
issy31

31st International Specialised Symposium on Yeast

Nova Gorica / Vipava

Slovenia

October 9 – 12, 2014

BOOK OF ABSTRACTS

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1. Piškur, Jure
275780352

Dear Colleagues,

It is our great pleasure to welcome you to the *31st International Specialised Symposium on Yeast* in Slovenia!

Through the years, ISSY has become a popular venue for the yeast researchers to meet and discuss the many aspects of their favourite study organism. This year's edition is dedicated to yeast fermentations, from the basic concepts to the applications, and from the aspects of biodiversity and evolution to genetic regulation. It is also dedicated to the new generation of yeast researchers, as all of them who have submitted an abstract have been given an opportunity to present their work to the scientific community in the form of a lecture. We hope that this format of the symposium will spark even more ideas, hypotheses and theories, and new collaborations.

Both, the venue and the topic of the conference, have been selected by late Jure Piškur, who, sadly, will only be present at this symposium in spirit. Jure felt at home in Slovenia, and in the science of yeast fermentations. The Law of Nature, to use his words, returned his physical existence into the grand metabolism of all living and non-living things on the planet, before he could enjoy the renovated Lanthieri palace in the company of his friends, colleagues and scientific competitors alike. He was deeply engaged and enthusiastic about this conference and planned it as a highlight of his life and career, and as a tribute to the numerous collaborations and friendships he had created during his productive life. Our wish for the ISSY31 is that together we will, through lectures, discussions and social events, celebrate Jure's life and work.

The ISSY31 Organizing Committee

Uroš Petrovič, Executive Chairman of the Committee

General information about the ISSY31 Symposium

The majority of participants will be accommodated in the Perla Hotel, the actual venue of all the plenary sessions of the symposium, as well as of the get-together on the first day of the programme and the conference dinner on Sunday evening. Perla is a casino hotel, so all the guests are granted access to the casino section on the ground level. You would need to register, however, before your first access to the premises.

Parallel sessions will be organized in Vipava, a countryside town about 35 km east of Nova Gorica. Transportation to/from Vipava will be organized by a total of 5 coaches which will depart from in front of the Perla Hotel in the direction of Vipava and from the Vipava Grammar School parking area in the direction of Nova Gorica. Please keep in mind that coaches will leave at hours listed in the programme and that there are very limited possibilities for commuting between the two towns in the evening hours.

For easier orientation in Vipava, please consult the map on page 216 of this booklet. Parallel sessions will take place at two different locations in Vipava, the Catholic Grammar School and the Lanthieri palace. The distance between the two is about 400 m. Lanthieri palace is the building where the University of Nova Gorica Centre for Wine Research is located, as well as the Centre for Biomedical Sciences and Engineering.

Light lunches on Friday and Saturday will be served in the Lanthieri palace, while coffee and tea will be served both at the grammar school and the palace.

In the case of serious problems, call emergency numbers which are:

- Mr. Boštjan Marcina: +386 31 628 477 (transportation)
- Mrs. Mija Rijavec: +386 41 766 367 (accommodation and general).

Symposium Programme Outline

Thursday, October 9, Nova Gorica **9**

from 12:00 Registration

18:00 – 18:10 Symposium Opening

18:10 – 19:55 Opening plenary lectures:
ICY lecture, Novozymes lecture, Pivovarna Laško lecture

from 20:15 ISSY31 Banquet

Friday, October 10, Nova Gorica and Vipava **15**

08:30 – 12:00: Plenary sessions (Nova Gorica)

Biodiversity – Basidioworld

Genetic Regulation – Metabolic Modelling

Industrial Applications – Bulk and Fine Chemicals

12:15 – 13:00 Transportation to Vipava – buses leave from in front of the Perla Hotel

13:00 – 14:00 Lunch at the Lanthieri Palace

14:00 – 19:00: Parallel sessions (Vipava)

Biodiversity & Wine

Genetic Regulation of Stress Response

Bioethanol & Bioenergy (*Visby* project)

Elucidating Regulation through Functional Genomics

YEASTCELL EU project session

19:15 – 20:00 Transportation to Nova Gorica – buses leave from in front of the Grammar School

Saturday, October 11, Vipava **89**

08:00 – 08:45 Transportation to Vipava – buses leave from in front of the Perla Hotel

09:00 – 12:30

Parallel sessions

Beverages 1 & 2

Biodiversity for Applications

2nd Generation Biofuels

Regulation of and by Lipids

Non-conventional Yeasts in Bioproduction

12:30 – 14:00 Lunch / Vipava sightseeing & meeting local food/wine producers

14:00 – 19:00

Parallel sessions

Ecology and Genomics

Cornucopia EU project session

Regulation of Metabolism and Nutrient Sensing

Biofactories: Tools, Physiology & Products

Polygenic Traits

YeSVitE EU project session

19:15 – 20:00 Transportation to Nova Gorica – buses leave from in front of the Grammar School

DETAILED DAILY PROGRAMMES AND ABSTRACTS

Thursday, October 9

Perla Hotel Auditorium

from 12:00 Registration in the Perla Hotel Lobby

18:00 Opening of the Symposium

Opening plenary lectures *(chair: Teun Boekhout)*

18:10 - 18:45 Wen-Hsiung Li

Evolutionary genetics and genomics of yeast carbon metabolism *(ICY lecture)* 11

18:45 - 19:20 Johan Thevelein

Polygenic analysis of commercially-important complex traits in yeast *(Novozymes lecture)* 12

19:20 - 19:55 Amparo Querol

Molecular bases of the regulation of the glycerol production in the *Saccharomyces* species and relevance for wine fermentations *(Pivovarna Laško lecture)* 13

from 20:15 ISSY31 Get-Together Banquet

EVOLUTIONARY GENETICS AND GENOMICS OF YEAST CARBON METABOLISM

Wen-Hsiung Li

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Department of Ecology and Evolution, University of Chicago, Chicago

Yeasts display highly diversified physiological characteristics. The most distinct physiological character in many yeasts is their special sugar metabolism. In particular, the baker's yeast *Sacharomyces cerevisiae* and its relatives predominantly ferment sugars into ethanol even in the presence of oxygen, which is known as Crabtree effect or aerobic fermentation. It has been postulated that this unusual metabolism provides these yeasts selective advantages in sugar-rich environments. However, it has long been a mystery as to genetically how these yeasts evolved a predominantly fermentative life style. The rapid accumulation of genomic, transcriptomic and epigenetic data in many yeast species in recent years has greatly increased our understanding of the genetic basis and molecular mechanism for the diversified sugar metabolisms among yeasts. In this talk, I shall provide a review of recent comparative genomics and evolutionary studies related to the metabolisms of glucose and galactose, whose metabolic pathways have been extensively studied in yeasts. A series of studies suggested that the evolution of aerobic fermentation involved many different factors, including increases in copy numbers of genes involved in glucose transport, glycolysis and ethanol production; sequence divergence; and transcriptional reprogramming of genes involved in mitochondrial functions through changes of *cis*-regulatory elements and promoter structures. It has also been found that the different abilities among yeasts to use galactose is strongly correlated with the presence of the galactose pathway genes in their genomes. These studies revealed that the adaptation of yeasts to specific niches has greatly shaped their genomic content and regulatory program.

POLYGENIC ANALYSIS OF COMMERCIALLY-IMPORTANT TRAITS IN YEAST

Johan M. Thevelein, María R. Foulquié-Moreno, Edgard Belo, Tom den Abt, Mekonnen Demeke, Françoise Dumortier, Annelies Goovaerts, Sylvester Holt, Yingying Li, Raquel Quintilla Mateo, Hygor Mezadri, Vaskar Mukherjee, Benjamin Offei, Dorota Radecka, Ben Souffriau, Marija Stojiljkovic, Bruna Trindade de Carvalho

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Most traits of industrial importance in yeast are polygenic traits, i.e. traits determined by multiple genes acting together. Screening of *Saccharomyces cerevisiae* strain collections has revealed a wide diversity for such traits, with certain strains often being superior for a desirable trait compared to industrial yeast strains. The genetic analysis of polygenic traits has been a major challenge for many years. We have developed pooled-segregant whole-genome sequence analysis to map all QTLs (Quantitative Trait Loci) determining a complex trait in such superior yeast traits relative to an industrial strain of interest. Reciprocal hemizygosity analysis and allele exchange are then used to identify and confirm the causative genes in the QTLs. The superior alleles found in this way are transferred into industrial yeast strains creating self-cloned, 'cisgenic' industrial yeast strains. This allows for predictable strain improvement with naturally occurring mutant alleles, minimizing the risk of side-effects on other essential traits. We have applied this technology platform to several yeast traits of prime industrial importance: ethanol tolerance of cell proliferation, maximal ethanol accumulation capacity, thermotolerance, reduced glycerol/enhanced ethanol production, acetic acid tolerance, xylose fermentation rate, osmotolerance, flavour compound production, etc. We have identified multiple causative alleles of which several had never been connected to the trait-of-interest. Different approaches have been used to improve the detection of minor loci. This work has also revealed the importance of mimicking as closely as possible the industrial conditions in phenotyping the trait of interest. For instance, we have found new genes responsible for ethanol tolerance at industrially relevant concentrations of 16-17% and we have shown that different superior alleles underlie maximal ethanol accumulation capacity compared to high ethanol tolerance of cell proliferation. Polygenic analysis of an industrial yeast strain with superior performance in bioethanol production with lignocellulose hydrolysates has revealed new genes controlling xylose fermentation capacity.

MOLECULAR BASES OF THE REGULATION OF THE GLYCEROL PRODUCTION IN THE S*accharomyces* SPECIES AND RELEVANCE FOR WINE FERMENTATIONS

Bruno M. Oliveira[1], Eladio Barrio[1,2], Roberto Pérez-Torrado[1] and Amparo Querol[1]

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The wine fermentation is a complex process produced as a result of the activities of a succession of microorganisms, being *Saccharomyces* yeasts (mainly *S. cerevisiae*) the responsible for the alcoholic fermentation. Although *S. cerevisiae* is the most frequent species in wines, and the subject of most studies, *S. bayanus* var. *uvarum* and natural hybrids between *Saccharomyces* species such as *S. cerevisiae* x *S. kudriavzevii* and *S. cerevisiae* x *S. bayanus* var. *uvarum* are also involved in wine fermentations and can be preponderant in certain wine regions.

Studies performed in our laboratory, which compared the properties of natural wine hybrids with their parent species, showed that strains of non-conventional *Saccharomyces* species, such as *S. bayanus* var. *uvarum* and *S. kudriavzevii,* exhibit physiological properties of potential interest in enology because they can respond to the new demands of the wine industry, such as their ability to ferment at low temperatures, their increased **production of glycerol**, their lower ethanol yield (1, 2, 3). By using DNA chips (4), we have also observed, a significant lower expression levels in *S. cerevisiae* compared to the rest of species for genes involved in the glycerol metabolism. These observations attracted our attention due to the potential use of these non-conventional *Saccharomyces* species as starters to solve some demands of the wine industry to improve wine quality.

The main objective of this work is to decipher the molecular mechanism responsible of the overproduction of glycerol in *S. kudriavzevii*, *S. bayanus* var. *uvarum* and natural strains of *S. cerevisiae*. To explain this observation at the molecular level we studied the expression of glycerol biosynthetic pathway genes (Oliveira et al, in preparation). We observed higher enzymatic activity of Gpd1p in *S. kudriavzevii* in response to osmotic and cold stress. Also, we determined that *S. kudriavzevii* Gpd1p enzyme presents increased catalytic properties that will contribute to increase glycerol production. (5).

References:

1. Gamero et al., (2014). J Appl Microbiol. 2013 May;114(5):1405-14.
2. González et al., (2007). Int J Food Microbiol. 2007 May 1;116(1):11-8.
3. Salvadó et al., (2011). Appl Environ Microbiol. 2011 Apr;77(7):2292-302.
4. Combina et al., (2012). Int J Food Microbiol. 2012 Jul 16;157(3):340-5.
5. Oliveira et al., (2014). PLoS One. 2014 Jan 30;9(1):e87290.

This work was supported by AGL2012-39937-C02 from Ministerio de Educación y Ciencia and the EU's Seventh Framework Programme (FP7) under grant agreement PITN-GA-2010-264717.

Friday, October 10

08:30 – 12:00

Plenary sessions (Nova Gorica)

Biodiversity – Basidioworld *(chair: Lorena Butinar)*

Genetic Regulation - Metabolic Modelling *(chair: Klaus Natter)*

Industrial Applications – Bulk and Fine Chemicals *(chair: Concetta Compagno)*

12:15 – 13:00 Transportation to Vipava – buses leave from in front of the Perla Hotel

13:00 – 14:00 Lunch in the Lanthieri Palace

14:00 – 16:15

Parallel sessions (Vipava)

Biodiversity & Wine *(chairs: Gianluigi Cardinali and Angelica Ganga)* *-- see p. 17 for details*

Genetic Regulation of Stress Tolerance *(chairs: Xinqing Zhao and Tilen Konte)* *– see p. 17 for details*

Bioethanol & Bioenergy – *Visby* project
(chairs: Volkmar Passoth and Andriy Sibirny) *– see p. 18 for details*

Coffee break 16:15 – 16:45

Bioethanol & Bioenergy – *Visby* project – continued
(chairs: Volkmar Passoth and Andriy Sibirny) *– see p. 18 for details*

Elucidating Regulation through Functional Genomics
(chairs: Mojca Mattiazzi Ušaj and Károly Kovács) *– see p. 20 for details*

YEASTCELL EU project session *(chair: John Morissey)* *– see p. 20 for details*

19:15 – 20:00 Transportation to Nova Gorica – buses leave from in front of the Grammar School

Friday, October 10 afternoon sessions in Vipava – parallel sessions in:

Aula Magna and Aula Parva – Lanthieri Palace

Grammar School Hall (5 min walk from the Lanthieri Palace)

Biodiversity & Wine / in Aula Magna

Genetic Regulation of Stress Response / in Aula Parva

Bioethanol & Bioenergy (*Visby* project) / in Grammar School Hall

15:10 - 15:20 Ievgeniia Tiukova: *Dekkera bruxellensis* competitive non-conventional ethanol production yeast 54

15:20 - 15:30 Iryna Salii: Deletion of *ADH1* gene, encoding alcohol dehydrogenase for the improvement of glycerol production in *Saccharomyces cerevisiae* 55

15:30 - 15:40 Iwona Kata: Metabolic engineering of *Hansenula polymorpha* for improving the conversion of glycerol to ethanol 56

15:40 - 15:50 Johanna Blomqvist: Ethanol and biogas from steam exploded ISP (integrated storage and pre-treatment) sawdust inoculated with *Arxula adeninivorans* 57

15:50 - 16:00 Jule Brandenburg: Bio-lipid production from lignocelluloses material with oleaginous yeasts 58

16:00 - 16:10 Justyna Ruchala: Construction of *CAT8* gene deletion strains in the yeast *Hansenula polymorpha* 59

(coffee break)

16:45 - 16:55 Krzysztof Berezka: Insertional tagging of the *Pichia stipitis* genes involved in xylose alcoholic fermentation 60

16:55 - 17:05 Marianna Yurkiv: Construction of glutathione overproducers in methylotrophic yeast *Hansenula polymorpha* by engineering of γ-glutamylcysteine synthetase 61

17:05 - 17:15 Marta Semkiv: Metabolic engineering of yeast *Saccharomyces cerevisiae* for rerouting of carbon flux towards glycerol production 62

17:15 - 17:25 Oleksii Lyzak: The role of transcription factor Sef1 in the regulation of flavinogenesis in yeast *Candida famata* 63

17:25 - 17:35 Olena Kurylenko: Metabolic engineering of the methylotrophic yeast *Hansenula polymorpha* for construction of the high-temperature ethanol producers from xylose 64

17:35 - 17:45 Orest Hryniv: Disruption of *ATG13* gene causes the increas of ethanol production during xylose alcoholic fermentation of the thermotolerant yeast *Hansenula polymorpha* 65

17:45 - 17:55 Taras Luzhetskyi: Improvement of high temperature alcoholic fermentation of *Saccharomyces cerevisiae* 66

Elucidating Regulation through Functional Genomics / in Aula Magna

***YEASTCELL* EU project session / in Aula Parva**

BASIDIOMYCETOUS YEASTS: PROSPECTS, DIVERSITY AND TAXONOMY

Teun Boekhout[1,2], Xin-Zhan Liu[2], Qi-Ming Wang[2], Bart Theelen[1], Marizeth Groenewald[1] AND Feng-Yan Bai[1,2]

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Our knowledge on the biodiversity of basidiomycetous yeast is rapidly expanding, but the taxonomy used is still largely outdated. Here we present novel insights based on a multigene phylogeny analysis of all described basidiomycetous yeasts of the Agaricomycotina, Pucciniomycotina and Ustilaginomycotina. Their taxonomy will be revised accordingly, based on this multigene phylogeny backbone as many well supported clades were observed. We will address some specific phenotypic features that are of applied interest, such as the ability to control the growth of pest organisms, the ability to ferment, the ability to cause disease, etc. highlighting the great applied potential of these fungi.

GENOME AND TRANSCRIPTOME ANALYSIS OF A YEAST *Pseudozyma antarctica* T-34

Tomotake Morita[1], Hideaki Koike[2], Shun Sato[1], Hiroshi Habe[1], Dai Kitamoto[1]

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Pseudozyma antarctica is a basidiomycetous yeast belonging to the Ustilagomycetes, a group including the smut fungus *Ustilago maydis*, and has been known as a producer of industry-relevant lipases. *P. antarctica* T-34 was isolated from phyllosphere as the producer of extracellular glycolipids, mannosylerythritol lipids (MELs). The MELs are known as multi-functional glycolipids exhibiting not only excellent properties as bio-surfactant but also unique properties for practical cosmetic ingredients. Recently, we reported the genome sequence (18Mb) of *P. antarctica* T-34, and found its oleaginous character. Among 6,543 of the total genes on the *P. antarctica* genome, 219 genes were categorized into the lipid transport and metabolism classification, while the ascomycetous yeast *Saccharomyces cerevisiae* (non-oleaginous) possessed a smaller amount of genes (140 genes) categorized into the same classification on the basis of KOG analysis. Particularly, the gene encoding an ATP/citrate lyase (ACL) related to acetyl-CoA synthesis commonly conserved in oleaginous strains was found in the *P. antarctica* genome. Moreover, the genome of *P. antarctica* (non-plant pathogen) exhibited a remarkable degree of synteny to the genome of *U. maydis* (plant pathogen), whereas the gene expression profiles of the two strains were significantly different by the DNA microarray method under the oily conditions. The expression of the gene sets relating fatty acid metabolism were markedly up-regulated under the oily conditions compared with glucose in *P. antarctica.* In addition, the MEL biosynthesis cluster was expressed at high intensity in *P. antarctica* regardless of the carbon source compared to *U. maydis.* These results indicate that *P. antarctica* shows oleaginous nature on the basis of the sequence analysis, and these genome and transcriptome analysis would facilitate developing the improved strains with customized properties for high production of the functional bio-based materials.

POLIEXTREMOPHILIC YEASTS FROM PATAGONIA ARGENTINA AND ANTARTCTIC SEA

de Garcia V.[1], Martina Turk[2], Polona Zalar[2], Nina Gunde – Cimerman[2], Giraudo M. R.[1]

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Microorganisms inhabiting cold environments have the ability totolerate freezing since they are exposed to low temperatures, dehydration, which is caused by water being bounded to ice crystals, and to survive in ultraoligotrophic conditions. Cellular adaptations to low temperatures and high salinity are generally similar and the species that inhabit these environments have specialized mechanisms for stress tolerance (osmotic and oxidative). These conditions trigger the synthesis of various metabolites that help the cellsto survive. Physiological adaptations of psychrophilic and psychrotolerant microorganisms include modifications of membrane lipid composition, the accumulation of cryoprotectants like polyols (glycerol, trehalose), synthesis of the enzymes that are active at low temperatures and the production of antifreeze proteins. The presence of yeasts in sea water and sea ice of Antarctic (Bellingshausen, Weddell and Scotia seas) was studied. From 26 samples (19 of sea water and 7 of sea ice), 281 yeast strains (65 species) were isolated and identified. On selected yeast strains (psychrophilic,psychrotolerant and halophilic) cellular stress was estimated by measuring phosphoglucomutase activity at various osmotic conditions and temperature (at 5 and 15 °C, with 3 % NaCl (w/v) and without NaCl). Results showed that strains have a higher phosphoglucomutase activity at 15 °C with 3 % NaCl (w/v). We also studied the capability of yeasts to grow on media with urea as the only carbon source at 4 °C, were only basidiomycetous yeasts were able to grow. Furthermore we have searched for the presence of antifreeze protein genesin psychophilic yeasts (*Glaciozyma, Mrakia* and *Rhodotorula*), and for all genera positive results were obtained. The results contribute to the general knowledge of the diversity, ecology, and biotechnological potential of these yeasts and will help to identify the impact of stress conditions on the major metabolic pathways of poliextremophilic yeasts from the marine environments of Patagonia and the Antarctic seas.

MAKING, MENDING, AND EXTENDING THE YEAST METABOLIC MODEL

Stephen G Oliver

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Stoichiometric (or constraint-based) models of microbial metabolic networks have provided new insights into metabolism and have enormous potential as tools with which synthetic biologists can design novel strains for the production of fuels, chemicals, and pharmaceutical agents. Such models are also of use to systems biologists in understanding the complex network of interactions that are required for the functioning of a single cell.

The metabolic model of the yeast *Saccharomyces cerevisiae* is the most complete for any eukaryotic organism and is very effective at predicting which gene deletions will have a lethal phenotype. However, despite early successes, it is very much less effective at predicting genetic interactions. At a coarse-grained level, the yeast metabolic model is capable of successfully predicting the overall topology of the network of interactions between genes involved with the organism's metabolism. However, the model only predicts < 3% of all the epistatic interactions revealed from synthetic genetic array (SGA) experiments. Moreover, predictions from the metabolic models for bacteria are much more accurate than is that for yeast. While, it is clear that the content and connectivity of the yeast model can be improved, can it really be that our view of yeast metabolism is so far behind that for bacteria, or is something else missing?

In this presentation, I shall demonstrate how machine-learning (sometimes assisted by a Robot Scientist), more careful strain construction, and the use of quantitative data on the localisation of individual proteins can all be used to improve the accuracy of the yeast metabolic model. I shall also discuss the impact of such improvements on the predictive power of the model and its use in strain design.

MODEL BASED ENGINEERING OF *Pichia pastoris* CENTRAL METABOLISM ENHANCES RECOMBINANT PROTEIN PRODUCTION

Justyna Nocon[1], Matthias G. Steiger[1,2], Martin Pfeffer[1], Seung Bum Sohn[3], Tae Yong Kim[3], Hannes Rußmayer[1,2], Stefan Pflügl[1], Christina Haberhauer-Troyer[2,4], Karin Ortmayr[4], Stephan Hann[2,4], Gunda Köllensperger[2,4], Brigitte Gasser[1,2], Sang Yup Lee[3], Diethard Mattanovich[1,2]

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Genome scale metabolic models have been successfully applied to predict genetic interventions to redirect metabolic fluxes towards desired products of the primary and secondary metabolism. Complex polymeric products like heterologous proteins equally demand redistributions of primary metabolic fluxes, their rational design however is far less obvious. Therefore cell engineering for protein overproduction has concentrated mainly to transcription, codon usage, protein folding and secretion. We have developed the first genome scale metabolic model for the yeast *Pichia pastoris* (PipMBEL1254), and integrated the synthesis of heterologous protein. The model could successfully predict the increase of protein production in oxygen limited conditions, as well as changes of central metabolic fluxes in production strains, as measured by ^{13}C flux analysis. Metabolic engineering targets for enhanced protein productivity were predicted with FSEOF for gene overexpression and MOMA for gene deletion. After deleting or overexpressing the respective genes as predicted more than 50 % of the interventions led to an enhanced production of cytosolic human superoxide dismutase (hSOD), and similarly of other proteins. Beneficial mutations were mainly related to reduction of the NADP/H pool and the deletion of fermentative pathways. We demonstrate that genome scale metabolic modeling is suitable to describe metabolic changes in recombinant strains and can be successfully applied to design genetic interventions to the primary metabolism to increase recombinant protein production.

