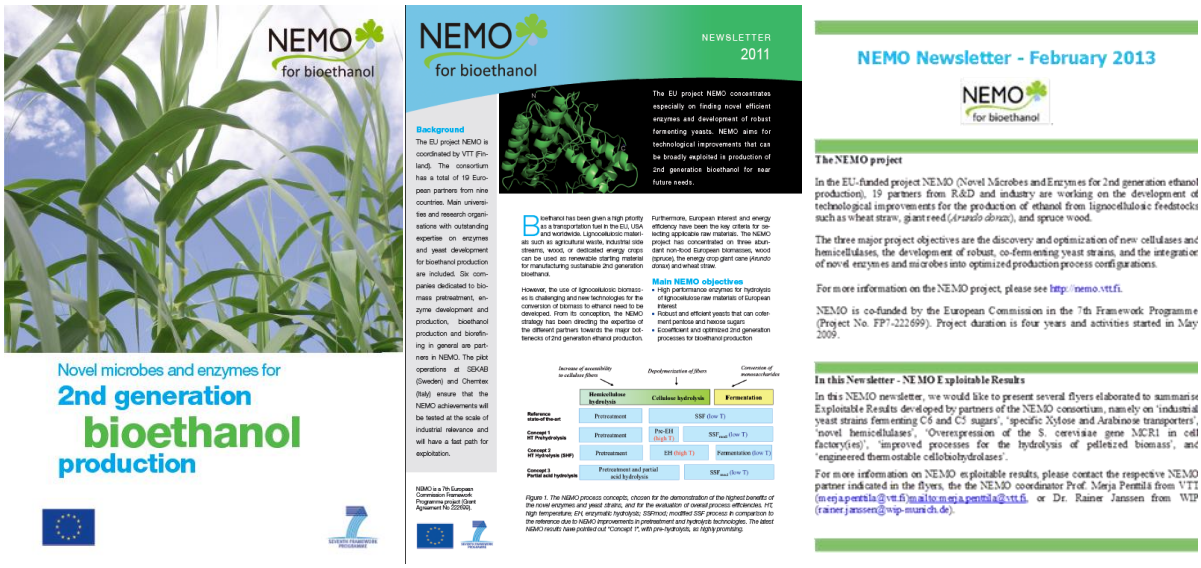

Figure 22: Front pages of NEMO project leaflets

In order to keep stakeholders from the scientific and industrial community updated on NEMO project activities and results, **regular NEMO info mails to stakeholders** have been sent. For this, a database with more than 1.600 stakeholders interested in the second generation bioethanol field was developed by WIP, the NEMO partner responsible for dissemination and exploitation. The database was continuously updated and extended during the project implementation.

Furthermore, NEMO project results have been disseminated via publications in scientific and trade journals as well as via presentations and posters at conferences and other events.

NEMO dissemination activities already during the project comprised an impressive number of close to **26 scientific peer-reviewed publications (+10 submitted)**, **33 oral presentations** and **35 posters** at international workshops and conferences, as well as **10 exploitation flyers**. The following tables present selected NEMO scientific publications, presentations and posters, whereas the exploitation flyers are presented in chapter 4.1.4.4.

Table 11: NEMO scientific peer-reviewed publications (a selection)

Title	Main author	Periodical	Year
Production of biofuels and biochemicals: in need of an ORACLE	Vassily Hatzimanikatis, EPFL	Trends in Biotechnology	2010
The laccase-catalyzed modification of lignin for enzymatic hydrolysis	Ulla Moilanen, UH	Enzyme and Microbial Technology	2011
Fungal enzyme sets for plant polysaccharide degradation	Joost van den Brink, CBS	Applied Microbiology and Biotechnology	2011
Challenges in enzymatic hydrolysis and fermentation of pretreated *Arundo donax* revealed by a comparison between SHF and SSF	Magnus Ask, CTH	Process Biochemistry	2012
Competition between pentoses and glucose during uptake and catabolism in recombinant *Saccharomyces cerevisiae*	Eckhard Boles, GUF	Biotechnology for Biofuels	2012
Effect of temperature on simultaneous saccharification and fermentation of pretreated spruce and arundo	Sarma Mutturi, ULUND	Industrial and Engineering Chemistry Research	2013
Quantitative trait analysis of yeast biodiversity yields novel gene tools for metabolic engineering	Johan Thevelein, VIB	Metabolic Engineering	2013