RECENT DEVELOPMENTS IN BIOFUELS AND BIO-BASED CHEMICALS PRODUCTION BY YEASTS

Charles A. Abbas, Ph.D.
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Archer Daniels Midland Research
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In the past decade, the demand for energy has fueled a worldwide resurgence in interest in the production of biofuels and bio-based chemicals from renewable feedstocks. The increased focus on the use of renewable feedstocks, such as lignocellulosics, is due in part to recent advances in pretreatment and hydrolysis technology. With the increased search for alternatives to fossil fuels, renewable feedstocks have also received governmental mandates and subsidies that aim to create incentives for their commercial production. Second generation biofuels include, in addition to cellulosic ethanol, long-chain alcohols, terpenoid hydrocarbons, and diesel-length alkanes. Advances in technology have also led the chemicals industry to leverage new feedstocks that provide a cost advantage while delivering a sustainable chemical product. Today, the vast majority of industrial chemicals are produced with fossil fuel-based feedstocks; but with the volatility of petroleum-based feedstocks, there is increased demand for more sustainable products.

The development of new yeast strains of *Saccharomyces cerevisiae* by genetic engineering and the use of other genera of yeasts that can utilize a broader range of feedstocks have been the focus of research efforts. The chemicals of primary interest are the C3 and C4 chemicals that represent the fundamental building blocks of key segments of the industrial chemical industry, which today have annual sales valued at more than $10 billion. Used for a broad range of industrial and consumer products, these chemicals are on the list of top ten chemicals developed by the U.S. Department of Energy (DOE). The production of these chemicals has been already demonstrated at bench- and pilot-scale and at demonstration-scale facilities.

ROBUST HIGH-PERFORMANCE *S. cerevisiae* STRAINS FOR C5 FERMENTATION IN LIGNOCELLULOSIC ETHANOL PRODUCTION

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The process consolidation of lignocellulose based ethanol production at industrial scale is now becoming a reality. Feedstocks, including agricultural crop residues, and wood and paper mill discards, represent a relatively cheap and abundant source of biomass. However, the sustainable production of ethanol requires the combination of efficient hydrolysis and successful fermentation. During biomass pretreatment and hydrolysis, several potential inhibitory compounds including weak acids, furaldehydes and phenolics are released, which reduces the microbial fermentation performance. While fermentation of lignocellulose based sugars such as glucose and xylose by recombinant yeasts have frequently been demonstrated in laboratory media, efficient fermentation in realistic process conditions is much more challenging.

The yeast *Saccharomyces cerevisiae* is currently the preferred microorganism for the fermentation step of lignocellulose ethanol production. While *S. cerevisiae* is naturally able to metabolize hexose sugars, genetic engineering is required to develop strains harboring heterologous pathways for the consumption of pentose sugars. However, lignocellulosic hydrolysates from different raw materials are very different in both sugar composition and inhibitor content. To meet the demand for strains suitable for different raw materials, we have developed a tool-box with a flexible modular system to generate collections of strains based on various *S. cerevisiae* strain backgrounds. Our xylose assimilation pathway based on a mutated xylose reductase (XR) eliminates xylitol formation and results in close to theoretical ethanol yields. C5/C6 fermentation in different lignocellulosic hydrolysates will be presented.

USING BAKER'S YEAST FOR PRODUCTION OF SUSTAINABLE INGREDIENTS IN HEALTH, NUTRITION AND WELLNESS

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Most valuable small molecules are made from fossil oils, from extraction of plant or other natural materials or obtained through specialized agriculture. The use of fossils for manufacturing has its obvious drawbacks, but less appreciated is the fact that extraction from natural sources may lead to exhaustion of these, and though some agricultural production systems are perfectly sustainable, not all are. Thus, growing the plant or raising the animal may take more land, more water or more energy than it really should. Finally, any extraction process may require solvents or other processes which generate significant waste.

Making the compounds by fermentation instead can improve the product's sustainability greatly, avoiding deleterious use of fossils, natural source extraction or freeing land or other resources for other uses. Evolva's Genetic Chemistry technologies [1,2] allow for this. State-of-the art methodologies for establishment of heterologous biosynthesis pathways in Baker's yeast allow for fast development of fermentation-based sustainable manufacturing routes. Added benefits are higher product quality (only one product formed at a time), improved supply chain stability (no seasonal variation) and the possibility for customization (blends of single components). Production of vanillin, resveratrol and Stevia sweeteners will be discussed.

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EXPLORING "PERIPHERAL" METABOLIC PATHWAYS IN BUDDING YEASTS

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The budding yeasts (phylum *Ascomycota*, sub-phylum *Saccharomycotina*) play a number of important roles in human society such as model systems for understanding basic cellular function, opportunistic pathogens, spoilage organisms and the production of fermented beverages to name but a few. From a metabolic perspective, budding yeasts have a much simpler metabolic circuitry than the filamentous ascomycetes (sub-phylum *Pezizomycotina*) or the basidiomycetes (phylum *Basidiomycota*). This metabolic simplicity in combination with the relative ease of genetic manipulation make the budding yeasts an ideal system to explore poorly characterized, non-universal metabolic pathways – so-called "peripheral" metabolism (to distinguish it from universally conserved central pathways such as glycolysis etc). Peripheral metabolism includes both catabolic pathways (the assimilation of non-conventional sources of carbon, nitrogen and sulfur) as well as anabolic pathways (for example the biosynthesis of sophorolipids, exopolysaccharides and volatiles). My research is devoted to characterizing these pathways and identifying the genes involved as well as discovering new pathways. An increased understanding of these pathways have a wide variety of applications including the improvement of substrate utilization in industrial fermentations, degradation of xenobiotics as well as production of biofuels and fine chemicals.

SPECIES DELIMITATION: DEFINING THE UNIT OF YEAST BIODIVERSITY

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Delimiting yeast species for reliable and rapid identification is a complex operation conditioned by philosophical, biological and technical problems that one can summarize in the three following questions: i. do yeast species exist?, ii. do the delimited species have a biological meaning?, iii. how to combine tools for the best classification and identification?.
The first issue is an exquisitely ontological question transformable in the other interrogation: "do yeast species present relevant discontinuities (gaps) among themselves?". Data from the literature are sometimes conflicting, maybe due to different behaviors of the analyzed taxa.
The evidence that molecular tools often based on very short DNA sequences (as barcoding), are increasingly popular in yeast identification, poses the question on whether the species delimited with these systems have also a biological meaning. This can be translated in the question: "do barcode clusters correlate with relevant biological factors?".
Finally, the presence of an overwhelming amount of data collected by yeast biologists over the decades suggests the urgent need to efficiently use existing databases, possibly developing next generation analytical tools to facilitate data mining.
Discontinuity analyses were carried out on various taxa, predominantly those involved in wine production, using LSU and ITS markers. Results showed that discontinuities are often not obvious, especially for species with many isolates like *Saccharomyces cerevisiae*. These results suggested that yeast taxa involved in the fermentation industry were subject to correlation between DNA markers and other available data. The high variability of these analyses outcome will be presented in detail. Finally, we will present an example of a state of the art database for yeast taxonomy and some lines of development for next generation bioinformatics tools focused on this topic.

YEASTS OF "*END OF WORLD"*: TECHNOLOGICAL POTENTIAL

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In the applied science, the main goal of our group has been the study of native yeasts isolated in different areas of Chile to determine its potential use in the wine industry. Our group has a collection of 1.800 yeasts isolates between *Saccharomyces* and *non-Saccharomyces* genera. During 8 years, we have researched about the potential use of *Saccharomyces* spp. species in winery industry. From this work, we have defined a native yeast named Fermicru XL (Oenobrands) to be used in young wines. This yeast is characterized to give fruity red wines, with enhanced mouth-feel and soft tannins.
In addition, we have performed a screening of different enzymatic activities xilanase, ß-glycosidase, etc.) using our non-*Saccharomyces* collection. Thus, we have obtained the strain *Metchiniscowia pulcherrim*a that produced ß glycosidase activity. During the wine fermentation, this yeast produces high thiols concentration, which have special interest for the oenologists because providing fruity aromas to wine, especially in white wine. As in the previous case, this yeast is being marketed as Flavia (Lallemand Inc).
On the other hand, our group has selected a native yeast from an typical wine of north of our country, named Pajarete. This wine is sweet and is produced with dried grapes. This native yeast provides fruits aromas, especially banana characteristic of this type of wine. At the moment, we are selecting more yeasts from this wine and we are studying to produce the yeasts at laboratory.

SURVEY ON YEAST BIODIVERSITY IN GEORGIAN VINEYARDS: A PRISTINE ENVIRONMENT FOR THE SELECTION OF WINE STRAINS

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The wine market is changing due to ever-growing demand for improved sensory and nutritional properties of the final product. New styles of wines and innovative ways of managing fermentations have intensified interest in the search for new starter strains. Here we investigate wine yeast biodiversity in Georgia, an ancient vine growing area where the use of starter cultures has not yet spread, to select non-conventional yeast species for wine-making. Yeast isolation was carried out from grapes, musts and wines in six different Georgian regions. One hundred and seventy six isolates were identified as belonging to 14 different species: *S. cerevisiae* (36.4%), *Hanseniaspora gulliermondi* and *Metshnikowia pulcherrima* (about 20%), *Cryptococcus flavescensis* (8%), *Cryptococcus carnescens* and *Torulaspora delbrueckii* (about 3%), *Candida intermedia*, *Pichia guilliermondi* and *Pichia kluyveri* (about 2%), *Candida gotoi*, *Issatchenkia terricola*, *Cystofilobasidium infirmominiatum*, *Kluyveromyces marxianus* and *Hanseniaspora osmophila* (≤ 1%). *S. cerevisiae* isolates were further submitted to whole-genome comparison to establish phylogenetic relationships and genome structure. Some non-*Saccharomyces* yeasts were investigated biochemically (alcohol and SO_2 tolerance, acetic acid, glycerol and H_2S production). Using micro-vinification experiments, one *K. marxianus* and one *T. delbrueckii* strain are proved to be promising for wine production.

MULTI-STRAIN INDIGENOUS YEAST STARTERS FOR 'WILD-FERMENT' WINE PRODUCTION

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Industrial wine-making is based on commercial *Saccharomyces cerevisiae* starter cultures, which are applied to confer manageability and reproducibility. However, the use of a single starter culture, which typically overwhelms the indigenous microbiota, deprives wines from the complexity and authenticity. Yet, global competition in the wine market calls for the production of quality wines with typical character. Indigenous *S. cerevisiae* and non-*Saccharomyces* (wild) yeast species may confer added value to wines, by adding to the wine complexity and authenticity. In the above context, the scope of the current project is to combine selected native *S. cerevisiae* with wild yeasts species in the development of novel multi-species yeast starter blends to serve as starters in induced 'wild' fermentations. Therefore, the biodiversity of key EU viticultural regions is being thoroughly surveyed to identify yeast strains of enological importance. With respect to the yeast populations from Peza and Nemea, two key Greek viticultural regions, a relatively high species as well as strain biodiversity was observed. Taking *S. cerevisiae* populations as an example, it was shown that the percentage of biodiversity ranged between 10 – 13%, with only 7 common genotypes (< 5%) between the two zones. Furthermore, present results clearly point to spatial structure of *S. cerevisiae* populations in separate regions, albeit the within-zone distribution seems rather random. Different genotypes were then evaluated as per their technological properties, e.g. SO_2 or ethanol resistance, and selected strains were applied in mixed fermentations. The best performing strain combinations will be examined in plant-scale fermentations. Project results are expected to allow the launching of 'wild ferment' technology in winemaking and the production of quality *terroir*-driven wines. By these means, the project is expected to assist the companies to enhance marketing abilities towards a more competitive and sustainable wine industry.
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EFFECT OF MIXED *SACCHAROMYCES* AND NON-*SACCHAROMYCES* STARTERS ON ALCOHOLIC FERMENTATIONS AND WINES

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Alcoholic fermentation is characterized by a succession of yeast species from grapes to final wines. In grapes, non-*Saccharomyces* yeasts are dominant, yet *Saccharomyces cerevisiae* soon takes over and dominates the fermentation process. Although non-*Saccharomyces* yeasts have often been considered as a source of microbial spoilage, there is substantial contrary evidence pointing to a positive contribution by these yeasts. The role of non-*Saccharomyces* yeasts in wine fermentation is therefore receiving increasing attention by wine microbiologists. In the present study, wine yeasts belonging to different species have been isolated from grapes and spontaneous fermentations in DOQ Priorat (Spain). After molecular yeast identification (RFLP-PCR of the ITS region and sequencing D1/D2 region of the 26S rRNA gene), isolates were typified at the strain level using different molecular techniques depending on the yeast species (analysis of inter-delta elements, PCR-based method of tandem repeat tRNA, RAPD-PCR, microsatelites, etc). *Hanseniaspora uvarum* was the dominating non-*Saccharomyces* species, followed by *Candida zemplinina*. Strains with good oenological characteristics have been then selected to be used as starters in mixed culture fermentations, performing sequential inoculation in pilot-scale fermentations. Natural grape musts were initially inoculated with either *Hanseniaspora uvarum*, *Candida zemplinina* or a mixture of them, and *S. cerevisiae* was added to the must after two days of fermentation. Fermentation kinetics development, yeast growth and evolution of the different species were followed through the process, and the main metabolites and aromatic compounds were determined at the end of the fermentation. Significant differences were observed in most of the parameters analyzed, confirming the higher complexity and diversity in flavor and aromatic compounds when yeast multi-starters are used. Thus, the development of yeast starter blends composed of indigenous, privileged strains, could ensure the production of elegant wines reflecting sense of place and compatible with *terroir*-driven approaches.

ADAPTIVE ROLE OF HORIZONTALLY TRANSFERRED OLIGOPEPTIDE TRANSPORTERS IN WINE YEASTS

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Wine yeasts have unique properties within the *Saccharomyces cerevisiae* species. However, the molecular mechanisms behind these features remain largely unknown. We previously showed that horizontal gene transfers (HGT) of three large regions (A, B and C) containing 34 genes from distant yeasts have shaped the genome of wine yeasts. These new genes potentially encode important metabolic functions in winemaking (sugar and nitrogen metabolism), suggesting a role in adaptation to the wine environment.
To assess the adaptive value of these HGT, we carried out a functional analysis of a mutant deleted for the region C (65kb). We found that this region confers a better cell viability and capacity to complete the fermentation in grape must. We showed that this phenotype is associated to a better assimilation of the oligopeptide fraction of grape must by the Fot1/2 oligopeptide transporters present in this region. We also showed that Fot-mediated peptides uptake affects the glutamate node and the NADPH/NADP+ balance, resulting in altered metabolite profile. By carry out competition assays, we demonstrated that the presence of *FOT* genes improves yeast fitness on grape must. Indeed, *FOT1/2* deletion mutants were completely outcompeted by the wild-type strain after three successive co-cultures. Finally, using population genomics data, we found a strong conservation of *FOT* genes in the genome of yeast strains possessing the region C, even when this region was incomplete. These findings demonstrate the adaptive value of *FOT* genes which improve nutrient resource utilization in a nitrogen-limited environment, and highlight the role of HGT in genome evolution and adaptation of yeasts to their ecological niche.

PHYSIOLOGICAL AND GENETIC CHARACTERISATION OF *Saccharomyces* STRAINS ISOLATED FROM ICE HARVEST OF CHARDONNAY GRAPES

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Specific ecological environment in the production of wine is ice harvest. Such harvest takes place in extreme temperature conditions while the grapes are still frozen and the microflora of those grapes has specific features like stress tolerance. Yeasts present during fermentation of ice wine have to be adapted to stressful conditions like high sugar content and low pH. In this study wine yeasts present during the Chardonnay ice wine must fermentation were identified and a physiological and genetic characterization of them was performed. In total, twenty-eight yeast strains were isolated during the fermentation and fourteen of them belonged to the *Saccharomyces* group. Identification at the species level was performed by restriction of the ITS region and six strains of *S. cerevisiae* as well as eight strains of *S. paradoxus* were identified. These strains were tested in more detail regarding physiological characteristics, which are important for fermentation of such specific substrate. Most of the isolates (79%) showed significant osmotolerance, which could result from adaptation or genetic inheritance. Flocculation capacity was tested as well but neither strain showed true flocculation in all conditions. A certain degree of flocculation was obtained for two strains, but only when grown in preculture containing calcium ions. SOY 28L showed interesting values and therefore investigation should be continued in the manner of detection and determination of regulation mechanisms leading to that pattern of behavior. The finding indicates that the indigenous isolates could really be competitors of other yeasts in ice wine fermentation and in the future used as a starter culture.

APPLICATION OF *Hanseniaspora vineae* STRAINS. SEARCHING FOR GENES TO EXPLAIN INCREASED FLAVOR COMPLEXITY IN WINES

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Use of novel yeast strains for winemaking is increasingly regarded as a way for improving quality and to provide variation including subtle characteristic differences in fine wines. Furthermore, spontaneous fermentations are associated with greater wine body, unusual or odd aromas and flavors, creamy texture and greater complexity.
More than 800 non-*Saccharomyces* strains were isolated from the Uruguayan winemaking environment over a 5 year period. We selected 11 strains of *Hanseniaspora vineae* to study sequential fermentations strategies with and without the presence of a conventional *Saccharomyces* wine strain.
Based on winemaking parameters, key chemical and sensory analysis we were able to define specific metabolic and sensory traits for each strain. In general, the co-fermentation strategy with *H.vineae* provided significant increases in glycerol and acetyl and ethyl ester flavour compounds and relative decreases in alcohols, fatty acids, and biogenic amines which correlates with wine differences found between these alternative fermentation procedures. In order to understand the genetic base of "flavour phenotypes" we also sequenced and assembled the genomes of two *H. vineae* strains. More than 4000 highly reliable coding sequences were predicted for *H. vineae* and the presence-absence of genes belonging to several metabolic pathways were analyzed. One hundred twenty eight *S. cerevisiae* genes associated with fermentation as those participating on Glycolysis/ Gluconeogenesis, Citrate cycle, Pentose pathway, Steroid biosynthesis, Fatty acid degradation and Fatty acid biosynthesis pathways were screened. Despite the great sequence divergence observed between *H. vineae* and industrial *S. cerevisiae* genomes, 87 of those genes (68%) were found in *H. vineae*. Interestingly, increased divergences in genes related to aroma compounds metabolism are discussed. For example, gene associated with the biosynthesis of acetates esters (ATF2) was highly divergent in *H. vineae* compared to that present in *S. cerevisiae* and genes involved in the regulation of higher alcohols (ARO8 and ARO9) had more than 3 copies compared to one present in *S. cerevisiae.*

EXPLORATION OF GENETIC RESOURCES FROM YEAST GENOME TO DEVELOP ROBUST YEAST STRAINS FOR BIOFUELS PRODUCTION

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Saccharomyces cerevisiae is widely used in brewing industry and for biofuels production and biorefinery. Industrial *S. cerevisiae* strains are exposed to various stressful conditions during bioethanol fermentation, such as toxic level of ethanol and inhibitors from cellulosic hydrolysates, high temperature and so on. Improvement of stress tolerance of yeast strains benefits economic production of fuel ethanol. In our previous work, the protective effect of zinc against ethanol toxicity and high temperature was revealed. When the effect of different concentrations of zinc ion on ethanol production of self-flocculating yeast SPSC01 in presence of high concentration of acetic acid was studied in batch fermentation, it showed that appropriate concentration of zinc ions significantly shortened the lag time of cell growth of the self-flocculating yeast. Transcriptome analysis revealed the remodeling of gene expression by zinc in presence of toxic level of acetic acid. Functional analysis of about 20 differentially expressed genes in the transcriptomic data showed that overexpression of these genes improved tolerance to toxic levels of acetic acid and ethanol. We proposed that exploration of differentially expressed genes under stressful conditions in different zinc status benefits development of robust yeast strains for biofuels production.

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THE WATER INSOLUBLE SOLIDS OF PRETREATED LIGNOCELLULOSIC MATERIALS INCREASES THE RESISTANCE OF *Saccharomyces cerevisiae* TO LIGNOCELLULOSIC INHIBITORS – A PROTEOMIC STUDY OF THE EFFECTS

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The pretreatment of lignocelluloses usually results in a material of semi-solid consistency containing liquid and solid fraction. The monomeric sugars and inhibitory compounds present in the liquid fraction have been extensively characterized and their effects on *Saccharomyces cerevisiae* based fermentation are well documented. However, there is no information on the influence of water-insoluble solid (WIS) fraction on the cellular performance. Here we show that when WIS was added to the minimal medium containing synthetic inhibitors, a reduced lag phase and enhanced volumetric ethanol productivity of *S. cerevisiae* CEN.PK 113-7D were observed compared to when no solid fraction was present in the medium. To investigate the underlying molecular reasons for the effects of WIS, acetic acid was used as an inhibitor to determine the response to WIS at the proteome level in yeast cells. Comparisons were made with cells grown in the presence of acetic acid but without WIS in the medium. Altogether, 729 proteins were detected and quantified, however when applying Student's t-test with 95% confidence we found 264 proteins were over-expressed and 280 proteins were under-expressed with a fold change≥1.2 in the presence of WIS compared to absence of WIS. The cells in the presence of WIS over-expressed several proteins related to glycolysis, electron transport chain, oxidative stress response, oxygen and radical detoxification and unfolded protein response; and under-expressed most proteins related to biosynthetic pathways including amino acid, purine, isoprenoid biosynthesis, aminoacyl-tRNA synthetases and pentose phosphate pathway. Differentially expressed proteins may indicate that the likelihood of increased ATP generation in the presence of WIS was used to defend against acetic acid stress at the expense of reduced biomass formation. Although, comparative proteomics of cells with and without WIS in the acetic acid containing medium revealed numerous changes, a direct effect of WIS on cellular physiology remains to be investigated.

TARGETING THE INTRACELLULAR REDOX STATE IN THE DEVELOPMENT OF MORE ROBUST *Saccharomyces cerevisiae* STRAINS FOR LIGNOCELLULOSIC BIOETHANOL PRODUCTION

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Bioethanol produced from lignocellulosic raw materials is a promising alternative to fossil fuels and to decrease greenhouse gas emissions, but several challenges still exist. When lignocellulosic biomass is pretreated, a number of undesired degradation products are generated, among which the furaldehydes furfural and hydroxymethylfurfural (HMF) have shown to impede growth and limit ethanol productivity of the yeast *Saccharomyces cerevisiae.* In the present study, a recombinant, xylose-utilizing *S. cerevisiae* strain was challenged with sub-lethal concentrations of furfural and HMF in anaerobic batch cultivations. By pulsing furaldehydes in either the glucose or the xylose consumption phase, perturbations in the intracellular NAD(P)H/NAD(P)$^+$ ratios could be demonstrated. A genome-wide study of transcription found that genes related to NADPH-requiring processes, such as nitrogen and sulphur assimilation, were significantly induced. Moreover, the protective metabolite and antioxidant glutathione was identified as the highest scoring reporter metabolite in the transcriptome analysis. *S. cerevisiae* strains overproducing glutathione were constructed and the resulting strains were evaluated in simultaneous saccharification and fermentation (SSF) of pretreated spruce. The results from the present study provide valuable insights of how *S. cerevisiae* responds to stress imposed by HMF and furfural and how such information could be used to engineer more robust yeast strains.

ROLE OF ANTIOXIDANT DEFENSE SYSTEMS ON AGING IN WINE YEAST

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Under aerobic and anaerobic conditions, biomass production and grape juice fermentation respectively, *S. cerevisiae* is subjected to many types of stress. Oxygen is responsible for the production of toxic reactive oxygen species (ROS) and the cells have defense mechanisms against oxidative stress. In *S. cerevisiae* there is a large group of ROS detoxifying enzymes where Tsa1 is a cytosolic peroxiredoxin (PRX) that reduce peroxides with the help of thioredoxins (TRX), usually acting as an electron donor. *S. cerevisiae* has two cytosolic thioredoxins, Trx1 and Trx2, that obtain the redox power of the thiorredoxin reductase Trr1 action and finally from NADPH. Furthermore, thioredoxin are involved in a reversible posttranslational modification mechanism, whereby a glutathione (GSH) is added to a cysteine residue of a protein to prevent its irreversible oxidation. Thioredoxins have a role of degluthationylation in stationary phase and this role may be related to the control of longevity. Tsa1 activity is regulated by sulfiredoxin Srx1. In our laboratory has shown the importance of *TRX2* gene in the biomass production. Due to this importance in the regulation of redox homeostasis and longevity, we studied the role of these proteins on chronological life span (measured as viability in stationary phase) and in protein glutathionylation in different environmental conditions. Double deletion of thioredoxins *TRX1* and *TRX*2 cause an increase in sensitivity to oxidants and shortening on CLS in aerobic conditions as expected, but it increases longevity in winemaking conditions. Peroxiredoxin Tsa1 is analyzed in these different environmental conditions too. Double deletion with nutrient regulator *GCN2* leads to a very short chronological longevity in all growth media. *SRX1* mutation extends longevity during winemaking and its combination with *GCN2* deletion extends it even further. The role of mitochondria in stress tolerance and longevity is also addressed. Mitochondrion is a key factor in the tolerance to drying.

TRANSCRIPTOME ANALYSIS OF WINE YEAST STRAINS UNDER SULPHITE-INDUCED STRESS CONDITIONS

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Sulphite is widely used in winemaking for its antimicrobial and antioxidant properties, although its toxic effect on human health is proven. For this reason strategies for reducing chemical preservatives in winemaking is strongly demanded. Wine yeasts can cope with SO_2 by different systems, such as acetaldehyde production, sulphite uptake and reduction or SO_2 export.
The aim of this work was to study the genes involved in sulphites response in *S. cerevisiae* and see how different strains respond to SO_2 addition in the early stage of fermentation.
In this study 10 strains has been chosen among those whose genome has been sequenced: 6 commercial yeasts, EC1118, AWRI796, AWRI1631, VIN13, QA23, VL3, and 4 isolated directly from vineyard, R008, R103, P301, P283. The strain S288C was added to the analysis, as reference. Fermentation trials in synthetic must were conducted at laboratory scale to assess the main technological and quality traits and to investigate strain behaviours towards sulphite. The 4 strains showing the strongest differences in terms of response to SO_2 were selected. To clarify the genetic basis of this complex enological trait we performed transcription profiling using SOLID technology. The analysis was conducted during fermentation process mimicking winemaking condition, in synthetic must supplemented with 25 mg/l of SO_2 in 1l-capacity bioreactors. For all strains, fermentation rate was determined overall the process, together with sulphite and acetaldehyde production. RNA seq was performed at early exponential phase (6 g/l of CO_2 produced) to investigate yeast adaptation under sulphite conditions. Gene expression analysis suggested that both specific gene activities and more general genetic pathways are involved in sulphite response.

MULTIPLE GLOBAL APPROACHES FOR DEPHICERING THE MOLECULAR BASIS OF LOW TEMPERATURE ADAPTATION IN WINE YEAST

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Low temperature alcoholic fermentations (10-15ºC) are becoming more frequent due to the winemaker's tendency to produce wines with more pronounced aromatic profile. However fermentation at low temperature presents some disadvantages: reduced growth rate, long lag phase, sluggish or stuck fermentations. The aim of this study is to phenotype a collection of 27 *S. cerevisiae* commercial wine strains growing within temperatures (4-45ºC) in both minimal media (SD) and synthetic must (SM) and, taking into account μ_{max} value, we selected two strains with divergent phenotype in their capacity to grow at low temperature. To confirm this differential phenotype, we design a competition between both strains during wine fermentations. As expected, at low temperature fermentation, the strain showing a good performance out-compete to the strain growing badly in cold, whereas the percentages of both strains were kept around 50% throughout the fermentation process at 28ºC.
Finally we aimed to decipher the molecular basis underlying this divergent phenotype by analyzing the genomic, proteomic and transcriptional differences between both strains at low temperature. The up-regulation of genes in the good strain implicated in biosynthesis of sulfur-containing amino acids and S-Adenosilmethionine (SAM) biosynthesis suggest the implication of this pathway in cold adaptation, likely for the requirement of SAM in the biosynthesis of phospholipids (Hickman *et al*., 2011). We also found genes implicated in glutathione redox reaction, together with the presence of thioredoxins proteins, could be indicating a bigger oxidative stress at low temperature. Moreover, we found glycolysis enzymes of the lower part of the pathway, the part that leads to ATP generation and enhances the glycolytic flux and the fermentative capacity of the strain.

THE ROLE OF YEAST TRANSCRIPTIONAL FACTOR Msn2 IN *Saccharomyces cerevisiae*

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During fermentation processes, yeast *Saccharomyces cerevisiae* cells are exposed to multiple environmental stresses, which often impair the cellular functions and limit their fermentation properties. To protect the cells from these harsh conditions, the transcriptional factor Msn2 plays a pivotal role in response to a variety of stresses in *S. cerevisiae*. When cells are exposed to such stresses, Msn2 is imported from the cytoplasm into the nucleus and binds to the stress response elements, normally found in the promoter regions, and subsequently activates the transcription of many stress-response genes. To improve the fermentation properties of industrial yeasts, we constructed the *MSN2*-overexpressing strain used for bioethanol production. The engineered strains exhibited reduced intracellular reactive oxygen species levels and showed rapid growth compared to the control strain in the presence of furfural, one of the major fermentation inhibitors generated by pretreatment of lignocellulosic biomass. Furthermore, a baker's yeast strain overexpressing *MSN2* accumulated trehalose in the cells and improved the gassing power in frozen dough compared with wild-type strain. These results suggest that overexpression of Msn2 is an effective strategy to enhance the stress tolerance of *S. cerevisiae* cells through affecting antioxidative mechanism and carbon metabolism. Intriguingly, we found that *MSN2*-overexpressing strains showed higher sensitivity towards several toxic amino acid analogues, such as azetidine-2-carboxylic acid, canavanine, and *o*-fluoro-DL-phenylalanine, than wild-type cells. Based on this result, we hypothesize that the overexpression of *MSN2* is involved in the regulation of amino acid transporters. Our preliminary data indicated that Gap1 and Gnp1 transporters were located on the plasma membrane of *MSN2*-overexpressing cells, whereas they were internalized in wild-type cells. Altogether, this study will aid in revealing a novel regulatory role of Msn2 in the uptake of amino acids, in addition to its function in stress response.

INVESTIGATION OF HOG SIGNALING PATHWAY ARCHITECTURE OF THE HALOPHILIC BASIDIOMYCETE *Wallemia ichthyophaga* WITH THE FUNCTIONAL COMPLEMENTATION OF *Saccharomyces cerevisiae* MUTANTS

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Exotic and uncommon model organisms are attractive research objects. Besides being a good source of potentially interesting new discoveries, they are hard to work with, because the complexity of their manipulation largely exceeds that of the well established models like *Saccharomyces cerevisiae*. High evolutionary conservation of signaling pathways allows yeast to be used as a system for investigation of heterologously expressed orthologous signaling proteins. We used *S. cerevisiae* knockout mutants to study the architecture of *W. ichthyophaga* HOG signaling pathway, with the focus on MAPKK WiPbs2 and its interacting proteins. We expressed in the yeast *hog1Δpbs2Δ* cells two paralogous MAPKs, WiHog1A or WiHog1B, along with the WiPbs2 kinase. When grown on salt, only the cells expressing WiHog1B displayed salt tolerance similar to that of the wt yeast cells, suggesting that most probably WiHog1B, but not WiHog1A, is the primary HOG pathway kinase also in *W. ichthyophaga*. The *pbs2Δste11Δ* cells, where MAPKKK Ssk2/22 is the only activator of WiPbs2, tolerated salt like the wt yeast, but in the *pbs2Δssk2/22Δsho1Δ* cells the salt-tolerance of the wt yeast cells could not be reached with the expression of WiPbs2 and WiSho1. In this strain, we only recovered the salt-tolerance by inserting the *S. cerevisiae* SH3-binding proline-rich motif to the N-terminal part of WiPbs2 kinase. Additionally, the polymyxin B sensitivity of the WiPbs2 expressing cells suggests, that the Ssk2/22 kinase of the SLN1 branch in the HOG pathway is the primary WiPbs2 activating MAPKKK also in *W. ichthyophaga*.

THE PROTEIN *O*-MANNOSYLTRANSFERASE PMT4 IS CRUCIAL FOR CELL WALL INTEGRITY AND STRESS RESISTANCE OF *Hansenula polymorpha*

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Protein *O*-mannosyltransferase (PMT) initiates the addition of mannose residue to serine or threonine residue of secretory proteins, which is an important post-translational modifications evolutionarily conserved from bacteria to human. PMTs are significant differences in their organization and properties, reflecting the diversity of *O*-mannosylation machineries. In this study we carried out functional and molecular characterization of the *PMT4* gene in the thermotolerant methylotrophic yeast *Hansenula polymorpha*. Deletion of *HpPMT4* did not affect thermotolerance but resulted in increased sensitivity to several cell wall destabilizers, and various salt stresses. Notably, the analysis of cell wall mannoproteins with lectin blotting indicated significant decrease in *O*-mannosylation in the *Hppmt4* mutant. Furthermore, just single deletion of *HpPMT4* generated severe glycosylation defects of the surface sensor proteins HpWsc1p and HpMid2p without alteration of the secreted glycoproteins, chitinase and HpYps1p lacking GPI anchor. Interestingly, the basal level of the phosphorylated HpMpk1p, a key kinase in the cell wall integrity pathway, was markedly reduced in the *Hppmt4* strain, although the phosphorylation of HpMpk1p was also increased to a high extent in the *Hppmt4* mutant upon treatment of cell wall disturbing agents. Altogether, our results indicate that Pmt4p plays important roles particularly in *O*-mannosylation of surface glycoproteins involved in signal pathways for cell wall integrity and stress resistance in *H. polymorpha*.

In vitro ANALYSES OF PHR1 AND PHR2 TRANSGLYCOSYLASES OF THE *Candida albicans* CELL WALL

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The yeast cell wall is an essential component that defines cell shape, preserves the cell´s osmotic integrity, is involved in flocculation, sporulation, mediates cell-cell interactions and plays a crucial role in host – pathogen interaction and in virulence. The individual structural components of the polysaccharide polymers of the *Candida albicans* cell wall, β-(1,3)-glucan, β-(1,6)-glucan and chitin, are mutually cross-linked by covalent bonds into large macromolecular complexes based on β-(1,3)-glucan backbone. We are focused on transglycosylases, specifically to pH-regulated enzymes Phr1 and Phr2 from *Candida albicans*. Phr1 and Phr2 proteins belong to β-(1,3)-glucanosyltransferases (family GH72) which catalyze the linking of part of β-(1,3)-glucan to another molecule of β-(1,3)-glucan.
In this part of our research, the biochemical characterization and determination of substrate specificity of Phr1 and Phr2 transglycosylases heterologously expressed in *Pichia pastoris* were found out. A fluorescence *in vitro* assay was used for biochemical characterization of Phr1p and Phr2p. The pH optimum was 5,6 for Phr1 and 3 for Phr2. The optimum temperature was 30°C for both. The transglycosylation activities of Phr1p and Phr2p were determinated by a fluorescent *in vitro* assay and size-exclusion chromatography (SEC). Laminarin was used as the donor and the oligosaccharides labeled by sulforhodamine (SR) as the artificial acceptors. The wide spectrum of (SR)-oligosaccharides (OS-SR) was tested as acceptors: laminari-OS-SR; N-acetyl-chito-OS-SR, cello-OS-SR and SR-oligosaccharides derived from xyloglucan, mixed-linkage β-(1,3/1,4)-glucan; β-(1,6)-glucan (pustulan), β-(1,4)-linked mannan and α-(1,4)-glucan (starch). Results based on relative rates of transglycosylation and elution profile of the reaction mixtures measured by SEC have shown that Phr1 and Phr2 are specific homotransglycosylases catalyzing transfer from β-(1,3)-linked laminarin to β-(1,3)-linked laminarioligosaccharides. The rate of transglycosylation mediated by Phr2 was higher than by Phr1. The products of the transglycosylation reactions were identified by MALDI-TOF mass spectrometry as the molecules composed of the acceptor and portions of the donor molecule attached to its non-reducing end.

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METHYLOTROPHIC YEAST *Hansenula polymorpha* AS A NOVEL TOOL FOR STUDYING MECHANISMS UNDERLYING PARKINSON'S DISEASE

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Several features of Parkinson's disease (PD) have been recreated in yeasts (e.g. formation of protein aggregates, cellular toxicity mediated by misfolded proteins), enabling researchers to use them as a powerful artificial model for investigation of molecular mechanisms of PD. We suggest expanding a small number of model yeast species using a methylotrophic yeast *Hansenula polymorpha.* This yeast as a new model of PD has several advantages, including its extreme thermotolerance with a consequent ability to grow at temperatures up to 45 °C. This allows to study PD-related mechanisms at physiological for both human organism and this yeast temperature, 37 °C, and also the general influence of the temperature on the amyloid formation. We have created novel yeast models of PD with regulated expression of α-synuclein gene (*SNCA*) fused with the gene of green fluorescent protein. These models are based on *H. polymorpha* wild type strain NCYC495 and mutant strain *gcr1-2* defective in glucose transport and metabolism. Epidemiological studies have identified diabetes mellitus as an independent risk factor for multiple diseases of the nervous system including PD. Combination of defective glucose metabolism and α-synuclein overproduction in single strain will help us to understand relationship between Parkinson's disease and abnormal sugar metabolism. Heterologous α-syn production was detrimental for yeast cells and decreased their growth rate on alternative carbon sources. We observed that NCYC495-*SNCA* strain did not form visible α-syn aggregates but exhibited plasma membrane perforations and cytoplasm leakage which are most probably caused by α-syn oligomers. *gcr1-2-SNCA* mutant strain exhibited enhanced aggregation of fluorescently tagged α-syn. However, such accelerated amyloid formation is not a direct consequence of the impaired glucose metabolism as were also observed in cells grown on glycerol. The question of what molecular mechanisms evoked by *gcr1* mutation result in α-syn aggregation will be addressed in our prospective studies.

NON-CONVENTIONAL YEASTS FOR BIOFUEL PRODUCTION

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Production of biofuels is a complex technology of handling and converting biomass. Yeasts represent a highly diverse group of organisms with an amazing metabolic potential. We have tested several non-*Saccharomyces* yeasts in biofuel production and as biopreservation and pre-treatment agents for both first generation raw materials and lignocellulose. The yeast, *Dekkera bruxellensis*, had outcompeted *Saccharomyces cerevisiae* in an industrial ethanol process, by this challenging the dogma that *S. cerevisiae* is always the most appropriate species for ethanol production. *Scheffersomyces* (*Pichia*) *stipitis* is the best known xylose-fermenting organism. Recently we discovered that the yeast, when used as biopreservative in airtight storage of moist wheat straw, inhibits moulds and at the same time improves the accessibility of polysaccharides to pre-treatment. Beyond ethanol production, the production of lipids as components of biodiesel or- as omega 3 fatty acids as feed- and food additives is a novel approach of using yeasts. Certain oleaginous species can accumulate more than 60% of their biomass as lipids, and some form substantial amounts of essential fatty acids (linoleic and linolenic acid). Apart from this, many of them can naturally assimilate sugars released from hemicellulose, which cannot be converted to ethanol by *S. cerevisiae* or *D. bruxellensis*. Utilising the metabolic diversity of non-conventional yeasts and integrating different steps of biofuel- and chemical production can push the development towards a biorefinery that is sustainable and competitive against the traditional, fossil based production systems.

CONSTRUCTION OF THE IMPROVED ETHANOL PRODUCERS FROM XYLOSE IN THE THERMOTOLERANT YEAST *Hansenula polymorpha*

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Hansenula polymorpha belongs to the best studied non-conventional yeasts and is apparently the most thermotolerant yeast known with maximal growth temperature of 50 ºC. It was also shown that this yeast is capable of xylose alcoholic fermentation though ethanol yield and productivity are low. Using methods of classical selection and metabolic engineering, the strains of *H. polymorpha* have been constructed which accumulate elevated amounts of ethanol from xylose, up to 10-12 g/L. For this, overexpression of the modified gene *XYL1m* (encoding xylose reductase) and the native genes *XYL2* (xylitol dehydrogenase), *XYL3* (xylulokinase) and *PDC1* (pyruvate decarboxylase) and, additionally, the isolation of 3-bromopyruvate-resistant mutants from metabolically engineered strains have been conducted. Additionally, the gene *CAT8* involved in regulation of gluconeogenesis has been deleted and role of this defect on xylose fermentation was studied. It was unexpectedly found by us that the *pex* mutants of the yeast *Hansenula polymorpha* which do not contain peroxisomes poorly grow on and do not ferment xylose. The mutants of this yeast deleted in genes *DAS1* and *TAL2* coding for peroxisomal dihydroxyacetone synthase and transketolase, respectively, show normal growth in xylose medium though fermentation of this sugar is severely damaged. Inversely, overexpression of *DAS1* and *TAL2* resulted in activation of xylose alcoholic fermentation whereas overexpression of cytosolic genes of pentose phosphate pathway *TKT1, TAL1, RPI1* and *RPE1* had only slight effect on this process. Derepression of *DAS1* and *TAL2* in the best ethanol producing strain further increased ethanol yield and productivity. Defect in peroxisomes autophagic degradation (pexophagy) due to insertion or deletion of the gene *ATG13* activated xylose alcoholic fermentation. All mentioned genetic changes have no effect on glucose alcoholic fermentation. Further improvement of high-temperature xylose alcoholic fermentation in *H. polymorpha* is discussed.

THE BIOCONTROL YEAST *Wickerhamomyces anomalus* IN MAIZE BIOPRESERVATION

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Preservation of moist crimped grain (barley, wheat) is made possible through fermentation by lactic acid bacteria (LAB) in an airtight environment, in a process similar to silage production. This yields a hygienic product with reduced levels of potentially pathogenic bacteria and spoilage moulds. Additional of the biocontrol yeast *Wickerhamomyces anomalus* (syn. *Hansenula anomala*, formerly *Pichia anomala*) further inhibits mould growth by production of ethyl acetate; this minimizes the risk for mycotoxin production. The biocontrol strain has also been shown to reduce *Enterobacteriaceae* by an unknown mechanism (1). Biocontrol with *W. anomalus* was tested for the storage of moist harvested maize in Cameroon, where key microbiota of maize kernels had been characterized earlier (2). The white maize cultivar 'Kasai' was harvested and stored in airtight plastic barrels, with and without inoculation of *W. anomalus.* Microbes were enumerated at harvest and after 2, 5 and 8 months of storage; nutritional contents were compared before and after storage. High levels of LAB naturally present on maize (10^8 cfu/g) were maintained throughout storage. Activity of LAB in both control and inoculated maize likely contributed to the decline in *Enterobacteriaceae* in both treatments to < 10 cfu/g after 2 months storage. The biocontrol yeast, *W. anomalus*, was not found in the inoculated treatment after 2 months, but a positive effect was seen in significantly reduced mould counts to < 100 cfu/g compared with the uninoculated maize. *W. anomalus* has previously been shown to improve protein and amino acid contents during storage of moist crimped grain. This was not observed for white maize, probably, due to the poor survival of the biocontrol yeast. Preliminary results suggested that *W. anomalus* survives better on the yellow ATP (acid-tolerant population) maize cultivar from Cameroon than on the white 'Kasai' cultivar; survival of the biocontrol yeast may be affected by cultivar.

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Dekkera bruxellensis COMPETITIVE NON-CONVENTIONAL ETHANOL PRODUCTION YEAST

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The outcompetition of an initial *Saccharomyces cerevisiae* inoculum by *Dekkera bruxellensis* has been reported in several ethanol production plants, while process efficiency has remained high. In most cases the co-occurrence of *D. bruxellensis* together with *Lactobacillus vini* has been observed. The outcompetition phenomenon was observed exclusively under fermentation conditions with glucose-limited continuous cultivation. Research was done to understand different aspects of *D. bruxellensis* competitiveness, recently reconsidered as essential trait for a production strain. Possible reasons for the competitive advantage of *D. bruxellensis* such as glucose uptake and metabolism, nitrate assimilation and interaction with *L. vini* were investigated. Global gene expression analysis allowed identifying genes responsible for efficient carbon metabolism of *D. bruxellensis*. Nitrate has been shown to be an important component of fermentation broth in Brazilian ethanol production plants. Ability to assimilate nitrate allows *D. bruxellensis* to utilize it as sole nitrogen source when ammonium is depleted. Regulation of genes involved in nitrate assimilation was investigated. Pseudomycelium structure and cell wall composition of *D. bruxellensis* were shown to be involved in the interaction with *L. vini*.

DELETION OF *ADH1* GENE, ENCODING ALCOHOL DEHYDROGENASE FOR THE IMPROVEMENT OF GLYCEROL PRODUCTION IN *Saccharomyces cerevisiae*

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Glycerol is widely used in cosmetics, food, tobacco, pharmaceutical, leather and textile industries. Glycerol is one the dominant by-product of glucose conversion during alcoholic fermentation by *S. cerevisiae*. In addition, it is considered as a cheap raw material for microbial fermentation. That is why the construction of yeast strains-producers of glycerol became an actual objective for modern metabolic engineering.

Our strategy comprises the deletion of *ADH1* gene, encoding alcohol dehydrogenase in *S. cerevisiae* in order to redirect the glycolytic pathway to a glycerol synthesis. Glycerol is synthesized in a two reaction process, consisting of the reduction of dihydroxyacetone phosphate by a NADH-dependent glycerol-3-phosphate dehydrogenase (GPD), followed by the dephosphorylation of glycerol-3-phosphate by a glycerol-3-phosphate phosphatase (GPP).

Deletion of *ADH1* gene will eliminate the alcohol dehydrogenase reaction during glycolysis and lead to the accumulation of NADH, which will further be used in glycerol-3-phosphate dehydrogenase reaction.

The deletion cassette pUC57_ADHpr_NTC_ADHterm, including terminal and promoter part of *ADH1* gene and *natNT2* gene was constructed. Plasmid pUC57_ADH1term_NTC was introduced into *S. cerevisiae* strain BY4742. As a result, several recombinants were obtained. The correct disruption of *ADH1* gene was confirmed by analytical PCR.

The specific activity of alcohol dehydrogenase in recombinant strains was decreased in comparison with wild type strain. The ethanol productivity was also significantly decreased in recombinant strains compared with wild type strain BY4742. A glycerol production was considerably enhanced in transformants if to compare with the wild type strain. Recombinant strains consumed less amounts of glucose than the wild type strain. An ethanol production was essentially reduced in transformants in comparison with the wild type strain.