Table 12: NEMO presentations (a selection)

Title	Partner	Location	Date	Audience
Bioethanol for sustainable transportation	VTT	Brussels, Belgium	10/06/2010	>100 scientific
Retrofitting complex systems for green growth	EPFL	Jeju, Korea	13/06/2010	>200 scientific & industry
Bioethanol for sustainable Transportation	VTT	Shanghai, China	14/06/2010	International scientific and industry audience; > 100 participants
Biotechnical conversion of biomass to biofuels	UH	Lyon, France	28/03/2011	>2500 scientific & industry
Novel high performance enzymes and micro-organisms for conversion of lignocellulosic biomass to bioethanol	VTT	Rimini, Italy	14/09/2010	International scientific audience; > 50 participants
Cell factories at the service of industrial biotechnology	UNIMIB	Gothenburg, Sweden	21/10/2011	>30 scientific
Long-term adaptation of an industrial *Saccharomyces cerevisiae* strain	ULUND	Belgirate, Italy	10/05/2012	>50 scientific
HMF and furfural stress results in drainage of redox and energy charge of *Saccharomyces cerevisiae*	CTH	Madison, USA	26/08/2012	>400 scientific & industry
Functional analysis of the *Myceliophthora thermophila* C1 xylanase machinery	DYADIC	Wageningen, Netherlands	07/04/2013	>150 scientific & industry
Development of superior xylose-fermenting and robust industrial yeast strains	VIB	Madrid, Spain	03/04/2013	>200 scientific & industry
Novel high performance enzymes and micro-organisms for conversion of lignocellulosic biomass to bioethanol	VTT	Madrid, Spain	03/04/2013	International scientific and industry audience, >200 participants

Table 13: NEMO posters (a selection)

Title	Partner	Location	Date	Audience
Screening of fungal diversity: a strategy to improve lignocellulolytic bio-ethanol production	KNAW	Rotterdam, Netherlands	01/06/2010	>300 scientific
Enzymatic modification of lignin for enhanced hydrolysis	UH	Rimini, Italy	16/09/2010	>100 scientific & industry
Lignin-derived inhibition of cellulases – effect of temperature and the origin of lignin	VTT	Naantali, Finland	07/06/2011	>50 scientific
Expression of the *Arabidopsis thaliana* lipocalin TIL in *Saccharomyces cerevisiae*	UNIMIB	Bruges, Belgium	08/06/2011	>100 scientific
Transcriptional response and alterations in adenonucleotides and redox cofactors in *S. cerevisiae*	CTH	Copenhagen, Denmark	28/02/2012	>100 scientific & industry
Novel recombinant xylose and arabinose fermenting industrial yeast strain for bioethanol production	GUF	New Orleans, USA	30/04/2012	>500 scientific & industry
Functional analysis of the *Myceliophthora thermophila* C1 xylanase machinery	DYADIC	Prague, Czech Republic	21/04/2013	>200 scientific & industry

In the framework of the NEMO project *specific training needs* have been regularly examined, such as training needs of students involved in NEMO R&D activities, and possibilities and suggestions how NEMO could contribute to biorefinery and biofuels related education at European universities and other forums. For instance, NEMO contributed to the **1st European Biorefinery Summer School** whose main organiser was the EU project Biocore. The course was held in Paris August 29–September 1, 2011. With special emphasis on Nordic biorefinery topics, NEMO organised on May 23-25, 2012 its own **NEMO Biorefinery Summer School** "Bioethanol in a Biorefinery Context" in Örnsköldsvik, Sweden (**Fig. 23**).

Figure 23: NEMO graduate level course, May 2012

Finally, the NEMO project established **close interactions with other EU projects** in the field, e.g. with respect to sharing technical know-how, promotion of seminars and the exploitation of project results. Cooperation links have been established with other EC projects such as Disco, Hype,

SAHYOG, BIOLYFE, BIOCORE, CaneBioFuel, SUNLIBB, ProEthanol2G, BABETHANOL, SWEETFUEL, and Global-Bio-Pact. Also information exchange has been with Eastern European countries, India, Latin America and Northern America.

4.1.4.4. Exploitation

In order to facilitate commercial exploitation of NEMO results, relevant companies and organisations with potential interest in the transfer of NEMO results into industrial application were contacted. The aim of initial contacts was to generally raise awareness about NEMO and existing and upcoming commercialisation opportunities.