METABOLIC ENGINEERING OF *Hansenula polymorpha* FOR IMPROVING THE CONVERSION OF GLYCEROL TO ETHANOL

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Decreasing fossil fuels, drastic increase in prices, global warming and environmental pollution caused that development of alternative renewable fuels, such as biodiesel, has become attractive. The increase in biodiesel production generates an excess of crude glycerol since this compound is a significant waste by-product accumulating directly from the trans-esterification of the plant oil. Glucose fermentation is efficiently carried out by microorganisms, at the same time; there are few microorganisms that are capable of converting glycerol to ethanol. Therefore, the tools of metabolic engineering along with classical selection should be used for construction of the recombinant strains with improved ethanol production.

Thermotolerant methylotrophic yeast *Hansenula polymorpha* is able to convert glycerol as well as glucose and xylose, to ethanol. However, the yield of ethanol from glycerol by wild-type strains is too low and needs to be substantially improved before introduction of those strains for production of ethanol on the industrial scale.

The aim of this work was to improve production of ethanol from glycerol using thermotolerant methylotrophic yeast *Hansenula polymorpha.* For this, vectors for multicopy integration of the overexpressed *PDC1* and *ADH1* genes under control of strong promoters have been constructed.

According to previous data, overexpression of a pyruvate decarboxylase gene (*PDC1*) improved the production of ethanol from glycerol. Compared to the wild type strain, overexpression of *PDC1* led to threefold increase of ethanol production. In addition, recent data show that overexpression of *ADH1* gene leads to production of higher amounts of ethanol. Therefore we suggest that overexpression of both *PDC1* and *ADH1* genes will further increase the yield of ethanol from by-product glycerol. The newest data of our experiments will be provided and discussed.

ETHANOL AND BIOGAS FROM STEAM EXPLODED ISP (INTEGRATED STORAGE AND PRE-TREATMENT) SAWDUST INOCULATED WITH *Arxula adeninivorans*

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To decrease the fossil fuel dependency and greenhouse gas emissions biofuels should be produced from second generation (lignocellulosic) biomass, such as forest residues. The lignocellulosic biomass needs to be pretreated, usually thermochemically, before enzymatic hydrolysis and fermentation for the production of biofuel. We have developed a method called integrated storage and pretreatment (ISP) where a biopreservation yeast is inoculated to the biomass prior storage. Ideally the yeast will both act as a biocontrol agent, inhibiting spoilage microorganisms, and start limited biomass degradation. As a result, a less harsh pretreatment is necessary leading to lower levels of inhibitors and lower energy requirements for thermochemical pretreatment (1). This has successfully been demonstrated on wheat straw but not on spruce sawdust, since the yeasts did not survive the storage (2). In this project we inoculated *Arxula adeninivorans* to birch sawdust and stored it for 6 weeks at 37°C. *A. adeninivorans* is a heat tolerant yeast that also has the capacity to degrade aromatic compounds. The storage flora of the sawdust was determined and the sawdust was pretreated using steam explosion. Then SSF (simultaneous saccharification and fermentation) with *Saccharomyces cerevisiae* as the fermentation yeast was performed followed by biogas digestion of the residue. Biogas digestion of steam exploded sawdust without prior ethanol fermentation was also performed. Preliminary results showed that *A. adeninivorans* did not have any biocontrol activity. The results from the microbial analyses and SSF and biogas digestion will be presented.

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BIO-LIPID PRODUCTION FROM LIGNOCELLULOSES MATERAL WITH OLEAGINOUS YEASTS

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Considering the limitation of fossil raw materials utilization of renewable resources for fuel and chemicals production is required. Biofuel production should be sustainable and not compete with food and feed production. Lignocellulose is the most abundant biomass resource on earth and its hydrolysates are commonly esteemed as promising substrates for biofuel production.
Lipomyces starkeyi and *Rhodotorula glutinis* are oleaginous yeasts, able to convert carbon sources present in lignocellulose to lipids, up to more than fifty percent of their biomass. We have tested lipid accumulation by these two yeasts when growing on birch hydrolysate. Using a fed batch strategy we obtained lipid contents of the biomass of more than 40%. The proportion of non-saturated fatty acids varied considerable between both species, with higher amounts of polyunsaturated fatty acids in *R. glutinis*, making this yeast apart from biodiesel production also to a good candidate for generating essential fatty acids for feed and food. However, commonly used strategies of acid lipid extraction may generate artifacts regarding the relation of saturated and non-saturated fatty acids. The impact of different lipid extraction methods on determined fatty acid composition and total lipid content are discussed.

CONSTRUCTION OF *CAT8* GENE DELETION STRAINS IN THE YEAST *Hansenula polymorpha*

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Efficient alcoholic fermentation of hexose and pentose sugars by yeast is essential to achieve maximal ethanol yield from lignocelluloses hydrolyzates. The yeast *Hansenula polymorpha* is capable of xylose alcoholic fermentation at elevated temperatures and therefore can be used in Simultaneous Saccharification and Fermentation Process (SSF).

The aim of this work was to isolate mutants of *H. polymorpha* with knock out of the gene *CAT8* which is known to be involved in regulation of gluconeogenesis in *S. cerevisiae* and regulation of alcoholic fermentation in the non-conventional yeast *Pichia guilliermondii.* We did hope that the mutants *H. polymorpha* defected in *CAT8* will be more efficient in ethanol production from glucose and especially from xylose. Such deletion strains were planned to be constructed in both *H. polymorpha* wild-type strain and in the previously isolated by us mutants with elevated ethanol production from xylose due to overexpression the genes encoded the key enzymes involved in xylose metabolism (xylose reductase, xylitol dehydrogenase and xylulokinase) and subsequently selected on resistance to 3-bromopyruvic acid (glycolysis inhibitor).

It is known that *CAT8* encodes carbon source-responsive transcriptional regulators that cooperatively control expression of genes involved in ethanol utilization. Near 200 genes involved in central metabolism pathways as well as several transcriptional factors are activated by *CAT8.* Deletion of *CAT8* gene affected all key gluconeogenic enzymes by defect in glucose derepression, glucose metabolism is systematically shifted in the direction of the fermentation at the transcriptional level.

Currently, the cassettes for *CAT8* gene deletion in wild-type strain and ethanol overproducing mutants have been constructed and the corresponding mutant strains have been isolated. The studying the glucose and xylose alcoholic fermentation in the constructed knock out strains is under way.

INSERTIONAL TAGGING OF THE *Pichia stipitis* GENES INVOLVED IN XYLOSE ALCOHOLIC FERMENTATION

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Among several exhaustively studied microorganism, the yeast *Pichia stipitis* seems to be particularly promising in terms of xylose alcoholic fermentation, the pentose which is the major constituent of lignocellulosic biomass hydrolyzates. Nevertheless, the ethanol yield and productivity are still insufficient to meet the requirements of a feasible industrial technology; hence the construction of the more effective *P. stipitis* ethanol producers is of great significance. Our aim was to isolate the insertional mutants with altered ethanol production from xylose and determine gene(s) affecting their fermentative capabilities. Mutants were obtained by random insertional mutagenesis, then screened for the altered growth abilities on solid media with different sugars and for their resistance to 3-bromopyruvate (3-BP). Shaking cultures in semianaerobic conditions were carried out in order to measure sugar consumption and ethanol formation rates. Subsequently, the most interesting strains were analyzed to determine the genetic background of the observed alterations.
Of more than 13,300 screened mutants, only 17 were characterized with significantly changed ethanol yields during the fermentation. More than 70% of the strains resistant to 3-BP exhibited improved fermentative capabilities. In the case of the two best fermenting strains, insertion and the corresponding structure disruption, which presumably led to the enhancement of ethanol formation, occurred within the ORFs of two genes: coding for calcium-binding protein and functionally unknown, not yet described mitochondrial transporter. Additional experiments aimed to prove the role of the mentioned genes in regulation of xylose alcoholic fermentation are in progress.

CONSTRUCTION OF GLUTATHIONE OVERPRODUCERS IN METHYLOTROPHIC YEAST *Hansenula polymorpha* BY ENGINEERING OF γ-GLUTAMYLCYSTEINE SYNTHETASE

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Tripeptide glutathione (gamma-glutamyl-L-cysteinyl-glycine, GSH) is the most abundant non-protein thiol that protects cells from metabolic and oxidative stresses and is widely used as food additives, in medicine and cosmetic industry. Although GSH can be produced by chemical or enzymatic synthesis, microbiological production using natural or engineered microorganisms is currently the most common method for the commercial production of GSH. Synthesis of GSH includes two consecutive reactions catalyzed by gamma-glutamylcysteine synthetase (GCS) and glutathione synthetase, respectively. Microbial GSH overproduction is limited by mechanisms of feedback inhibition of GCS, the first and rate-limiting enzyme of GSH biosynthesis, by the end product. In addition the expression of gene coding for GCS is repressed by GSH.Thermotolerant methylotrophic yeast *H. polymorpha* with naturally high content of GSH and resistance to different kinds of stress is considered as promising organism for genetic modification and design of competitive GSH producer.The modified versions of *GSH2* gene, coding for GCS in *H. polymorpha*, obtained by error prone PCR were cloned under the control of strong constitutive promoter of glyceraldehyde-3-phosphate dehydrogenase in replicative plasmid pYT3. The selection of Gsh2 mutant forms was done on the basis of deletion strain *Δgsh2 H. polymorpha*, dependent on exogenous GSH. Transformants with modified versions of *GSH2* gene were obtained on medium without GSH. Selected transformants were analyzed for their resistance to different prooxidant agents (1,2,3-triazole, diethylmaleate, ethionine) as compared to strains carrying unmodified *GSH2* gene. Selected strains possessed growth on medium supplemented with triazole. This compound is able to chemical interaction with GSH reducing intracellular concentration of the tripeptide. Strains providing more intensive growth on the selective medium revealed higher GSH accumulation as compared to strains carrying unmodified *GSH2* gene, indicating the reduction of Gsh2 feedback inhibition. Identification of the mutations in frame of modified alleles of *GSH2* gene is under the progress.

METABOLIC ENGINEERING OF YEAST *Saccharomyces cerevisiae* FOR REROUTING OF CARBON FLUX TOWARDS GLYCEROL PRODUCTION

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Glycerol is used in cosmetic, paint, automotive, food, tobacco, pharmaceutical industries. Currently there is an interest in development of microbial or yeast strains effectively converting cheap feedstocks to glycerol. Facultative anaerobic yeast *Saccharomyces cerevisiae* can be a good platform for development of recombinant strains overproducing glycerol under low-aeration conditions. In *S. cerevisiae* glycerol synthesis from dihydroxyacetone phosphate is catalyzed by glycerol-3-phosphate dehydrogenase (Gpd1) and glycerol-3-phosphate phosphatase (Gpp2). But, in physiological conditions the major part of intracellular dihydroxyacetone phosphate is converted to glyceraldehyde-3-phosphate by triose phosphate isomerase (Tpi1), resulting in ethanol formation. We aimed to construct recombinant strains with reduced Tpi1 activity and increased Gpd1 and Gpp2 activities to redirect Carbon flux towards glycerol production. In order to decrease *TPI1* gene expression recombinant *S. cerevisiae* strains with shortened versions of *TPI1* gene promoter were constructed. Obtained strains contain 100, 50 or 25 base pairs of native *TPI1* gene promoter before *TPI1* ORF, and revealed corresponding sequential decreases in Tpi1 activity. Glycerol production by strains with 100 bp version of *TPI1* promoter wasn't substantially higher than that by WT strain, whereas strains with 50 or 25 bp version of *TPI1* promoter revealed up to 2 times increase in glycerol production in comparison with WT strain. In order to enhance the activities of enzymes involved in glycerol synthesis, we transformed *S. cerevisiae* with vectors containing genes *GPD1*, or *GPP2*, or hybrid *GPD1-GPP2* ORF (encoding artificial fusion of both enzymes) under the control of strong constitutive promoter of the alcohol dehydrogenase gene (*ADH1*). Recombinant strains overexpressing *GPP2* gene possessed similar glycerol titer to WT strain. Recombinant strains overexpressing *GPD1* gene or *GPD1-GPP2* fusion showed diverse increase in glycerol production, probably depending on gene copies number. Glycerol production reached 4 folds increase in the best of studied strain as compared to WT.

THE ROLE OF TRANSCRIPTION FACTOR Sef1 IN THE REGULATION OF FLAVINOGENESIS IN YEAST *Candida famata*

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Riboflavin (vitamin B2) is one of the most important vitamins required for human and animals. This vitamin is metabolic precursor of flavin nucleotides, FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide) involved as coenzymes in numerous enzymatic reactions, mostly of oxidative metabolism. Riboflavin deficiency causes retardation of growth, skin, nervous system and eye diseases. Although the riboflavin biosynthesis pathway is well studied it regulation is not known well. Only one regulatory gene *SEF1* was found in flavinogenic yeast *C. famata* so far. The *SEF1* is involved in positive regulation in riboflavin synthesis as additional copies of the gene leads to improvement of riboflavin production in *C. famata*. One-hybrid system on base of *Saccharomyces cerevisiae* was used to identify DNA binding sites of Sef1. The strains BY4742 of *S. cerevisiae* with plasmid harboring *SEF1* gene of *C. famata* under the control of galactose-inducible *GAL1* promoter of *S. cerevisiae* and reporter plasmid bearing *LAC4* gene of *Kluyveromyces lactis* under control of *RIB1* and *SEF1* promoters of *C. famata* were constructed. Shortened sequences of promoter *RIB1* lacking hypothetical Sef1 binding sites was used. β-galactosidase assay was used for detection of the Sef1 binding capacity of the target sequences. To study the expression of *SEF1* gene the chimeric genes were constructed. For this purpose, *SEF1* gene was fused with Myc-tag or *GFP* sequence on 3'- or 5'- end. Obtained sequences were cloned under the control of own or *TEF1* promoters. Constructed plasmids were used for transformation of *C. famata Δsef1* strain to provide complementation test. The study of the constructed strains is under the progress.

METABOLIC ENGINEERING OF THE METHYLOTROPHIC YEAST *Hansenula polymorpha* FOR CONSTRUCTION OF THE HIGH-TEMPERATURE ETHANOL PRODUCERS FROM XYLOSE

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The nonconventional thermotolerant yeast *Hansenula polymorpha* naturally ferments xylose, the second most abundant sugar in lignocellulosic hydrolysates, to ethanol at elevated temperatures. However, the efficiency of this process using wild-type strains is low and not sufficient for industrial ethanol production. Several metabolic engineering approaches have been successfully developed to improve ethanol production from xylose in *H. polymorpha*. Overexpression of engineered xylose reductase with decreased affinity toward NADPH (*XYL1m*) together with native genes encoding xylitol dehydrogenase (*XYL2*) and xylulokinase (*XYL3*) led to an elevation in ethanol yield from xylose in the wild-type strains during xylose alcoholic fermentation at high temperatures (45-48^{0}C). Also multicopy overexpression of *DAS1* and *TAL2* genes, encoding peroxisomal enzymes transketolase (dihydroxyacetone synthase) and transaldolase, respectively, had positive effect on ethanol production from xylose. In the current work both approaches were combined to improve ethanol production from xylose using earlier described *H. polymorpha* an ethanol-non-utilizing mutant (2EthOH$^-$) as a host. First the transformants overexpressing modified *XYL1m* and native *XYL2* genes were isolated and characterized to possess 1.5-fold higher ethanol production from xylose as compared to the parental strain. The additional overexpression of *XYL3* gene resulted in further 2.3-fold improvement of ethanol production and completely reduced xylitol formation during xylose fermentation. The best ethanol producing strain 2EthOH$^-$ /XYL1m/XYL2/XYL3 obtained by metabolic engineering approaches was subjected to selection for resistance to the known inhibitor of glycolysis, the anticancer drug 3-bromopyruvate. The best isolated mutant accumulated up near 10 g/l of ethanol from xylose at 45^{0}C. Additional overexpression of *DAS1* and *TAL2* genes revealed further increase up to 30 % in ethanol production during alcoholic fermentation of xylose as compared to the parental strain.

DISRUPTION OF *ATG13* GENE CAUSES THE INCREAS OF ETHANOL PRODUCTION DURING XYLOSE ALCOHOLIC FERMENTATION OF THE THERMOTOLERANT YEAST *Hansenula polymorpha*

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Fuel ethanol production from the renewable raw material, such as plant biomass or lignocellulose, has a great economic and ecological significance. However, the production of fuel ethanol from non-starch, lignocellulosic materials is not developed yet. The main reason is the absence of robust microorganism capable of efficient alcoholic fermentation of all main sugars of lignocellulose, most importantly, xylose. Thermotolerant yeast *Hansenula polymorpha* is a promising organism which ferments glucose and xylose even at 48°C. But for cost-effective ethanol production, molecular mechanisms of regulation of xylose fermentation need to be studied and available strains need to be substantially improved.
Method of insertional mutagenesis, based on obtaining mutation by integrating a nucleotide sequence in the genome of recipient organism leading to alteration of gene expression, was used for identification of genes involved in xylose alcoholic fermentation.
3-Bromopyruvate inhibits hexokinase, leading to the glycolysis disruption. Yeast mutants resistant to 3-bromopyruvate could possess increased glycolysis and elevated amount of synthesized ethanol. Several 3-bromopyruvate resistant mutants of yeast *H. polymorpha* were selected on xylose containing medium via insertional mutagenesis. One of the selected strains possesses double increase in ethanol production from xylose. Insertion cassette disrupted *ATG13* gene which is involved in conserved process of autophagy initiation. Disruption of the *ATG13* in mutant strain has no effect on peroxisome degradation. However the deletion of *ATG13* gene caused the block of this process. The Δ*atg13* strain of *H. polymorpha* produced elevated amount of ethanol from xylose, similar to insertional strain. *ATG1/ATG13* complex is responsible for initiation of autophagy processes. Deletion of *ATG1* gene in *H. polymorpha* has no influence on ethanol production from xylose.
Autophagy-related gene *ATG13* is somehow involved in the regulation of xylose alcoholic fermentation pathway in the yeast *H. polymorpha.* The role of peroxisomes in xylose alcoholic fermentation is discussed.

IMPROVEMENT OF HIGH TEMPERATURE ALCOHOLIC FERMENTATION OF *Saccharomyces cerevisiae*

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Ethanol is currently the most common liquid fuel derived from biomass. In recent years, the volume of industrial production of ethanol increased by the use of alcohol in the transport sector. Ethanol is used in automobile engines as a supplement to gasoline-ethanol blends or pure in specialized engines. The yeast *Saccharomyces cerevisiae*is is dominant industrial producer of ethanol via alcoholic fermentation of sugar substrates. Efficiency of alcoholic fermentation strongly depends on the resistance of the yeasts strains to the stress conditions, which can appear during fermentation, e. g. temperature increase or high ethanol concentration. Disaccharide trehalose is one of the major stress protectants in *Saccharomyces cerevisiae* cells. It is synthesized through two sequential steps. Trehalose-6-phosphate synthase (encoded by *S. cerevisiae* gene *TPS1*) catalyzes trehalose-6-phosphate synthesis from glucose derivatives glucose-1-phosphate and UDP-glucose. Trehalose-6-phosphate is further dephosphorylated by trehalose-6-phosphate phosphatase (encoded by gene *TPS2*) to produce trehalose. The aim of this work is improvement of high temperature alcoholic fermentation of yeast *S. cerevisiae* by overexpression of the genes *TPS1* and *TPS2*. For overexpression of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, the vector for multicopy integration was constructed, in which ORFs of *TPS1* and *TPS2* genes were placed under the control of strong constitutive *ADH1* promoter. The resulting vector was linearized and used for transformation of *S. cerevisiae* industrial strain. Recombinant strains overexpressing genes for trehalose synthesis possessed increased intracellular concentration of trehalose. Thermotolerance and tolerance to the higher ethanol concentrations of the constructed strains were also increased. Recombinant strains with higher trehalose concentration produce more ethanol during fermentation at 42°C. This difference is particularly noticeable in the first hours of the fermentation. Recombinant strains with increased intracellular concentration of trehalose produce more ethanol during alcoholic fermentation at 42°C. Constructed strains are promising for industrial implementation.

EXPLORING THE ENDOCYTIC PATHWAY BY COMBINING HIGH-THROUGHPUT GENETICS AND HIGH-CONTENT MICROSCOPY

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Endocytosis is a highly conserved fundamental cellular process that controls the lipid and protein composition of the plasma membrane, and the exchange of the majority of molecules between a cell and its environment. It is a complex process that depends on an intricate network of interacting proteins and precise coordination of molecular events, and serves as a link between many intracellular signalling networks. We have combined synthetic genetic array (SGA) analysis with high-throughput confocal fluorescence microscopy and quantitative image analysis to assess the phenotypes of cortical actin patches, endosomes and vacuoles for yeast *S. cerevisiae* deletion mutants and temperature sensitive (TS) essential gene mutants, covering approximately 5400 open reading frames (~90% of the yeast genome). Our systematic and automated approach confirmed previously known components of the endocytic pathway and identified new genes, including some previously uncharacterized, involved in the process of endocytosis. Here, the work pipeline, results and ongoing efforts will be presented.

COMPLEX GENETIC INTERACTION PROFILES REVEAL EVOLUTIONARY FATES OF DUPLICATED GENES

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The *Saccharomyces cerevisiae* genome retained 551 genes after whole genome duplication and the deletion of 35% of these sister paralogs results in a synthetic sick or lethal (SS/SL) genetic interaction, suggesting that they buffer each other's loss. The remaining duplicates do not show an SS/SL and may be redundant with a third gene, which masks their functional relationship. The digenic interaction profiles of individual paralogs provide insight about their degree of functional divergence; whereas, the functions that are shared between the paralogs can be interrogated through their trigenic interaction profiles. We are using an automated form of yeast genetics called Synthetic Genetic Array (SGA) analysis to explore trigenic interactions involving paralog pairs for the purpose of understanding paralog gene function and testing models of how paralog pairs are maintained through evolution. In its simplest form, the fate of duplicated genes has been either one of functional divergence, for functional specialization by sub- or neo-functionalization, or functional redundancy, for back-up compensation or dosage amplification. To differentiate between these possibilities, we are generating genetic interaction profiles for single mutants of sister paralogs and double mutants lacking both paralogs. So far we have analyzed over 250 paralog pairs and we have identified a functionally divergent set, as well as a set displaying a gradient of trigenic interaction frequency, suggesting that these paralogs retained varying degrees of functional redundancy. Physiological and evolutionary features, such as the double mutant fitness defect, average digenic interaction degree, expression level, rate of divergence and coding sequence similarity, correlate with trigenic interaction frequency and can be used for its prediction using a machine learning algorithm. Thus, the assessment of trigenic interactions offers a novel approach to study the functional relationship and buffering capacity of sister duplicates.

CHAPERONE OVERLOAD IS A MAJOR DETERMINANT OF DOSAGE SENSITIVITY

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Why are proteins harmful when produced in excess? While protein burden and associated energetic costs have long been suggested as an important cause of dosage sensitivity of highly expressed genes, the underlying molecular mechanisms have remained largely unclear in eukaryotes. Here, we ask how the costs of an unneeded fluorescent protein (yEVenus) vary across genetic backgrounds and environmental stresses. By integrating chemical perturbation assays and a genome-wide genetic interaction study in yeast *Saccharomyces cerevisiae*, we mapped three major subsystems with critical roles in buffering gene expression costs: the cellular machinery involved in the speed and accuracy of translation, amino acid metabolism and the Hsp70-prefoldin chaperone system. While protein expression cost occurs in all tested environments, it was especially pronounced under amino acid starvation and high temperature. Taken together, our work indicates two major sources of gene expression costs. First, translation of unneeded proteins tie up amino acids that could be incorporated into other beneficial proteins. Second, by perturbing cellular protein homeostasis, gratuitous gene overexpression causes both protein mistranslation and aggregation on a global scale.

GENERAL PRINCIPLES OF TRANSCRIPTOMIC CHANGES FOLLOWING SINGLE-GENE DELETION IN *Saccharomyces cerevisiae*

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What are the general patterns and principles behind transcriptomic responses following genetic perturbations? Our work is the first to analyse this question in a systematic manner. What gene features determine the extent of transcriptomic changes following a single-gene deletion? How specific are these changes to the deleted gene? Are they mostly adaptive or rather deleterious? A recent systematic dataset, provided by the Holstege lab, allowed us to address these questions on an unprecedented scale. This dataset contains information on genome-wide mRNA expression changes for 1484 viable knock-out strains of *Saccharomyces cerevisiae*. Our results indicate that the number of up- or downregulated genes show strong positive correlation with both the fitness contribution and the degree of pleiotropy of the deleted gene. While we revealed a significant overrepresentation of functional relatedness between the deleted and the responsive genes, this effect is small and most changes do not occur within functional modules. By overlaying the gene expression data on a genetic interaction map of yeast, we demonstrate that most expression changes are unlikely to provide compensation following gene deletion.

RNA FUNCTIONAL PROFILING BY GENE DELETION IN *S. cerevisiae*

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Developments in genome-wide analysis, such as next generation sequencing technologies and high-resolution tiling arrays, have revealed that the eukaryotic genome is extensively transcribed. Recent studies have shown that a large proportion of the transcriptome contains RNAs which are not translated in to protein; non-coding RNA (ncRNA). The focus of current debate is into how many of these non-coding RNAs are functional, and what their function is. Since its release in 2000, the Yeast Knock-out (YKO) deletion collection has proven to be a valuable tool for research teams studying cellular processes and the mechanisms of drug action. However, pervasively transcribed RNA-coding genes are absent from the collection, preventing the study of ncRNA. We have constructed ~500 barcoded ncRNA gene deletion strains. These strains include annotated snoRNAs, tRNAs and recently identified stable unannotated transcripts (SUTs) and cryptic unstable transcripts (CUTs).Confirmed diploid heterozygotes were sporulated and dissected to create haploid and diploid homozygote deletion strains. For continuity, we replicated the methodology used to create the original YKO deletion collection. Any phenotypes observed during the construction process were noted. Furthermore, the ncRNA deletion strains have been used for RNA fitness profiling, utilizing next generation sequencing of the included barcodes. This will help us to understand the role of individual ncRNAs in different growth conditions.

DISCOVERY OF NOVEL GENETIC INTERACTIONS BY CHROMOSOMAL SEGMENTAL DELETIONS AND ITS APPLICATION TO ETHANOL FERMENTATION IN *Saccharomyces cerevisiae*

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It has been recognized these days that most of the industrially useful traits are controlled not by a single gene but by multiple genes. Thus, a technology that allows large scale manipulation of a genome and chromosomes is strongly required. In this context, we have developed chromosome engineering technology called PCR-mediated chromosome splitting (PCS) in *Saccharomyces cerevisiae*. PCS enables splitting of yeast chromosome at any desired site and generates two functional chromosomes. PCS can be applied to many derivative technologies. One such application is PCR-mediated chromosomal deletion (PCD), which enables deletion of any genomic regions as we desire. Using PCD technology, we deleted 110 genomic regions in a haploid *S. cerevisiae* strain. These 110 regions were selected not to contain any essential genes and are larger than 9 kb. Surprisingly, we discovered that deletion of almost half of the regions lead to lethality, suggesting that these regions contain synthetic lethal combinations. In many cases, there were no known synthetic lethal combinations in such regions. This result implied that there are a lot of unidentified synthetic lethal combinations in adjacent genomic regions beyond expectations predicted from large scale genome analysis conducted so far. Hence, our result will uncover a novel landscape of genetic interactions in *S. cerevisiae* genome. Furthermore, we demonstrated that the PCD technology can also be applied to breeding of yeast strains since some segmental deletion mutants showed improved tolerance to bioethanol fermentation-related stresses such as high temperature, high concentration of ethanol, and weak acid. In conclusion, the PCD technology can be exploited not only for revealing genomic interactions but also for breeding of industrially useful yeast strains.

USE OF PROTOTROPHIC DELETION LIBRARIES AS A TOOL TO IDENTIFY NITROGEN RELATED GENES IN LAB AND WINE YEAST

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Nitrogen present in grape juice promotes yeast biomass formation and shortens fermentation duration. Insufficient nitrogen often causes slow or sluggish fermentation and can result in wine with microbial contamination. Problems due to nitrogen deficiency can be rectified by optimizing vineyard fertilization or more commonly supplementing the fermentation with ammonium salts. An alternative to supplementation is to use wine yeast that can efficiently assimilate nitrogen and complete fermentation in low nitrogen conditions. In order to generate "nitrogen efficient" strains it is important to investigate gene functions related to nitrogen assimilation under fermentative conditions. Several genome wide studies in the past used of lab yeast deletion libraries (collection of strains each with a single gene deleted) to identify gene functions. However, these libraries contain auxotrophic markers (requiring amino acid supplementation) that make quantitative physiological studies harder especially under nitrogen limited conditions. Therefore, to reduce the bias caused by auxotrophic markers we have used the prototrophic deletion mutant collection in both lab and wine yeast backgrounds to identify genes related to nitrogen assimilation. The methodology of this project involves firstly the screening of the prototrophic lab yeast deletion library (BY4741) and the partial wine yeast library (AWRI 1631) for growth, nitrogen and sugar consumption rates under limiting and non-limiting nitrogen concentrations. The second part of the study aims to identify the function of the candidate genes highlighted in the screen using bioinformatic tools. The last part of the study seeks to compare the identified genes in both laboratory and wine yeast backgrounds and to understand the role of the identified genes in cell growth and metabolism. The outcomes of this study will increase the knowledge on the yeast genome in relation to nitrogen assimilation. This knowledge will then be used to formulate new strategies to produce improved strains with high nitrogen efficiency to benefit the wine making process.

DEVELOPMENT OF *Kluyveromyces marxianus* FOR CELL FACTORY APPLICATIONS

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The yeast *Kluyveromyces marxianus* is widely used in biotechnology for applications that include production of ethanol, enzymes and single cell protein. The ability to produce ethanol is of particular interest because of the growing bioethanol sector. Certain traits, namely thermotolerance, high secretory capacity, GRAS/QPS status, and the fastest growth rate of any eukaryotic microbe render *K. marxianus* especially suitable for industrial exploitation. Industrially and for research applications, a range of different wild-type strains are used and research from our group, and by others, has found that there is extensive phenotypic variation between strains. At one level, this is advantageous as it allows screening to identify natural variants with specific traits of interest. It also creates challenges, however, as variation in stress response and other parameters can hinder strain development for commercial application. Recent years has seen significant advances, especially with regard to the development of molecular and genomic tools. In addition, there has been renewed interest in using *K. marxianus* as a cell factory for the production of specific chemicals, especially those with applications as flavour or fragrance metabolites. This presentation will review the progress in the field in general towards developing *K. marxianus* as a cell factory and will discuss specific work from our group in the areas of stress tolerance and bioflavour production.

ENGINEERING THE YEAST mRNA METABOLISM FOR FISHING THE DESIRED PHENOTYPE

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Biotechnological processes are of increasing significance for industrial production of fine and bulk chemicals, including biofuels. Under operative conditions microorganisms meet multiple stresses such as non optimal pH, temperature, oxygenation and osmotic stress, among others. Moreover, they have to face inhibitory compounds released during the pre-treatment of lignocellulosic biomasses, which constitute the preferential substrate for second generation production processes. All together these factors impair cellular metabolism and growth and, as a consequence, reduce the productivity of the process. The highly desirable evolution of robust cell factories is rarely ascribable to a single molecular element, since it requires a complex cellular reprogramming, implying the simultaneous modification of many regulatory and operative elements. In the last years, the cellular engineering approach termed "global transcription machinery engineering" (gTME) has been proposed, in association with specific screening protocols, as a method for reaching the above mentioned purpose. In addition to transcription, cells can modulate their complex phenotype by controlling mRNA metabolism and trafficking, translation and finally post-translational modifications. Here we present our approach for manipulating post-transcriptional events in the yeast cell factory *Saccharomyces cerevisiae* by modulating and mutagenizing one of the key regulatory element, *PAB*1. Data related to one of the improved mutant selected from the screening will be also presented and discussed.

CHARACTERIZATION OF BEER-RELATED PHYSIOLOGICAL PROCESSES IN *Saccharomyces cerevisiae, Saccharomyces eubayanus* AND THEIR INTERSPECIFIC HYBRID, THE BEST OF BOTH WORLDS

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The yeast strains used for industrial fermentation of lager beers are taxonomically classified as *Saccharomyces pastorianus* natural, aneuploid species arising from the hybridization of *S. cerevisiae* and a non-*cerevisiae Saccharomyces* species. The first complete genome sequence of a lager brewing strain, *S. pastorianus* Weihenstephan 34/70 (WS34/70) was released in 2009 confirming the hybrid nature of lager yeast genome. The recent discovery and genome analysis of *S. eubayanus* revealed that this cryotolerant yeast, whose genome sequence shows a 99.5% identity with that of the '*bayanus*' part of lager strains, contributed the non-*cerevisiae* part of the lager brewing strain *S. pastorianus* genome.

The hybrid genomes of lager brewing strains raise intriguing questions about the relative contribution and regulation of genes in the *S. cerevisia*e and *S. eubayanus* subgenomes involved in cellular processes relevant for lager brewing. It is, for example, unclear whether the production of specific flavours and off-flavours is predominantly encoded by one of the subgenomes. To study in more details how each subgenome may contribute respectively to specific phenotypic traits of lager strains, a de novo interspecies hybrids between the two proposed *S. pastorianus* parents, *S. cerevisiae* and *S. eubayanus*, was created. We have subsequently investigated and characterized the performance of the hybrid strain in environmental conditions relevant for the brewing process. The physiological characteristic of the hybrid strain was then compared with those of the parental strains as well as with the one of a *S. pastorianus* strain. The analysis of these data provided a deeper understanding of the relative contribution of *S. cerevisiae* and *S. eubayanus* in *S. pastorianus* phenotypic behavior.

PRODUCTION OF SPECIALIZED FATTY ACIDS IN *Saccharomyces cerevisiae*

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The primary aim of this project is to design strains of *S. cerevisiae* to produce specialized fatty acids that are precursors for biosynthesis of commercially interesting compounds. In the first stage of the project metabolic enzymes that are required to redirect fatty acid metabolism will be identified. Simultaneous expression of large gene sets using eYACs (expression Yeast Artificial Chromosomes) will then be applied to functionally clone new enzymes for fatty acid synthesis. Using this information rational engineering will be employed to tailor make novel GM strains that produce relevant fatty acids.

DECIPHERING THE GENETIC AND METABOLIC BASES OF YEAST AROMA PROPERTIES

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The yeast species *Saccharomyces cerevisiae* plays a vital role in the formation of aroma compounds during wine fermentation. Wine aroma includes volatile compounds that are produced de novo by yeast, like esters, higher alcohols and organic acids, and compounds that are formed from precursors present in the must, like terpenols or DMS.
The main aim of this work is to identify the genomic and metabolic bases for the formation of these molecules. For this purpose a crossing will be performed between two wine yeast strains selected because of their difference in the need for nitrogen during fermentation. Approximately 130 F2-segregants will be genotyped by next-generation sequencing and individually phenotyped by measuring extracellular metabolites using high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). Based on the metabolic data, the intracellular fluxes in central metabolism will be estimated using a constraint-based model of yeast metabolism. Quantitative trait locus (QTL)-mapping will be used to identify specific allele variations influencing the aroma profile and the metabolic fluxes. Finally the role of detected alleles will be validated by allele replacement and studied by functional genomics.
This will lead to a more profound knowledge about the correlation between genetic properties and phenotypic characteristics and will enable the exploitation of the natural diversity of yeast to generate novel aromatic wine yeast strains.

MULTI-STRESS RESISTANCE AND LAG-PHASE TIME REDUCTION IN WINE FERMENTATION

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Saccharomyces cerevisiae has been used for several millennia to perform wine fermentation due to its endurance and unmatched qualities. Nevertheless, at the moment of inoculation, wine yeasts must cope with specific stress factors that still challenge wine-makers nowadays by slowing down or even compromising the fermentation process. The objective of the present work is to improve the multi-stress resistance of commercial yeast strains specifically during the lag-phase in order to reduce fermentation start period and possibly allow direct wine inoculation. Red and white wine fermentations present distinguished sets of stress factors at the moment of inoculation: for instance high osmotic pressure and lack of vitamins (*e.g.* thiamine) in red and considerable SO_2 concentrations, lack of important lipid components due to wine clarification and low temperatures (14ºC) in white. Due to these differences, several commercial yeast strains will be preliminary tested and the best candidates chosen according to their best suitability for each type of fermentation. Next, two parallel approaches will be conducted: an evolutionary engineering approach where for several generations specific selective pressures will be applied in order to promote yeast adaptation to stressful conditions as well as a decrease in lag-phase duration. Concomitantly a QTL approach will also be conducted with the aim of identifying important genes and loci responsible for stress resistance and/or for yeast improved growth during lag-phase. It is expected to obtain optimized wine yeasts with improved stress resistance and reduced lag-phase while keeping the desirable metabolic and fermentation traits. A deeper knowledge about the genetic and molecular bases responsible for yeast adaptation and multi-stress resistance is intended as well.

REPROGRAMMING OF *Saccharomyces cerevisiae* SHIKIMATE PATHWAY FOR THE PRODUCTION OF BULK CHEMICALS

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The production of bulk chemicals is often associated to energy waste and it is not an environmental friendly process. Therefore, finding new alternatives to produce commodity chemicals is now a major interest.
S. cerevisiae can be engineered as a microbial cell factory in order to produce interesting compounds and it has many advantages if compared to other microbial hosts.
In particular, we are focusing on the Shikimate pathway, which is the central pathway involved in the production of aromatic amino acids.
Through metabolic engineering approaches we intend to reprogram *S. cerevisiae* and redirect its metabolism towards the production of bulk chemicals that might be derived from Shikimate pathway intermediate metabolites.

EVALUATION OF *Zygosaccharomyces bailii* FOR LACTIC ACID TOLERANCE

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Nowadays an industry searches for a cheap and environmentally friendly way to produce fine chemicals. The cell biofactories are an attractive system to fulfill modern requirements for an industrial production of chemicals and among those lactic acid is considered as an oldest and high-volume produced chemical. However, bacterial strains used in lactic acid fermentation suffer from low acid tolerance making downstream purification of final product troublesome and expensive. Exploitation of acid tolerant yeast strains can effectively eliminate the obstacle. *Zygosaccharomyces bailii* is a well-known food spoilage yeast usually found in can and wine industry. *Z. bailii* possess a significant low pH tolerance and organic acid tolerance which can be considered as an advantage in lactic acid production. Therefore we would like to elucidate and understand the mechanism underlying lactic acid tolerance in *Z. bailii* not thoroughly investigated up to now. Data resulting from several fermentations using both flasks and bioreactor with different pH, oxygen supply and organic acid concentrations will be presented and discussed, together with evaluations regarding viability profile measured at single cell level.

ELUCIDATING MECHANISMS THAT DETERMINE DIFFERENTIAL MALTOTRIOSE UTILIZATION BETWEEN STRAINS OF THE HYBRID YEAST *Saccharomyces pastorianus*

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The lager brewing yeast *Saccharomyces pastorianus* is an allotetraploid hybrid of *S. cerevisiae* and *S. eubayanus*. Its hybrid condition allows it to benefit from the strong fermentative capacity of *S. cerevisiae* and the cryotolerance of *S. eubayanus*. These properties make it suitable for the production of lager beer which requires low fermentation temperatures. Possibly as a result of different hybridization events, two genetically distinct groups are present within the *S. pastorianus* taxon, Saaz and Frohberg. The groups display some functional differences, e.g. cold tolerance is more evident in the Saaz group, while the Frohberg group has greater fermentation performance. It has been observed that the Saaz group (as well as the parental *S. eubayanus*) has limited ability to utilize the maltotriose present in brewer's wort and this may be the cause of its relatively poor fermentation performance. The mechanisms responsible for this limitation are still to be uncovered. It is hypothesized that the inability to utilize maltotriose is related to insufficient transmembrane transport. To test this hypothesis a comprehensive study of wort fermentation performance, sugar utilization and membrane transport activity in Saaz strains is being conducted. The expression of maltotriose transporter genes (namely *MTT1*, *MPH2/3* or *AGT1*) in Saaz strains will determine whether transmembrane transport is the main factor limiting fermentation performance of Saaz strains or if other factors, yet to be identified, are contributing to this property.

GALLIC ACID PRODUCTION BY THE TWO TANNASES Atan1p AND Atan2P USING THE YEAST *Arxula adeninivorans*

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Tannin acyl hydrolase (tannase, EC 3.1.1.20) is an inducible enzyme and belongs to the group of hydrolases. It hydrolyses ester bonds between sugar and gallic acid, as well as depsides bonds between two molecules of gallic acid present in tannins. The most wanted product of this reaction is gallic acid that is used as additive for colorants, as an antioxidant in food, as well as precursor for trimethoprim production and others. The presented studies focus on the development of more efficient and cheaper methods of tannase production by genetically modified *Arxula adeninivorans*. This dimorphic, non-pathogenic, thermo- and osmotolerant yeast, which exhibits a very broad spectrum of usable substrates, is the only organism known, which synthesizes two different tannases. Atan1p (encoded by *ATAN1*) is secreted and the other one Atan2p (encoded by *ATAN2*) is localized in the cell wall. Two vectors containing *ATAN1* and *ATAN2* genes with hexahistidine encoding sequence were constructed and transformed into an *A. adeninivorans* yeast strain. The respective recombinant proteins expressed by the trangsgenic strain were purified by IMAC and enzymatic parameter were determined. Furthermore the large scale protein production was optimized. The characterization of Atan2p protein is in progress. Deletion mutants generated by gene disruption: *A. adeninivorans* G1236 [Δ*atan1*], G1237 [Δ*atan2*], and a double mutant MS1031 [Δ*atan1,* Δ*atan2*]) were produced for comparison of the function of both enzymes in the cell.

AUTOMATED ANNOTATION, STRUCTURAL VALIDATION, AND METABOLIC MODELING OF INDUSTRIAL YEAST GENOMES

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Unicellular fungi (yeasts) have been used for the production of food and beverages since long before we possessed a detailed description of the cellular processes underlying such practices. Today, a vast amount of knowledge has been accumulated and utilized with both academic and industrial implications, shaping the field of yeast biotechnology. Modern yeast biotechnology depends heavily on genome sequencing technologies and the bioinformatic methods that are used to interpret genome sequences. Therefore, we will optimize and improve a computational system for automated gene identification and *de novo* genome sequence annotation, and apply it to industrially-relevant yeasts sequenced using next-generation genome sequencing technologies. The system (Yeast Genome Annotation Pipeline; YGAP) was developed in the Wolfe lab in 2011 and is based on using interspecies synteny data from the Yeast Genome Order Browser. A second aim of this project is to develop automated methods for validating the structural integrity of genome sequence assemblies. Commonly, next-generation *de novo* genome sequence assemblies consist of multiple contigs that are physically linked together into scaffolds. Our goal is to obtain scaffolds that correspond as closely as possible to the structures of complete chromosomes. A third aim is to use newly-annotated yeast genomes to construct metabolic models of the corresponding species, by cross-referencing from existing metabolic models for other species. It will be of particular interest to focus on interspecies differences in predicted metabolic networks. As a starting point, we will use new public genomic sequences for two strains of *Kluyveromyces marxianus* to test the performance of the YGAP annotation pipeline and identify its current weaknesses relative to author-supplied annotations and benchmark gene sets from other species. This research project receives funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n° 606795, project acronym: YEASTCELL.

TOWARDS A GENOME SCALE METABOLIC MODEL OF *K. marxianus*: A KEY TOOL TO IMPROVE ITS INDUSTRY-RELEVANT PROPERTIES.

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The redirection of metabolic fluxes towards the production of a desired compound usually requires an overall understanding of cell metabolism. One effective strategy to obtain this view is by constructing a Genome Scale Metabolic (GSM) model. For yeasts, different GSM models have been built and were shown to be effective in designing metabolic engineering experiments. The yeast *Kluyveromyces marxianus* has special traits (lactose utilization, thermotolerance and high growth rate) that make it attractive for different biotechnological applications. Although this yeast has been already used in industry, there is a growing interest in enhancing its special properties. The main aim of this research project is to generate a GSM model for *K. marxianus*. For this purpose, first a metabolic-based genome annotation will take place using the tool MERLIN. Then, the metabolic reconstruction and model building will be carried out using RAVEN. To facilitate this process, previous GSM models from *S. cerevisiae* and *K. lactis* will be taken into account. In parallel, comparative genomics analyses will be done to serve as a general tool to assist the orthology-based reconstruction. Finally, in order to obtain a final high quality model, manual curation and experimental data will be considered. The generated model will be used to compare the *K. marxianus* metabolic network with those of other yeasts to understand the basis of its unique traits. At the same time the model will serve as a framework for metabolic engineering of this yeast enhancing its potential as a cell factory for the production of industry-relevant compounds.

SYNTHESIS OF WAX ESTERS IN *Saccharomyces cerevisiae* BY ENZYMATICALLY CATALYZED ESTERIFICATION OF LONG-CHAIN FATTY ACIDS AND PRIMARY LONG-CHAIN FATTY ALCOHOLS

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The objective of this project is the enzymatic synthesis of wax esters by the esterification of primary, long-chain fatty alcohols and long-chain fatty acids with both components varying in their carbon chain length. Naturally, wax esters occur in pro- as well as eukaryotes where they fulfill different purposes, e.g. they can serve as a storage compound or have a protective or structural function (Röttig *et al*. 2013). Wax esters have a broad application range and can be used for many commercial purposes. These applications include e.g. personal care products, lubricants, varnishes, inks, waxes, detergents, resins & plastics. Wax esters can also be used for coatings (for fruits & pills), as an oil phase in formulas containing active compounds to enhance the efficiency of topical drugs or even as a replacement for diesel fuel. Unfortunately, so far most of the possible applications are limited to cosmetic and medical products due to the high price of production (Hassan et al. 2009). Because of this fact, a renewable approach for wax ester synthesis in a well-studied organism like *S. cerevisiae* is desirable. Just recently it was shown that wax ester synthases of different origin can be used for the production of wax esters in *S. cerevisiae* (Shi *et al*. 2012). Dependent on the enzyme source, different alcohols were preferred as a substrate when testing the esterification of palmitoyl coenzyme A. Because of this promising observation, further enzymes shall be tested for their ability to enable wax ester biosynthesis in *S. cerevisiae*.

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KINETIC ANALYSIS AND GAS–LIQUID BALANCES OF THE PRODUCTION OF FERMENTATIVE AROMAS DURING WINEMAKING FERMENTATIONS

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In winemaking, higher alcohols and esters produced during the alcoholic fermentation have a major impact on wine quality, especially for young wines. Accessing to their kinetics of synthesis is therefore of primary importance. To reach this objective, we developed an online monitoring system of the concentrations of the main volatile compounds in the off gas during the alcoholic fermentation. The high acquisition frequency of this specific device makes it possible to calculate the rates and specific rates of production, which are of direct interest for a better understanding of yeast metabolism. We also determined the gas–liquid balances of aroma production to take losses into account and to distinguish between microbial and physicochemical phenomena. We have thus demonstrated that the lower liquid concentrations of esters at high temperature resulted mostly from large losses by evaporation, with only limited changes in yeast metabolism. Then, we investigated the impact of both initial nitrogen concentration and temperature on the kinetics of production of the fermentative aromas. Nitrogen availability appeared as the main parameter affecting the kinetics of higher alcohol and ester synthesis, but the magnitude of this effect depended on temperature. The production of fermentative aromas was closely linked to central carbon metabolism, except for propanol. Indeed, two successive linear production phases (with yields dependent on initial nitrogen content and temperature) were obtained when plotting volatile compound synthesis versus sugar consumption. Rates and specific rates of isobutanol and isoamyl alcohol production were affected differently by nitrogen and temperature, indicating major differences in the regulation of synthesis for these two compounds, despite their partially shared metabolic pathways. Finally, propanol was produced exclusively during the nitrogen consumption phase, and the amount produced increased with initial nitrogen concentration. This compound was, therefore, identified as a quantitative, metabolic marker of the availability of intracellular nitrogen.