In order to inform industry stakeholders about **concrete NEMO exploitable results** and cooperation opportunities, the following **10 NEMO Exploitation Leaflets** have been elaborated:

1. Industrial Yeast Strain Fermenting C6 and C5 Sugars, Contact: Goethe-University Frankfurt (GUF), Prof. Eckhard Boles
2. A specific Xylose Transporter evolved by using a newly developed yeast-based screening system, Contact: Goethe-University Frankfurt (GUF), Prof. Eckhard Boles
3. A specific Arabinose Transporter for improved fermentations of lignocellulosic hydrolysates with recombinant yeasts, Contact: Goethe-University Frankfurt (GUF), Prof. Eckhard Boles
4. Novel hemicellulases derived from *Myceliophthora thermophila* C1 for the degradation and modification of plant materials, Contact: Dyadic Nederland BV (DNL), Dr. Sandra Hinz
5. Overexpression of the *S. cerevisiae* gene MCR1 in cell factory(ies), Contact: Univ. of Milano-Bicocca, Dept. of Biotechnology and Biosciences (UNIMIB), Prof. Danilo Porro, Dr. Paola Branduardi
6. Procedure and apparatus for hydrolysis of pelletized biomass using hydrogen halides, Contact: Green Sugar GmbH (GS), Dipl.-Ing. Biotech. Frank Kose
7. Engineered thermostable cellobiohydrolases for improved lignocellulose hydrolysis, Contact: VTT Technical Research Centre of Finland (VTT), Dr. Anu Koivula, Prof. Merja Penttilä
8. Industrial Yeast Strain combining inhibitor and temperature tolerance, Contact: Lund University, Sweden, (ULUND), Prof. Marie F Gorwa-Grauslund
9. Robust xylose fermenting industrial yeast strain for bioethanol production, Department of Molecular Microbiology, KU Leuven, Belgium (VIB), Prof. Dr. Johan Thevelein
10. Optimized temperature profile in simultaneous saccharification and fermentation, Contact: Lund University (ULUND), Prof. Gunnar Liden
11. Strains with enhanced intracellular glutathione pool and enhanced robustness. Contact: Chalmers University of Technology (CTH), Prof. Lisbeth Ohlsson
12. Methods to overcome the detrimental effects of lignin in lignocellulose hydrolysis Contact: University of Helsinki (UH), Prof. Liisa Viikari

Widespread dissemination of these NEMO Exploitation Leaflets to industry stakeholders was done via several dedicated mailing initiatives as well as on the occasion of international events, such as the Third International Conference on Lignocellulosic Ethanol (3ICLE) which took place on 3-5 April 2013 in Madrid, Spain. Several positive responses have been received from the companies in the field.

Several positive expression of interest to NEMO results have been obtained from companies. **Evaluation of the NEMO results by outside companies**, such as the novel enzymes and pentose fermenting yeasts strains, are ongoing within Europe, Brazil, Chile and India. Since several companies have shown interest a follow-up with industry stakeholders interested in NEMO exploitable results will be performed by the NEMO partners responsible for the respective results after the end of the NEMO project.

Furthermore, **Table 14** presents **patent applications** which have been filed based on results obtained in the framework of the NEMO project.

Table 14: NEMO patent applications

Subject or title of application	**Inventor(s)**
Specific arabinose transporter of the plant *Arabidosis thaliana* for the construction of pentose-fermenting yeasts	Boles, E. and T. Subtil (GUF)
Specific alleles important for ethanol tolerance	Swinnen S., A. Goovaerts, M.R. Foulquié-Moreno and J.M. Thevelein (VIB)
Procedure and apparatus for hydrolysis of biomasses which can be pelleting using hydrogen halides	Kose, F. (GS)
Mutant yeast strain with decreased glycerol production	Thevelein, J.M., G. Hubmann and M.R. Foulquié-Moreno (VIB)
Yeast alleles involved in maximal ethanol accumulation capacity and tolerance to high ethanol levels	Goovaerts A., T. Pais, S. Swinnen, M.R. Foulquié-Moreno, F. Dumortier and J.M. Thevelein (VIB)
Identification of thermotolerance genes in yeast	Yang Y., M.R Foulquié-Moreno and J.M Thevelein. (VIB)