Saturday, October 11 – Vipava

08:00 – 08:45 Transportation to Vipava – buses leave from in front of the Perla Hotel

09:00 – 12:30

Parallel sessions (Vipava)

Beverages 1 & 2 *(chairs: Florian Bauer / Sylvie Dequin)* *see p. 91 for details*

Biodiversity for Applications *(chairs Ian Roberts and Cene Gostinčar)* *see p. 92 for details*

2nd Generation Biofuels *(chairs Akihiko Kondo and Takayuki Mizuno)* *see p. 92 for details*

10:30 – 11:00 Coffee Break & Meeting Local Food/Wine Producers

Regulation of and by Lipids *(chairs Peter Gajdoš and Martin Kavšček)* *see p. 93 for details*

Non-conventional Yeasts in Bioproduction
(chairs Gotthard Kunze and Janja Zajc) *see p. 93 for details*

12:30 – 14:00 Lunch / Vipava sightseeing & Meeting local food/wine producers

14:00 – 19:00

Parallel sessions (Vipava)

Ecology and Genomics *(chairs: Duccio Cavalieri and Joseph Schacherer)* *see p. 95 for details*

Cornucopia EU project session *(chair Hana Sychrova)* *see p. 95 for details*

Regulation of Metabolism and Nutrient Sensing
(chairs: Hyun Ah Kang and Marta Maria Rubio-Texeira) *see p. 97 for details*

16:10 – 17:00 Coffee break & Meeting local food/wine producers & Vipava sightseeing

Biofactories: Tools, Physiology & Products

(chairs: Hiroshi Takagi and Anne Gschaedler) *see p. 98 for details*

YeSVitE EU project session *(chair: Ileana Vigentini)* *see p. 98 for details*

Polygenic Traits *(chairs: Gianni Liti and Ed Louis)* *see p. 99 for details*

19:15 – 20:00 Transportation to Nova Gorica – buses leave from in front of the Grammar School

Saturday, October 11 morning sessions in Vipava – parallel sessions in:

Aula Magna and Aula Parva – Lanthieri Palace

Grammar School Hall (5 min walk from the Lanthieri Palace)

Beverages 1 / in Aula Magna

Beverages 2 / in Aula Magna

Biodiversity for Applications / in Aula Parva

2nd Generation Biofuels / in Grammar School Hall

Regulation of and by Lipids / in Aula Parva

Non-conventional yeasts in bio-production / in Grammar School Hall

Saturday, October 11 afternoon sessions in Vipava – parallel sessions in:

Aula Magna and Aula Parva – Lanthieri Palace

Grammar School Hall (5 min walk from the Lanthieri Palace)

Ecology and Genomics / in Aula Magna

14:00 - 14:20 Duccio Cavalieri: Population genomics of *Saccharomyces cerevisiae* human isolates: passengers, colonizers and invaders 136

14:20 - 14:40 Joseph Schacherer: Population genomics reveals the evolutionary fate of a large-scale introgression in a protoploid yeast 137

14:40 - 15:00 Ursula Bond: Distinctive chromosomal end loss and lager specific genes define two different *Saccharomyces cerevisiae* lineages for *S. pastorianus* group I and II strains 138

15:00 - 15:20 Jürgen Wendland: Genome sequence of *Saccharomyces carlsbergensis*, the world's first pure culture lager yeast 139

15:20 - 15:35 Samina Naseeb: Role of chromosomal inversions on yeast fitness and gene expression 140

15:35 - 15:50 Qamar Almutava: Evolutionary impact and environmental adaptation of natural yeast isolates bearing karyotypic rearrangements 141

15:50 - 16:05 Kobchai Dungrattanalert: Evolutionary effect of mitochondrially encoded proteins on global gene retention in yeast hybrids 142

Cornucopia **EU project session / in Aula Parva**

14:00 - 14:20 Hana Sychrova: Opening remarks

14:10 - 14:22 Benjamin Offei: Polygenic analysis of low pH tolerance in *Saccharomyces cerevisiae var. boulardii* 143

14:22 - 14:34 Honeylet Ochangco: Intracellular pH of potential probiotic yeasts during the passage through an in vitro gastrointestinal tract model 144

Regulation of Metabolism and Nutrient Sensing / in Grammar School Hall

Biofactories: Tools, Physiology & Products / in Aula Magna

***YeSVitE* EU project session / in Aula Parva**

Polygenic Traits / in Grammar School Hall

ENVIRONMENTAL PERTURBATIONS, NUTRIENTS AND THE YEAST MICROBIOME: CAN WE PREDICT THE OUTCOME OF WINE FERMENTATIONS?

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Wine fermentations are continuously evolving ecosystems that are adapted to juices of variable nutritional composition and respond to continuously changing environmental conditions. The species and strains of microorganisms that constitute this ecosystem, some of which will conduct alcoholic fermentation, are primarily responsible for many of the aromatic features of the final product. For the most relevant fermentative microorganism in wine, the yeast *Saccharomyces cerevisiae*, many of the genetic and metabolic regulations that lead to the fermentation-derived aromatic and flavour profile are relatively well understood. However, the complexity of interactions between microorganisms, the differing chemical composition of grapes and grape musts across cultivars and vintages, and the variable and changing fermentation conditions, have not previously allowed for the prediction of a specific aromatic outcome for any given fermentation. Here we present data on the environmental factors that control the fermentation performance of mixed culture fermentations, and the impact of changes in nutritional composition and fermentation conditions on the aromatic features of wine made from synthetic must. The data show that simple nutrient compositions lead to predictable outcomes, with frequently approximately linear correlations between a precursor and a derived aromatic compound, but that more complex nutrient compositions result in non-linear and strain-dependent changes to the aromatic output. Changes to specific environmental factors such as temperature and osmotic pressure similarly lead to sometimes unexpected changes in aroma composition. However, the data suggests that multivariate models will be able to predict some of the relevant aromatic features of a wine based on the nutritional composition of the must, the fermentation conditions and the relevant yeast microbiome.

LOWERING ETHANOL CONTENT OF WINES BY SUGAR RESPIRATION

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During the last decade, the wine industry has been demanding the development of new approaches to reduce alcohol content of wines, which had been steadily increasing due global climate change and consumer preferences towards well structured, full body wines. We recently proposed respiration of sugars by non-*Saccharomyces* yeasts for lowering ethanol yields. As a first step in order to develop an industrial process based in this proposal we characterized a collection of non-*Saccharomyces* yeast strains according to respiratory quotient under aerated conditions, at low pH and high sugar concentrations, yields of ethanol and other relevant metabolites, and growth responses to the main stress factors found during the first stages of alcoholic fermentation. One strain of *Metschnikowia pulcherrima* was selected for a proof of concept. The optimized procedure involved the simultaneous inoculation of one strain of *Saccharomyces cerevisiae* and that strain of M. *pulcherrima*, and controlled aeration during the first two days after inoculation of a natural white grape must. One essential optimization criterion was controlling acetic acid production, which tended to increase with increasing aeration of the must. We have demonstrated the crucial role of oxygen availability and respiratory metabolism in order to reduce alcohol levels. By choosing and optimizing the appropriate gassing conditions we managed to find a good balance between alcohol level reduction (3-4 degrees), the increase in volatile acidity mostly associated to growth of *S. cerevisiae* under aerobic conditions, and levels of dissolved oxygen during the process. The implementation at the industrial level of a strategy to lowering ethanol content of wine based on the respiratory breakdown of sugars by non-*Saccharomyces* yeasts would probably require further optimization involving yeast species and strain selection, inoculation strategies, oxygenation conditions, or fermentation nutrients.

CHENIN BLANC WINE PRODUCTION WITH *Torulaspora delbrueckii* AND *Saccharomyces cerevisiae* YEASTS

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Non-*Saccharomyces* yeasts can have a positive role to play in wine fermentation and *Torulaspora delbrueckii* was one of the first species to be commercialised. In this study, two *T. delbrueckii* isolates were compared to commercial *T. delbrueckii* strains for small-scale production of Chenin Blanc wines over four vintages. The fermentation treatments included a *S. cerevisiae* reference, *T. delbrueckii* only, and *T. delbrueckii* and *S. cerevisiae* co-inoculated at time zero and 24 hours into fermentation. Fermentations were monitored by CO_2 mass loss and yeast cell counts. Fermentation speeds were the fastest for the co-inoculated fermentations and slowest for the *T. delbrueckii* on their own. Analyses on wines included standard chemical, sensory and volatile aromatic compounds. The *T. delbrueckii* only fermentations usually had higher residual sugar levels than the co-inoculated fermentations. Principle component and discriminant analyses of the data showed that vintage was a greater driver of differences between wines than fermentation treatments. However, per vintage, single, co-inoculated wines at zero hours, and co-inoculated wines at 24 hours grouped separately, indicating stylistic difference between fermentation treatments.

SELECTED *Saccharomyces cerevisiae* AND *Torulaspora delbrueckii* STRAINS TO IMPROVE THE ANALYTICAL PROFILE OF CRAFT BEER

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In the present study it was investigated on the influence of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* strains on the analytical and sensorial profiles of craft beer.
In the first part of the work we investigated on the influence of the yeast cultures used in the refermentation process. After a preliminary screening of 33 *S. cerevisiae* strains, four were selected and used in bottle refermentation trials. Molecular typing, carried out to attribute the flavour profile of the refermented beers to the inoculated strains, confirmed the dominance of these inoculated strains. While the main analytical characters did not show differences among the bottle-refermented trials, distinctive and significant variations in the volatile compounds were shown. Subsequently in the second part of work, we evaluated the potential use of *Torulaspora delbrueckii* selected strains to give a peculiar bioflavoring to craft beer. Indeed this species exhibits some positive features particularly in mixed fermentations under winemaking condition (Bely et al. 2008; Comitini et al. 2011). From a bunch of 28 strains of *T. delbrueckii*, eight (able to ferment maltose) were evaluated for their fermentative aptitude on wort. A strain that exhibited the better fermentative performance was selected and used in mixed fermentation with *S. cerevisiae* starter strain S-05 (Fermentis Lesaffre, France) at different ratio (1:1, 1:10 and 1:20) to evaluate the influence of this yeast on the analytical profile of beer. Preliminary results showed that *T. delbrueckii* in mixed fermentation positively influence the analytical profile of beer.

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Comitini F., Gobbi M., Domizio P., Romani C., Lencioni L., Mannazzu I., Ciani M. (2011) Selected non- Saccharomyces wine yeasts in controlled multi starter fermentations with Saccharomyces cerevisiae. Food Microbiol, 28: 873–882.

LABORATORY BREWING UNDER LARGE-SCALE PRESSURE VS. INDUSTRIAL BREWING: MICROARRAY PHENOTYPING OF YEAST DURING SERIAL REPITCHING

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Serial recycling of yeast biomass, i.e. repitching, has been a common practise in brewing, mainly due to economic reasons. Repitching is usually performed 6 to 12 times, because of possible yeast aging effects on beer quality. However, the rationale behind these aging effects is not understood completely. There have been reports of extensive repitching in laboratory conditions, which did not show any significant cell damage in yeast. Our aim was therefore to monitor any changes in yeast physiology along the repitching cycles. Moreover, the monitoring was performed in a large-scale fermenter up to 3250 hL along 15 repitching cycles and in small-scale laboratory fermenters of 35 ml along 20 re-pitching cycles. The laboratory fermenter system was constructed with the aim to mimic the industrial process as much as possible. Fermentations were performed in conical Falcon tubes at 14 °C. To mimic hydrostatic and CO_2 pressure of the industrial fermenter, the system was connected to a gas supply (20 % CO_2 in nitrogen) at the pressure of 1.1 atm. One day after each repitching, the following tests on yeast biomass were performed: cultivability (CFU), viability (methylene-blue staining), vitality (Live-Dead kit), optical density, cell counting (haemocytometer and ImageJ software), and at the selected repitching cycles also the phenotypic microarray analysis using the Omnilog system. At the end of fermentations the level of fermented sugars, pH and flocculation were determined. Regarding the cautiousness around the eighth repitching in breweries, we observed no significant changes in yeast physiology in either industrial or laboratory experiments at this point. In fact, first deficiencies in metabolism were observed after the fifteenth repitching.

EXPLORATION OF YEAST BIODIVERSITY FOR POTENTIAL IMPROVEMENTS OF *RIBOLLA GIALLA* WINE AROMA PROFILE

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During the last decades only a limited number of yeast species have been used in commercial wine fermentations. The EUITN project Cornucopia has recently attempted to reverse this trend and screened a few hundred yeast isolates to identify novel species with a promising aroma potential, which could be employed in wine fermentations. We applied some of these yeasts in *Ribolla Gialla* must fermentations, with the future aim to intensify the aroma profile of this wine. In our experiments, we performed sequential micro-fermentations starting with a novel yeast species, and after 60 hours a commercial *Saccharomyces cerevisiae* wine strain was added. Growth rate, competition between the inoculated yeasts, sugar consumption, ethanol accumulation and aroma profile were monitored during these experimental fermentations. Sensory analyses of young wine showed that particularly one of the novel yeasts, *Kazachstania gamospora*, enriched the aroma profile of the fermented *Ribolla* must, and has also fermentative properties, which qualify it for future large-scale wine fermentation trials.

EVOLUTIONARY AND REVERSE ENGINEERING OF COMMERCIALLY IMPORTANT METABOLIC TRAITS IN WINE YEAST

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The wine industry faces a series of challenges related to globalization, new market trends, technological and climate change. In this context, there is a high demand for the development of microbial strategies to develop *Saccharomyces cerevisiae* yeast strains with novel traits. Non-targeted approaches such as evolutionary engineering are alternative strategies to genetic engineering for reprogramming metabolic networks. Using this approach, we obtained evolved wine yeasts with improved gluconate utilization. The evolved strains had increased flux through the pentose phosphate pathway and produced higher amount of esters, which contribute to the fruity and floral aroma of wine. Another example was the selection, by a combination of experimental evolution and conventional breeding, of an evolved strain that produce substantially more glycerol and less ethanol. In pilot-scale experiments, this strain efficiently decreases the ethanol content of wines by 0.9 to 1.3%, while producing less acetate and more 2,3-butanediol. These evolved strains represent novel tools to improve the sensorial quality of fermented beverages. Besides, a major challenge is to elucidate the genetic bases of the evolved phenotypes, which will enable their transfer to other industrial strains. Using genome-wide approaches, we identified the evolutionary genomic variations of evolved strains with improved gluconate utilization. We showed that pleiotropic metabolic phenotypes have been acquired by a dominant, loss-of-function mutation of *BCY1*, a gene controlling the cAMP-dependent protein kinase (PKA) nutrient signaling pathway that play major roles in yeast growth, metabolism and stress response.

CHARACTERIZATION OF GENES INVOLVED IN THE ADAPTATION OF FLOR STRAINS TO BIOLOGICAL AGEING CONDITIONS OF SHERRY WINES

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Saccharomyces cerevisiae is responsible of a lot of biotechnological processes essential for human feeding, as fermentation of wine, beer and bread. In contrast with these fermentation lifestyles, during the biological ageing of Sherry wines *S. cerevisiae* flor strains grow aerobically at the wine surface forming a biofilm. This distinctive feature allows cellular population to overcome stress conditions, like nitrogen and sugar depletion, after the end of fermentation. With the aim to detect alleles involved in flor yeast adaptation to biological ageing, the genomes of 7 flor and 9 wine strains have been sequenced. The comparison of flor and wine genomes revealed several divergent regions. These regions include genes from different functional categories such as cell wall proteins, *FLO11* regulation, homeostasis of metal ions which present numerous allelic variations, including SNPs and recombination. Strikingly, the many genes involved in metal ions homeostasis suggest an important role in the adaptive evolution of flor strains. We have performed the allelic replacement of different flor and wine alleles for several divergent genes and evaluated the effect of the allelic replacement on their phenotypes, respectively, in wine and flor haploid strains.

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THE INFLUENCE OF DIFFERENT YEAST INOCULUM LEVELS ON THE QUALITY OF CIDER

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In this study, the impact of the different inoculum levels of commercial dry yeast on the yeast growth, fermentation rate, ethyl alcohol and flavour compounds formation was investigated. Increasing the inoculum level increased the fermentation rate. Higher inoculation rate led to higher maximum yeast cell number in the culture. By using commercial Actiflore PM and Uvaferm CM yeasts, ethyl alcohol levels ranged from 5.84% (v/v) to 6.27% (v/v) and from 6.08 % (v/v) to 6.41% (v/v), respectively. The concentrations of flavour compounds of ciders depend on the commercial yeast strain and inoculum levels. The highest amounts of higher alcohols were found in the experiments inoculated with $1x10^7$ cells/mL using Actiflore PM commercial yeast, esters inoculated with $1x10^6$ and $1x10^7$ cells/mL and carbonyl compounds pitched with $5x10^6$cells/mL. The highest concentrations of higher alcohols, esters and carbonyl compounds were determined in the ciders inoculated with $1x10^5$ cells/mL using Uvaferm CM commercial yeast.

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IMPACT OF INOCULUM PREPARATION ON MICROBIAL CONSORTIA, FERMENTATION KINETICS AND PRODUCT COMPOSITION IN TRADITIONAL MEZCAL FERMENTATIONS

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Mezcal, a traditional Mexican spirit obtained by distillation of fermented cooked agave juices is nowadays gaining more popularity on the market of beverages. The principal problem of most mezcal producers consists in carrying out spontaneous fermentations that last sometimes more than two weeks affecting strongly the quality of the product. These fermentations are characterized by the presence of complex microbial consortia, where bacterial populations exceed up to 100 times the concentration of yeasts. As a consequence of this fact it is commonly observed that the acidity of the final product exceeds the maximum levels fixed by the official Mexican Norm for mezcal.

In order to control the contribution of the bacteria to the chemical composition of the agave musts, a simple "artisan" inoculum was prepared with the native microflora applying conditions that favor the growth of yeast rather than bacteria. In three different mezcal factories in Guerrero state, two fermentations were carried out in parallel (with and without inoculum) in order to study the effect of this practice on the microbial consortia, fermentation kinetics and chemical composition of the fermented agave musts. The evolution of bacterial and yeast populations and of the generated aroma compounds as organic acids, esters and alcohols was monitored with PCR-DGGE and HPLC/GC, respectively.

Although the maximum yeast concentrations could apparently not be increased by this practice and the bacterial populations still were dominant, clear differences were observed in the production of organic acids and other compounds important for the organoleptic profile of the mezcal, particularly, the production of lactic acid could be clearly reduced. Moreover the fermentation time was shorter and the conversion rates of sugars into ethanol were improved when an inoculum was prepared. This work exemplifies the possibility to improve traditional fermentations introducing modern practices without ruining the artisan image of these processes.

EFFECT OF THE AGAVE SAPONINS ON THE CELL WALL COMPOSITION OF *Saccharomyces cerevisiae* AND *Kluyveromyces marxianus* YEASTS STRAINS IN CONTINUOUS CULTURE

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The main secondary metabolites isolated from the *Agave* leaves, are steroidal saponins, these metabolites are also found in less proportion in the agave juice that is used in the fermentation process for the production of alcoholic beverages in Mexico. Although the saponins are present in less proportion in the agave juices they inhibit the growth of yeast during the fermentation process, however the mechanisms of the cell growth inhibition are poorly understood. The aim of this work is to study the effect of saponins on the cell wall composition (β1-3 glucan, β1-6 glucan and mannoproteins) of the fermentative yeast *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* isolated from the tequila and mezcal fermentations process respectively. The analysis was performed in a continuous culture with saponins pulses (0.75, 1.5 and 3 mg/ml final concentration in reactor medium) extracted from agave leaves of *Agave durangensis* and *A. salmiana* ssp. *crassispina*. It was observed changes on the cell wall composition in *S. cerevisiae* at 3 mg/ml of *A. salmiana* saponin extract where the ratio of mannan/glucan change from 1:15 to 1:6, and in *K. marxianus* at 3 mg/ml of *A. durangensis* saponin extract where the ratio of mannan/glucan change from 1:8 to 1:50, these difference compare with scanning eletron microscopy observation shown that *K. marxianus* is more tolerant and have the ability to growth in stressful environment.

NEW METHOD FOR FAST SEPARATION OF MAGNETIZED YEAST *Saccharomyces bayanus* IN SPARKLING WINE PRODUCTION

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A novel method for fast magnetic separation of *Saccharomyces bayanus* yeast cells from sparkling wine was developed. The wine yeast cells were made responsive to a magnetic field with absorption of superparamagnetic nanoparticles of iron oxide maghemite (γ-Fe_2O_3) coated with a thin layer of silica and grafted with (aminoethylamino) propylmethyldimethoxy silane (APMS). The terminal amino groups of the APMS molecules provides a positive charge at the nanoparticles' surfaces and promotes their electrostatic absorption onto the negatively-charged surfaces of the yeast cells. The influence of the magnetic nanoparticles on yeast metabolism, as well as separation of magnetic yeast cells biomass in the magnetic field was studied. The optimal mass ratio between the magnetic nanoparticles and the wine yeast was determined to be 1:10. Scanning and transmission electron microscopy showed that the magnetic nanoparticles remained fixed at the microbial cell surfaces even after fermentation. The results of chemical analysis showed that besides faster microbial kinetics there were no other influences on the cell metabolism. The same results were confirmed in sensorial analyses of sparkling wine. Separation of the magnetized waste yeast biomass into the neck of the bottle using a relatively weak magnetic-field gradient can be successfully completed in approximately 15 minutes.

EXPLORING A YEAST GENETIC RESOURCE COLLECTION TO AID BIOREFINERY PRODUCTION OF BIOBASED CHEMICALS FROM AGRIFOOD FEEDSTOCKS

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Biorefinery operations have generally focussed on production of biofuels to reduce dependency on fossil fuels. However, replacement fuels, whilst produced in high volume, are currently of low financial value. A further biorefining objective therefore focusses on achieving economic sustainability through co-production of higher value biobased chemicals. Production of such chemicals using yeast has already proved economically viable. Various organic acids, industrial surfactants and carotenoids are already produced commercially. However, the challenge remains to improve production efficiency and develop a wider range of biorenewable chemicals and feedstocks using approaches that do not conflict with food production. Here, we explore whether the UK National Collection of Yeast Cultures (NCYC) contains hitherto untapped genes and pathways that could play a role in yeast strain development for such purposes. We describe methodologies for screening the entire NCYC collection to make improved use of agrifood feedstocks such as Oil Seed Rape Straw (OSRS) and comparable bio-waste material.

CHARACTERIZATION OF A NOVEL NON-GMO YEAST FOR FUTURE LIGNOCELLULOSIC BIOETHANOL PRODUCTION

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Concerns about climate change and the uncertainty about future fuel supply make renewable biofuels, such as bioethanol, attractive alternatives to fossil fuels in the short/medium term. Lignocellulosic biomass (for example spruce, wheat straw and corn stover) is an abundant raw material that can be utilized to produce ethanol with the help of a fermenting microorganism. Traditionally the yeast *Saccharomyces cerevisiae* is used for industrial ethanol production. *S. cerevisiae* can be metabolically engineered to consume xylose (the second to glucose most prevalent monosaccharide in lignocellulose). However, despite many years of intensive research, it can still not ferment xylose in a satisfying way which affects the overall ethanol yield negatively. We have isolated a non-genetically modified (non-GMO) yeast species (here called C5-yeast) that has the natural ability to efficiently produce ethanol from glucose and xylose. The aim of the project is to further characterize the growth and fermentation capacities of this novel microorganism to elucidate its' potential for lignocellulosic bioethanol production. We can show that besides glucose and xylose, the C5-yeast can also consume the pentose arabinose and the disaccharide cellobiose; both present in lignocellulosic hydrolysates. The C5-yeast rapidly converts the inhibitory sugar degradation products HMF and furfural formed during the conversion of lignocellulosic material into fermentable sugars.

MALTOTRIOSE UTILIZATION IN BREWER'S YEAST WITH RESPECT TO TRANSPORTER GENE PROVENANCE

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During wort fermentation, transmembrane transport is the rate limiting factor for efficient utilization of maltotriose. Quite often residual maltotriose remains after fermentation, causing economic losses and compromising quality. The lager yeast (*Saccharomyces pastorianus*) taxon apparently arose through interspecific hybridization of an ale (*S. cerevisiae*) and an *S. eubayanus* strain and possesses sugar transporter genes derived from both parents. However, usually only one of the sugar transporter orthologues is functional, whereas the other has gained mutations leading to pseudogenization. Transporters able to carry maltotriose are therefore distributed differently between ale and lager yeast strains as well as between the two subgroups within the lager yeast, namely Saaz and Frohberg. This affects the strains' fermentation capacity, e.g. in ale strains ScAgt1 type transporters, which are efficient maltotriose carriers, dominate, whereas in lager strains of the Frohberg type the presence of Mtt1 and SbAgt1 transporters is crucial for maltotriose utilization. Lager strains of the Saaz type do not possess any functional maltotriose transporters and leave this sugar unfermented. Fermentation capacity of brewer's yeast strains can be significantly improved by modifying characteristics of maltotriose transporters. Characterization of properties of individual maltotriose transporters and their distribution among brewer's yeast is helping to elucidate the factors that limit maltotriose utilization as well as suggesting methods to improve strains for superior performance.

DELIMITATION OF *Meyerozynma guilliermondii* FOOD ISOLATES FOR IMPROVED SAFETY IN FOOD INDUSTRY

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Meyerozyma guilliermondii is a yeast species widely isolated from several natural environments and from fruits. In medical microbiology it is known as the telomorph of the opportunistic pathogen *Candida guilliermondii*, which causes about 2% of the human blood infections. This yeast is promising in a variety of biotechnological applications as vitamins production and post-harvest control. The question if isolates from different sources are physiologically and genetically similar, or if the various environments induced significant differences, is crucial for the understanding of this species structure and to select strains appropriate for each application. This question was addressed using LSU and ITS sequencing for taxonomic assignment, i-SSR ($GACA_4$) for the molecular characterization and FTIR for the metabolomic fingerprint. All data showed that fruit and environmental isolates cluster separately and a general good agreement between metabolomics and molecular analysis. An additional RAPD analysis was able to discriminate strains according to the isolation position within the pineapple fruit. Although all strains are members of the *M. guilliermondii* species according to the current standards, the distribution of large variability detected suggests that some specialization occurred in the niches inhabited by this yeast and that food related strains can be differentiated from the medical isolates.

OPPORTUNITIES ARISING FROM THE GENOME SEQUENCING OF FOUR *Aureobasidium* VARIETIES

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Aureobasidium pullulans is a black yeast from the order Dothideales. The species is of considerable interest due to several reasons: (i) polyextremotolerance and occurrence in numerous habitats from plant surfaces and household dust to coastal ponds of hypersaline water and Arctic glaciers; (ii) many biotechnological applications (production of polysaccharides, enzymes, antimicrobials) and use in agriculture as a biocontrol agent; (iii) emerging medical relevance.

We performed a *de novo* genome sequencing and analysis of the four recognised varieties of the species (*A. pullulans* var. *pullulans*, var. *melanogenum*, var. *subglaciale*, var. *namibiae*). Annotation of the genes was performed with the help of the transcriptome sequencing. The differences between varieties and with other phylogenetically related fungi were analysed on the genomic and (predicted) proteomic levels. The 25.43-29.62 Mb genomes encode between 10266 and 11866 predicted proteins. These include genes for various enzymes involved in the degradation of the plant material (and possibly also of plastic and aromatic compounds) and many sugar transporters. Proteins believed to be involved in the synthesis of the polysaccharide pullulan and of the siderophores are predicted. Putative stress-tolerance genes include those encoding several membrane channels and transporters, melanin synthesis enzymes, all of the components of the high-osmolarity glycerol pathway, and bacteriorhodopsin-like proteins.

The structure of the mating-type locus is homothallic, although no sexual reproduction has been observed in this species before. The differences between the genomes are large enough to justify their redefinition as separate species.

The availability of the genomic sequence of *A. pullulans* is expected to facilitate research into this interesting species and promote its use in the fields of biotechnology and agriculture.

DEVELOPMENT OF MICROBIAL CELL FACTORIES FOR BIOREFINERIES

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To build an energy and material secure future, a next generation of renewable fuels produced from lignocellulosic biomass is required. Although lignocellulosic biomass, which represents an abundant, inexpensive and renewable source for bioethanol production, is of great interest as a feedstock, the complicated ethanol production processes involved make the cost of producing bioethanol from it higher compared to corn starch and cane juice. Therefore, consolidated bioprocessing (CBP), which combines enzyme production, saccharification and fermentation in a single step, has gained increased recognition as a potential bioethanol production system. CBP requires a highly engineered microorganism developed for several different process-specific characteristics. The dominant strategy for engineering a CBP biocatalyst is to express multiple components of a cellulolytic system from either fungi or bacteria in the yeast Saccharomyces cerevisiae. The development of recombinant yeast strains displaying cellulases and hemicellulases on the cell surface represents significant progress toward realization of CBP. Regardless of the process used for biomass hydrolysis, CBP-enabling microorganisms encounter a variety of toxic compounds produced during biomass pretreatment that inhibit microbial growth and ethanol yield. Systems biology approaches including disruptome screening, transcriptomics, and metabolomics have been recently exploited to gain insight into the molecular and genetic traits involved in tolerance and adaptation to the fermentation inhibitors. In this review, we focus on recent advances in development of yeast strains with both the ability to directly convert lignocellulosic material to ethanol and tolerance in the harsh environments containing toxic compounds in the presence of ethanol.

BREEDING INDUSTRIAL *Saccharomyces cerevisiae* STRAIN FOR LIGNOCELLULOSIC ETHANOL PRODUCTION

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Using an industrial *Saccharomyces cerevisiae* strain as the parental strain, strain for lignocellulosic bioethanol production was constructed. By introducing *XYL1, XYL2,* and *XKS1* genes, strain with xylose-fermenting ability was constructed. The strain could grow using xylose as the sole carbon source. Under normal batch fermentation condition, 50 g/L of xylose could be consumed within 36 h by the strain. By overexpression of *HXT7* and *GXS1* genes, xylose consumption rate of the strain was improved. Continuous fermentation fed with 75 g/L of xylose kept stable at a dilution rate of 0.1 h^{-1}. The xylose-fermenting capacity maintained stable during one-year continuous operation. By site directed mutagenesis, the affinity of xylose reductase to NADH was increased and strains with improved xylose consumption and ethanol yield were obtained. Tolerance of these strains to inhibitors (weak acids, furans, aldehydes, phenols) generated during pretreatment process was assessed systematically. The inhibitor-tolerance of strains was improved by overexpression of key genes in PPP pathway and acclimation under inhibitor-stressed conditions.

DETOXIFICATION AS A STRATEGY FOR DEVELOPING TOLERANCE IN *Saccharomyces cerevisiae* TO PHENOLIC COMPOUNDS

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Several phenolic compounds are formed as products of lignin breakdown during pretreatment of lignocellulosic biomass. These phenolic compounds are inhibitory to cell growth and function as biocatalysts in the production of second generation biofuels from degraded lignocellulosic biomass. Our research is focused on developing a *Saccharomyces cerevisiae* strain with improved resistance to phenolic compounds.

As part of our study, we have focused on understanding the ability of *S. cerevisiae* to tolerate and convert phenolic compounds. We aim to understand the conversion mechanisms of phenolic compounds and adapt the knowledge to the engineering and use of *S. cerevisiae* on a biotechnological platform for bioethanol production and prospective, novel bio-based chemicals.

We have investigated toxicity of various phenolic compounds against *S. cerevisiae.* Our results showed that phenolic compounds have varied toxicity against *S. cerevisiae* and the toxicity may be dependent on the structure of the compound involved. Under aerobic batch cultivation conditions, we have also studied the conversion of phenolic compounds by *S. cerevisiae* using coniferyl aldehyde, ferulic acid and *p*-coumaric acid as representative phenolic compounds. We compiled a list of conversion products of the three starting compounds under investigation and we proposed a possible conversion pathway, currently being investigated.

In this talk, we present the proposed conversion pathway through which *S. cerevisiae* converts and detoxifies coniferyl aldehyde, ferulic acid and p-coumaric acid under aerobic cultivation condition.

ENGINEERING GLUTATHIONE BIOSYNTHESIS TO ENHANCE REDOX ROBUSTNESS OF *Saccharomyces cerevisiae*

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The focus for biofuel production shifts to using lignocellulose biomass from forest and agricultural by-products since it does not compete with food and feed production. Polysaccharides must be pretreated to be made accessible to hydrolytic enzymes to generate monomeric sugars for the following fermentation. In this pretreatment step inhibitors of fermenting microorganisms are generated, mainly furan derivates, weak acids and phenolics. Although *Saccharomyces cerevisiae* is more robust than bacteria, there is demand for improvement and the development of novel yeast strains with increased inhibitor tolerance is highly desirable.
Furan derivates and other inhibitors have been shown to induce the formation of reactive oxygen species. Engineering of the redox metabolism of *S. cerevisiae* in terms of increasing the intracellular levels of glutathione by overexpressing glutathione synthetase *GSH1* resulted in increased strain robustness in a simultaneous saccharification and fermentation (SSF) process. Cell survival and final ethanol concentrations were increased in the recombinant strains compared to the wild type in industrial media [Ask et al. 2013].
To show a correlation between the intracellular concentration of glutathione and the resulting effect on robustness, strains accumulating different amounts of glutathione will be created. GshF is a bi-functional enzyme found in several bacterial species, that catalyzes the formation of glutathione from its precursors without accumulation of the intermediate product γ-glutamylcysteine and without any relevant feedback inhibition. *GshF* will be overexpressed in a CEN.PK strain, followed by deletion of the native GSH1 and GSH2 enzymes catalyzing the two-step reaction in *S. cerevisiae*.

Reference:
Ask, M., Mapelli, V., Höck, H., et al., 2013, Microbial Cell Factories 12, 87-97.

Candida glabrata SHOWS VERY HIGH RESISTANCE FOR HIGHLY CONCENTRATED SULFURIC ACID AND THE TOLERANCE IS USEFUL FOR ETHANOL PRODUCTION

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Candida glabrata, an imperfect fungus, resembles *Saccharomyces cerevisiae* in morphology and gene sequencing. However, *C. glabrata* is only a haploid cell and is unable to mate with another. It is known that *C. glabrata* is yeast bringing opportunistic infection. There are increasing concerns over *Candida*, because it causes not only mucocutaneous but also systemic infections in transplant and immunosuppressed patients. The other side of *C. glabrata*, is a superior strain for fermentative production of pyruvate.

C. glabrata has loud fermentation power and is very useful industrially. The growth rate is extremely fast in comparison with *S. cerevisiae*. *C. glabrata* can also grow under high temperature condition (42-44 degrees). Furthermore, surprisingly *C. glabrata* shows strong tolerance for the sulfuric acid. When the ethanol is made from cellulose, highly-concentrated sulfuric acid is used for saccharification of the cellulose. We found that *C. glabrata* has tolerance to strong sulfuric acid, and at the same time, increase of the production of ethanol efficiency was seen. Furthermore, the *C.glabrata* can produce ethanol without being neutralized using the cellulose which I saccharified with strong sulfuric acid.

CHALLENGES AND POSSIBILITIES WITH HIGH SOLIDS LIGNOCELLULOSE FERMENTATION

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One current research trend in lignocellulose based ethanol production is to increase the solid substrate loading in order to increment the final ethanol concentration, leading to decreased distillation costs and improved water economy. Even though several challenges have to be addressed, this is an important research direction with broad implications towards development of lignocellulose based biorefineries.

At high solids loading of typically 15-30 (w/w) %, high viscosity prevails leading to poor mixing and therefore large deviation occur in local conditions in the fermentor. The high solid content has a direct influence on the enzymes and microorganisms. Furthermore, high concentrations of inhibitory compounds will typically be present leading to poor cellular performance and cell viability problems. In our research group we address these problems by gaining detailed physiological insights, followed by developing fermentation strategies and applying strategies for strain improvement. In this presentation, insights from physiological behavior at high solids condition will be discussed in light of designing fermentation strategies suitable for high solid conditions. We have in particular focused on how to increase the cell viability in lignocellulose based processes and to gain understanding on the influence of solid material on cellular performance.

Yarrowia lipolytica – USEFUL TOOL FOR ENGINEERING LIPID BIOSYNTHESIS PATHWAYS

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Yarrowia lipolytica is oleaginous yeast with metabolism adapted for life in protein and oil rich environments. It possesses protease and lipase activities that enable it to utilize such substances as a source of nutrients and energy. On the other hand *Y. lipolytica* lacks amylolytic and invertase activities, which are common for other microorganisms from Fungi kingdom. Its orientation towards hydrophobic substrate utilization is underlined by presence of both *DGAT1* and *DGAT2* enzymatic activities. This makes that *Y. lipolytica* is a yeast with unique storage lipid formation machinery. Taking an advantage of *Y. lipolytica*'s natural ability of lipid accumulation strains with improved substrate flow towards lipid biosynthesis and storage were constructed. In this study strains derived from Q4 strain (Δ*dga1* Δ*dga2* Δ*lro1* Δ*are1*) were constructed. Because Q4 strain is unable to synthesize storage lipids it provides us a good platform for studying impact of single acyltransferase gene on lipid synthesis. Thus was shown that proteins encoded by *DGA1* and *DGA2* genes have major TAG forming potential. Both proteins are able to mediate accumulation of significant quantities of TAGs in yeast cells, but with slightly different fatty acid composition. Based on this findings potential single cell oil producing strains were constructed. These strains were constructed from Q4 by reintroduction of either *DGA1* or *DGA2* under TEF promoter. Newly constructed strains accumulated lipids up to 50% of biomass, which was more than two times more compared to wild type. By heterologous expression of *SUC2* from *Saccharomyces cerevisiae* an invertase activity was introduced into *Y. lipolytica* which enables it to grow on sucrose based substrates such as molasses.

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OPTIMIZATION OF CULTIVATION CONDITIONS FOR LIPID PRODUCTION WITH A GENOME-SCALE MODEL OF *Yarrowia lipolytica*

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In recent years *Yarrowia lipolytica* has become an important non-conventional yeast for basic research and for biotechnologically relevant processes. As an oleaginous yeast, it might be used for single cell oil production. Besides, it is a good host for the production of citric acid and other intermediates of the tricarboxylic acid cycle. The availability of a sequenced genome and tools for genetic engineering are promising even greater possibilities for usage of this GRAS species in metabolic engineering. By using a validated genome scale model of baker's yeast as a scaffold and information from two recent network reconstructions of *Y. lipolytica*, we reconstructed a new metabolic network of this yeast, named iMK799, that describes the metabolism of *Y. lipolytica* very accurately. After qualitative validation, we used flux balance analysis (FBA) to confirm the accuracy of our model regarding growth performance on glucose and glycerol and product formation kinetics for lipid and citrate. Experimental and simulated growth rates differed only by 8% and 1.6% on glucose and glycerol, respectively. In addition, dynamic FBA showed good correlation of biomass production with fermentations. We simulated oxygen limitation conditions and formation of citrate and designed two new fermentation strategies to induce lipid accumulation. As for the growth characteristics, computational predictions and experimental results for neutral lipid synthesis were in good agreement. We expect that further optimization of fermentation techniques together with metabolic engineering strategies will additionally enhance lipid accumulation, provide a better understanding of lipid metabolism in *Y. lipolytica* and will contribute to the development of cost efficient strategies for lipid production for biodiesel.

METABOLIC ENGINEERING TO MAXIMIZE LIPID CONTENT IN *Yarrowia lipolytica*

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The oleaginous yeast *Yarrowia lipolytica* is an attractive non-conventional yeast that is used in basic research to study dimorphism and lipid metabolism. In addition, it is intensively investigated for its potential in industrial applications such as bioremediation, production of single cell protein, single cell oil and citric acid. It is known that, under optimal growth conditions *Y. lipolytica* can store about 36-40% neutral lipid in the form of triacylglycerol (TAG). The trans-esterification of TAG yields alkyl esters and glycerol, wherein alkyl esters can be used as major biodiesel component. The development of a genome scale model (GSM) and the available genetic tools provide the possibility to exploit *Y. lipolytica* as a platform for the production of TAG by metabolic engineering of the required pathways. The aim of our research is to optimize and maximize TAG content in this yeast. The classical approach is to target the *de novo* lipid synthesis pathways. In addition, channeling carbon flux towards lipid synthesis by engineering upstream pathways has a potential to achieve higher lipid storage, as predicted by computational simulations with a GSM of *Y. lipolytica*. The first targets are metabolites from glycolysis. During glucose catabolism, some carbon flux is also channeled to the formation of storage bodies. We eliminated this pathway to achieve a higher carbon flux into the tricarboxylic acid cycle for biomass and/or *de novo* lipid synthesis. Further modifications to increase the production of precursor metabolites for the *de novo* lipid synthesis pathway as well as the deletion of genes coding for TAG lipases and the β-oxidation pathway will result in an additional improvement of lipid content in *Y. lipolytica*. Hence, the engineered strains will be optimized for maximum lipid yield on glucose or glycerol as carbon source.

THE INFLUENCE OF MEMBRANE COMPOSTION ON ACETIC ACID PERMEABILITY AND POTENTIALLY ACETIC ACID TOLERANCE

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Compounds entering the cell do so either by passive diffusion over the plasma membrane or through transporters in the membrane. The specific lipid composition of the plasma membrane influences both the passive diffusion rate but also the activity of membrane proteins. Acetic acid, a major hurdle in fermentation processes using lignocellulosic material, is believed to pass through the membrane in its protonated from mainly by passive diffusion [1].
Sterols and sphingolipids are lipid classes thought to contribute to membrane rigidity. Sterols are often found to be involved in stress resistance [2, 3] and in our previous work sphingolipids were pointed at as an important constituent of the plasma membrane of the yeast *Zygosaccharomyces bailii*, known to be very tolerant to acetic acid, suggesting a possible link between acetic acid tolerance and sphingolipid relative abundance in the membrane [4].
Here we will provide supporting evidence of the importance of sphingolipids and sterols in acetic acid membrane permeability. We have combined biochemistry techniques with *in silico* membrane modeling to answer the question how membrane engineering can be used to decrease acetic acid membrane permeability.

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Tum1 AFFECTS LIPID METABOLISM IN *Saccharomyces cerevisiae*, HOWEVER NOT THROUGH tRNA THIOLATION

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Most, but not all genes involved in lipid metabolism have been functionally characterized. Our research group has been studying new genes that regulate lipid metabolism in *S. cerevisiae*. We found decreased amount of triacyglycerols (TAG) and increase of sterol esters (SE) in *tum1*Δ deletion mutant. The only hitherto known biological role of Tum1 is that it is one of the five proteins in the tRNA wobble uridine modification pathway. Three tRNAs specific for Gln (tQ^{UUG}), Lys (tK^{UUU}), and Glu (tQ^{UUC}) have the 2-thiouridine derivative mcm^5s^2U as the wobble nucleoside. Tum1 is not essential for this thiolation, but has been described as a component involved in this pathway. We showed that in *tum1*Δ strain a small fraction of tRNAs still remained thiolated, whereas no thiolation of tRNA was observed in the control *uba4*Δ strain. Uba4 is downstream of Tum1 in the pathway and is essential for tRNA 2-thiolation. This result indicates that not the whole sulfur flux passes through the Tum1-mediated pathway branch. The amount of TAG and SE in *uba4*Δ cells was the same as in the wild type strain, indicating that the role of Tum1 in tRNA modification is not linked to its role in lipid metabolism. To test this further, we over-expressed tK^{UUU}, tE^{UUC} and tQ^{UUG} which have been shown to partially suppress the inhibitory effect of the lack of mcm^5s^2U in these tRNAs in *uba4*Δ strain. Elevated levels of these three tRNA did not suppress the *tum1*Δ lipid phenotype. We thus conclude that Tum1 affects lipid metabolism through another, as yet unknown pathway, and thus has an additional molecular function that remains to be discovered.

Blastobotrys (*Arxula*) *adeninivorans* – A SUITABLE BIOCATALYST FOR NEW BIOTECHNOLOGICAL PRODUCTS

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The industrially important yeast *Blastobotrys (Arxula*) *adeninivorans* is an asexual hemiascomycete phylogenetically very distant from *Saccharomyces cerevisiae*. Its unusual metabolic flexibility allows it to use a wide range of carbon and nitrogen sources, while being thermotolerant, xerotolerant and osmotolerant. Based on the completely sequenced and annotated *Arxula* genome combined with gene expression data, numerous so far non-described pathways in yeasts were explored and exploited such as the metabolism of n-butanol, tannic acid and purine. The obtained data provide new knowledge to the exceptional broad substrate spectrum and robustness of this yeast. In addition *A. adeninivorans* is used as suitable host for synthesis of special products such as recombinant functional human receptors or glycosylated secretory tannases and it serves as a suitable biocatalyst for the synthesis of biotechnologically interesting products such as n-butanol and polyhydroxyalcanoate (PHA), because all essential prerequisites and components for heterologous gene expression are available. A lot of special protocols have been established (transformation/expression platform Xplor®2, gene disruption, protoplast fusion, mitotic segregation) and industrial strains were constructed. These strains are suitable producers of enzymes and enzyme mixes to degrade plastic material and lignocellulose (1), to synthesize enantiometrically pure alcohols (2) and to produce food with low purine content (3). Other biotechnological application fields for *Arxula* cells are bioremediation by accumulation of metal ions and production of biobutanol. Furthermore *A. adeninivorans* is used as microbial sensor compound to detect hormone activities (estrogenic, androgenic, glucocorticoidic, gestagenic activities), dioxins as well as pharmaceuticals in tap water, mineral water, waste water, urine and blood serum.

BLACK YEAST *Aureobasidium pullulans*: A SOURCE OF GENES FOR THE IMPROVEMENT OF INDUSTRIALLY IMPORTANT TRAITS OF *Saccharomyces cerevisiae*

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Black yeast *Aureobasidium pullulans* (de Bary) G. Arnaud is characterized by an exceptional stress tolerance to a wide variety of stresses (*i.e.* polyextremotolerance), such as hypersaline, acidic, basic, cold and oligotrophic. It is of substantial biotechnological importance (production of pullulan, enzymes, antimicrobials) and has a potential for numerous novel applications in medicine, pharmacy, the food industry, and other fields. The recent availability of genomes of four *A. pullulans* varieties opened exciting new possibilities for harvesting its rich resources. For example it offers an opportunity for a fresh approach to improving the stress tolerance of *S. cerevisiae*. In this study, we have cloned selected genes (cation transporters, extracellular enzymes, and metabolic enzymes) from *A. pullulans,* expressed them in *S. cerevisiae* and finally evaluated the performance of the transformants at increased concentrations of salts or other relevant conditions. Yeast strains with improved capability to degrade complex substrates and/or with improved stress tolerance will be valuable to biofuel industry and agriculture.

OPTIMIZATION OF GLUCOSE OXIDASE PRODUCTION BY RECOMBINANT *Kluyveromyces lactis*

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Successful production of recombinant protein does not rely on genetic engineering alone, and often requires the optimization of fermentation processes. Here, we describe the conjugate effect of both approaches on the heterologous production of *Aspergillus niger* glucose oxidase (GOD) by *Kluyveromyces lactis*. The secretion signal peptide of KlPho5p led to a higher secretion of GOD compared to that of the □-subunit of zymocin complex. The control of fermentation processes had an extended influence on GOD production. Indeed, contrary to a complex broth with any fermentable carbon source, a selective mineral medium with lactose greatly increased the yield of GOD production from 18.9 to 341.6 U g DW^{-1} in flask cultures. The optimization of fermentation processes in bioreactor led to 501.8 U g DW^{-1} of GOD production. The control of *K. lactis* growth rate has proven essential to improve GOD secretion.

INTERACTION OF *Pseudomonas fluorescens* AND DIFFERENT SPOILAGE YEASTS

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Metabolic activity of microorganisms plays an important role in food spoilage. Bacteria predominate mainly in the food spoiling microbiota but yeasts are also present. Population dynamics of the bacteria during storage is well documented, though less is known about the behaviour of yeast species. Investigation about interactions of bacteria and yeasts during spoilage is sparse. Our aim was to study the interaction of *Pseudomonas fluorescens* of different origin and yeast strains isolated from spoiled food. Several strains of the tested *P. fluoerscens* inhibited the growth of certain yeast species but the antagonistic effect was strain dependent. Isolates of *Rhodotorula mucilaginosa* and *Cryptococcus curvatus* were highly sensitive to the effect of *P. fluoerscens*, while the growth of *Metschnikowia pulcherrima* strains was not affected by this bacterium. Testing the influence of yeasts on the growth of *P. fluorescens* only *M. pulcherrima* had inhibiting activity. When co-culturing of *P. fluorescens* and *M. pulcherrima* was performed we observed that the inhibitory effect of *M. pulcherrima* was still detectable if it was inoculated in two orders of magnitude smaller than the bacterium. We concluded that the antagonistic effect is due to some extracellular metabolic products because cell free supernatants showed also inhibition. Although *M. pulcherrima* can produce low amount of ethanol, *P. fluorescens* was not sensitive to this alcohol content. It has already been published that both *Pseudomonas* and *M. pulcherrima* can produce iron chelating compounds (siderophores and pulcherrimin, respectively); therefore we tested the effect of elevated iron concentrations on the interaction. In case of iron excess the inhibitory effect of *M. pulcherrima* against *P. fluorescens* abolished, which indicated that iron availability is one of the key factors in the interaction. Acknowledgement: This work was supported by the Hungarian project OTKA 101716.

IS β-CAROTENE INVOLVED IN THE ANTIOXIDANT RESPONSE OF CAROTENOGENIC YEASTS? AN ANSWER FROM THREE *Rhodotorula spp*. STRAINS

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Red yeasts ascribed to the genus *Rhodotorula* produce carotenoids, mainly β-carotene, torulene and torularhodin. The production of β-carotene may restore hydrogen peroxide resistance in yeast cells lacking cytosolic catalase. However, β-carotene works as a prooxidant in the presence of high oxygen concentrations and determines an increase in the sensitivity to reactive oxygen species when it accumulates inside yeast cell. Thus β-carotene ROS scavenging activity *in vivo* is rather controversial. Here, to further elucidate the involvement of β-carotene in yeast cellular response to oxidative stress, a wild type strain of *Rhodotorula* spp. (C2.5t1) and two of its primary mutants, that differ in the amount and type of carotenoids produced, were treated with H_2O_2 without and with diphenylamine (DPA), a known inhibitor of phytoene desaturase and the progression of carotenoids biosynthesis. While the parental strain C2.5t1, that produces the three main carotenoids, and the colorless mutant 200A6, unable to produce β-carotene, torulene and torularhodin, showed comparable resistance to H_2O_2, the β-carotene overproducing mutant 400A15 was more sensitive to this prooxidant. DPA-mediated inhibition of carotenoids biosynthesis restored growth of the three strains in the presence of H_2O_2 and resulted in a decrease in the intracellular content of superoxide anion, also in the albino mutant 200A6. H_2O_2 treatment determined comparable oxidative damage to proteins in the three strains, in spite of the different carotenoid content. In addition catalase activity induced by H_2O_2 was comparable in cells grown without and with DPA. Thus that β-carotene, but also torulene and torularhodin, do not seem to be directly involved in H_2O_2 detoxification in *Rhodotorula* spp..

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DIFFERENTIAL PROTEOMIC ANALYSIS OF RHODOTORULA SPP. STRAINS FOR THE IDENTIFICATION OF ENZYMES INVOLVED IN CAROTENOGENESIS

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With the aim of identifying the enzymes involved in red yeasts carotenogenic pathway, a wild strain of *Rhodotorula* spp. (C2.5t1) and two mutants (400A15 and 200A6), were subject to comparative proteomic analysis by means of 2D-DIGE. These three strains differ in the amount and type of carotenoids produced. In particular, C2.5t1 produces β-carotene, torulene and torularhodin, the yellow mutant 400A15 overproduces β-carotene, and the colorless mutant 200A6 does not produce any of these carotenoids. Comparison of the proteomic maps of the three strains after 24 growth on glycerol containing medium, highlighted 38 spots as differentially abundant in the three strains. Spots showing a higher abundance in C2.5t1 and/or 400A15 in respect to 200A6 were analyzed by Tandem Mass Spectrometry and seven different databases were utilized for their identification. Sixteen peptide sequences were identified upon database searching and blastp alignment as likely belonging to carotenogenic enzymes; unfortunately, however, no more than one sequence per enzyme could be detected, possibly due to differences in the amino acid sequences of these proteins and those available in the databases utilized for the identification. In perspective, the recent publication of the genome of *Rhodorula glutinis* and the assembly and the annotation of C2.5t1 genome is expected to provide useful tools to re-analyze the dataset generated by this study. Such analysis could result in a significant increment in the number of identifications as already experienced by other authors on the red yeast *Rhodosporidium toruloides* (Zhou et al. 2012, Nature Communications 3:1112).

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Rhodotorula glutinis AS A MODEL FOR STUDY OF CAROTENOIDS AND LIPID BIOSYNTHESIS

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Rhodotorula glutinis as an oleaginous yeast accumulates large quantity of lipids. *R. glutinis* is also known as a producer of various pigments, especially β-carotene and unusual torulene and torularhodin. Carotenoids are natural antioxidants with significant immunostimulatory and anticancer effects with the capacity to protect cells against free oxygen and peroxide radicals. These benefits allow their widespread application in pharmaceutical, food and feed industry. Due to high demand for these biologically active molecules, the work was focused on mechanisms of carotenoid overproduction by yeasts. One of the main physiological target for regulation of pigment accumulation is selection of optimal C:N ratio of the cultivation medium. Therefore, four different C:N ratios, 20:1, 50:1, 70:1 and 100:1 have been applied to study their impact on carotenoid biosynthesis and profile. In addition, further important factor for pigment production is carbon starvation. It was found that yeasts after complete carbon assimilation utilized storage lipids as an energy sources and converted fatty acids into acetyl-CoA via the β-oxidation. Such acetyl-CoA served as a substrate for pigment biosynthesis in mevalonate pathway. C:N ratio 50:1 was found to be optimal for the highest carotenoid yield (21.0 mg/L) and their accumulation in cells (1.9 mg/g cells). It should be noted that β-carotene and torularhodin were the main pigments during the first growth stage. However pigment profile was rapidly changed during carbon starvation where rapid accumulation of □-carotene and torulene was detected. Moreover, the yeast was able to accumulate over 30% storage lipids in cells (4.3 g/L) that consisted of mainly oleic and palmitic acids.

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POPULATION GENOMICS OF *Saccharomyces cerevisiae* HUMAN ISOLATES: PASSENGERS, COLONIZERS AND INVADERS

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The quest for the ecological niches of *Saccharomyces cerevisiae* has led to research in areas as diverse as wineries, oak and more recently the gut of *Crabro* wasps. Here we propose the role of the human gut in shaping *S. cerevisiae* evolution, presenting the genetic structure of a previously unknown population of yeasts, associated with Crohn's disease, providing evidence for clonal expansion within the human gut. To understand the role of immune function in human-yeast interaction we classified strains according to their immunomodulatory properties, discovering two sets of isolates: the first inducing anti-inflammatory signals via regulatory T cell proliferation and the latter, made up of strains with a mosaic genome, eliciting inflammatory, IL-17 driven immune response. The approach integrating genomics with immune phenotyping showed selection in genes involved in sporulation and cell wall remodeling to be central in the evolution of *S. cerevisiae* Crohn strains from passengers to commensals and then to pathogens.

POPULATION GENOMICS REVEALS THE EVOLUTIONARY FATE OF A LARGE-SCALE INTROGRESSION IN A PROTOPLOID YEAST

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Yeast species represent an ideal model system for population genomics but large-scale polymorphism surveys have only been reported for two closely related yeast species, *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* so far. Hence, little is known about intraspecific diversity and evolution in yeast. To obtain a new insight into the evolutionary forces shaping natural populations, we sequenced the genomes of a expansive worldwide collection of isolates of a distant species relative to *S. cerevisiae*: *Lachancea kluyveri*. We identified 6.5 million SNPs and showed that a large introgression event of 1-Mb of GC-rich sequence in the chromosomal arm occurred in the last common ancestor of all *L. kluyveri* strains. Our population genomic data clearly revealed that the introgressed region underwent a molecular evolution pattern very different from the rest of the genome. It is characterized by a higher recombination rate, with a dramatically increased A:T→G:C substitution rate, which is the signature of a increased GC-biased gene conversion. The presence of two distinct recombination and substitution regimes within the same genome demonstrates that the chromosome-scale compositional heterogeneity will persist after the genome has reached mutational equilibrium. Altogether, the data presented herein suggest that large DNA introgressions resulting from interspecific hybridizations lead to different evolutionary patterns within a given genome.

DISTINCTIVE CHROMOSOMAL END LOSS AND LAGER SPECIFIC GENES DEFINE TWO DIFFERENT *Saccharomyces cerevisiae* LINEAGES FOR *S. pastorianus* GROUP I AND II STRAINS

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The lager yeast species *Saccharomyces pastorianus* is a natural hybrid arising from the fusion of two parental species, a *Saccharomyces cerevisiae-like* yeast and the newly discovered *Saccharomyces eubayanus*. Genome sequencing of the prototype Group I lager yeast strain, Weihenstephan 34/70, identified eight telomere-associated lager-specific (LgS) genes that were not present in other *Saccharomyces* species. We re-evaluated the origins of the LgS genes through bioinformatic analysis of 32 sequenced *Saccharomyces* genomes combined with PCR analysis of a further 48 un-sequenced strains. Nearest neighbour and SNP analyses confirm that the *S. cerevisiae* portion of the lager yeast genome originates from Ale-like yeasts. The analysis also revealed that four of the LgS genes originate from *S. cerevisiae* while the other four originate from *S. eubayanus*. Furthermore, a pattern of presence or absence of the LgS-genes was identified within the *S. cerevisiae* strains. We analysed the sub-telomeric regions of all 16 *S. cerevisiae*-like chromosomes in the *S. pastorianus* genomes and in the 32 sequenced *S. cerevisiae* strains and identified a pattern of loss of chromosome ends that allows distinction between Group I and II lager yeasts and between *S. cerevisiae* strains. Based on this analysis we identify two distinct classes of Ale yeasts with patterns of LgS-gene loss and chromosome end loss identical to *S. pastorianus* Group I and II yeasts respectively. These findings lead us to speculate that *S. pastorianus* Group I and II strains originated from separate hybridisation events with two distinct Ale yeasts. Using the combined bioinformatics and PCR data, we describe a potential classification map for lager, wine, distillers, coconut, laboratory and sake yeasts.

GENOME SEQUENCE OF *SACCHAROMYCES CARLSBERGENSIS,* THE WORLD'S FIRST PURE CULTURE LAGER YEAST

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Lager yeast beer production was revolutionized by the introduction of pure culture strains. The first established lager yeast strain is known as the bottom fermenting *Saccharomyces carlsbergensis*, which was originally termed *Unterhefe* No.1 by Emil Chr. Hansen and used in production in since 1883. *S. carlsbergensis* belongs to group I/Saaz-type lager yeast strains and is better adapted to cold growth conditions than type II/Frohberg-type lager yeasts, e.g. the Weihenstephan strain WS34/70. Here, we determined the draft genome sequence of *S. carlsbergensis* based on Illumina sequencing of 8 kb mate-pair libraries. Lager yeasts such as *S. carlsbergensis* are descendants from hybrids formed between a *S. cerevisiae* parent and a parent similar to *S. eubayanus*. Accordingly, the *S. carlsbergensis* 19.5 Mb genome is substantially larger than the 12 Mb *S. cerevisiae* genome. Based on the sequence scaffolds, synteny to the *S. cerevisae* genome, and by using directed PCRs for gap closure we generated a chromosomal map of *S. carlsbergensis* consisting of 29 unique chromosomes. We present evidence for genome and chromosome evolution within *S. carlsbergensis* via chromosome loss and loss of heterozygosity specifically of parts derived from the *S. cerevisiae* parent. Based on our sequence data and via FACS analysis we determined the ploidy of *S. carlsbergensis*. This inferred that this strain is basically triploid with a diploid *S. eubayanus* and haploid *S. cerevisiae* genome content. In contrast the Weihenstephan strain is essentially tetraploid composed of two diploid *S. cerevisiae* and *S. eubayanus* genomes. Evolutionary implications for type I and type II lager yeasts will be discussed.

ROLE OF CHROMOSOMAL INVERSIONS ON YEAST FITNESS AND GENE EXPRESSION

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The non-random gene organisation in eukaryotes is thought to play an important role in genome evolution and function, however the effect of rearrangements on global transcription and fitness is still controversial and not yet fully understood. To investigate the effect of gene order and centromere location on phenotype, fertility and global gene expression, we engineered 16 *S. cerevisiae* strains carrying different pericentric and paracentric inversions between TY1 elements, since such repetitive regions are a natural substrate for rearrengments. We found that four inversions were lethal, while the others did not have a detectable impact on mitotic fitness. In meiosis spore viability didn't correlate with either size or type of inversion, however lower fertility was seen in heterozygote carriers with 2 or 3 recombination hotspots present in the inverted region. The amount of transcriptional changes varied among the strains and altered expression was observed throughout the whole genome rather than being over-represented within the inversions or around the breakpoints. However, in the strain XIV-inv, the chromatin remodeling gene *ARP5*, positioned near the inversion break point, was significantly up-regulated. This strain showed the largest expression changes and an up-regulation of genes involved in nuclear pore complex. Interestingly, such genome-wide transcriptional alteration was not translated in a detectable fitness change, highlighting the robustness of the expression network in yeast and supporting the notion that transcriptome rewiring may compensate for changes introduced by rearrangements in order to maintain a constant phenotype.

EVOLUTIONARY IMPACT AND ENVIROMENTAL ADAPTATION OF NATURAL YEAST ISOLATES BEARING KARYOTYPIC REARRANGMENTS

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Yeast is a powerful eukaryotic model organism that has been studied extensively and successfully in the cell biology and physiology fields as well as in evolution studies, population genetics and speciation. *Saccharomyces* '*sensu strico'* species are reproductively isolated via post-zygotic barriers; hybrids are readily formed but they are sterile upon meiosis. The major reproductive isolation barrier is due to sequence divergence and the inability of homologous chromosomes to pair up in meiosis. Surprisingly, *Saccharomyces paradoxus* and *Saccharomyces cariocanus* have low sequence divergence but are two different species. They differ in their genome by four chromosomal translocations and it is possible that such rearrangements are the cause of the very low spore viability. In fact, hybrid heterozygote for just one translocation produces only 50% of viable progeny. This project aims to understand the impact chromosomal translocations have on reproductive isolation and fitness in these two yeast isolates. Two out of the four chromosomal translocations present in *S. cariocanus* were created in *S. paradoxus* YPS138 strain to render this part of the genome collinear to *S. cariocanus* by PCR-mediated gene replacement system and Cre-*loxP* mediated recombination event. Translocated and non-translocated *S. paradoxus* strains were crossed with *S. cariocanus.* Hybrids were selected on minimal medium and meiosis was induced to score fertility. Our results showed that there is significant increase in the total spore viability from 3.3 % in the engineered hybrids carrying 4 translocations (4T) to 12% in the engineered hybrids carrying 2 heterozygote translocations (2T) ($p<0.05$). While the 3T engineered hybrids shows an intermediate spores viability (6.9%). These results suggested that chromosomal translocation could be the source behind the reproductive isolation between *S. paradoxus* and *S. cariocanus* species. However, from these data the expectation for complete collinear crossing could be around 40%, indicating that multiple factors may contribute to spore viability.

EVOLUTIONARY EFFECT OF MITOCHONDRIALLY ENCODED PROTEINS ON GLOBAL GENE RETENTION IN YEAST HYBRIDS

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In eukaryotes, mitochondria take important roles in aerobic respiration, apoptosis and cellular metabolism of several metabolites such as fatty acids, amino acids and heme. Mitochondria DNA is inherited since it cannot be synthesised anew. In yeast hybrids, it has been observed that mitochondrial inheritance is uniparental that only the mitochondrial DNA of one parent is maintained. This is perhaps to avoid incompatibility which reduces overall fitness. The mithocondrion is composed mainly by nuclear-encoded proteins, and there are only 8 mitochondrial genes. Here we investigate the impact of different types of mitochondria on the transcriptome in interspecific *Saccharomyces* hybrids. We want to understand whether the expression of the nuclear parental alleles changes according to the mitotype. We produced independent lines of hybrids between *S. cerevisiae* strain BY4741 and *S. uvarum* strain NCYC2669 that possess a single type of mitochondria: either *S. cerevisiae* or *S. uvarum* mitochondria. The hybrids were cultured in rich YPD medium or YP medium with glycerol and at two different temperatures (28C and 16C). Their total RNAs were extracted for transcriptome analysis by sequencing on Illumina platform. Preliminary results in YP-glycerol at 28C shows that the twice as many *S. cerevisiae* alleles changes expression in the hybrids containing *S. uvarum* mitochondria compared to the hybrids with S. cerevisiae mitochondria. Further experiments are on-going to study interactions and incompatibilities between nucleus and mitochondria in different nutritional media and temperatures.

POLYGENIC ANALYSIS OF LOW PH TOLERANCE IN *Saccharomyces cerevisiae var. boulardii*

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Saccharomyces cerevisiae var. boulardii (*S. boulardii*) is a probiotic yeast strain. The ability of *S. boulardii* to withstand low pH environments better than other *S. cerevisiae* strains has been demonstrated through various studies and this phenotype is believed to contribute directly to *S. boulardii's* ability to act as a probiotic. Our results have also confirmed the superiority of *S. boulardii* strains in terms of tolerance to extreme low pH environments, when compared to *S. cerevisiae*. To date no genetic element has clearly been linked to this phenotype in *S. boulardi.* A possible bottleneck that may hamper genomic studies to unravel the genetic basis of low pH tolerance in *S. boulardii* is its inability to sporulate. We have obtained results indicating that an *S. boulardii* strain, isolated directly from a pharmaceutical product, becomes mating competent through overexpression of an early sporulation gene. Through propidium iodide staining and flow cytometry, we have established the ploidy of the mating competent cells as diploid. However, being mating competent, we have succeeded in generating a triploid hybrid strain after crossing our *S. boulardii* with ER7A, a haploid low pH sensitive strain isolated from a bioethanol production wildtype *S. cerevisiae* strain. We are currently evaluating offspring from the triploid strain exhibiting similar levels of low pH tolerance to the superior *S. boulardii* strain. Such a segregant will be crossed further with a laboratory *S. cerevisiae* strain to obtain segregant pools for Quantitative Trait Loci (QTL) analysis and ultimately identify the causative alleles/mutations , behind the low pH tolerance in *S. boulardii*. Successful identification of such alleles can have important applications in improving probiotic yeast strains that are sensitive to low pH environments similar to that of the gut as well as industrial strains that are frequently subjected to acid wash to combat bacteria contamination.

INTRACELLULAR pH OF POTENTIAL PROBIOTIC YEASTS DURING THE PASSAGE THROUGH AN *IN VITRO* GASTROINTESTINAL TRACT MODEL

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The aim of our study was to investigate the different strategies of yeast to regulate intracellular pH (pHi) upon exposure to gastrointestinal stresses. Fluorescence ratio imaging microscopy and CDCFDA-SE dye was employed to determine the pHi of yeasts. Two strains of *Debaryomyces hansenii* DI02 and D18335, with respectively strong and weak survival capacities under *in vitro* gastrointestinal conditions, were compared to the known probiotic yeast *Saccharomyces boulardii* with regard to pHi response using an *in vitro* gastrointestinal tract model. Our findings suggest different approaches in regulating pHi in response to gastrointestinal stresses. While *D. hansenii* DI02 adapt to high (pH 6.5) or low (pH 2.5) extracellular pH by respectively increasing or decreasing its pHi, both *D. hansenii* D18335 and *S. boulardii* maintained pHi in the range of 4.5-6 and 4-6.5 respectively. Interestingly, *D. hansenii* DI02 had wide range of pHi in sub-populations which correlated to a better survival of yeast upon exposure to gastrointestinal stress whereas *D. hansenii* D18335 had narrow range of pHi in sub-populations which correlated to a poor survival of yeast upon exposure to gastrointestinal stress. These findings give insight to survival strategies of yeasts exposed to gastrointestinal stress.

PHYSIOLOGY AND OSMOTOLERANCE OF TWO *Dekkera bruxellensis* STRAINS

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Yeast *Dekkera bruxellensis* is associated with various fermentation systems like bioethanol, beer and cider production. In winemaking process, *Dekkera* is one of the non-*Saccharomyces* yeasts which contributes to the final quality of red wine. *D. bruxellensis* produces various phenolic compounds and their concentration influences the organoleptic properties of wine, sometimes improving them, sometimes causing spoilage and large economic losses. *D. bruxellensis* genome is very dynamic, several chromosome sets can be present and copy number of genes can determine physiological properties. In this study we compare basic physiological parameters and osmotolerance of two *D. bruxellensis* strains Y879 (diploid/polyploid?) and Y881 (ploidy is not known), both isolated from wine. Though the cells have similar morphology and size, they differ in growth parameters, tolerance to various stresses, plasma-membrane potential and survival upon hyperosmotic conditions. An important property for survival of yeasts during the fermentation is their osmotolerance in which the intracellular accumulation of glycerol is involved. Our aim is to characterize *Dekkera* systems for glycerol uptake which are supposed to play a key role in counterbalancing high extracellular osmotic pressure. On the basis of homology with a transporter from *S. cerevisiae* (encoded by the *STL1* gene) we identified two genes encoding putative glycerol transporters in *Dekkera* genome, cloned them and expressed them in an osmosensitive *S. cerevisiae* mutant strain to verify their function and role in osmotolerance.

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STUDIES ON ACETIC ACID STRESS RESPONSE AND GALACTOSE METABOLISM IN *Dekkera bruxellensis*

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Recent studies have highlighted how *D. bruxellensis* shares with *S. cerevisiae* the ability to survive in challenging environments, such as industrial bioethanol production processes and wine. Beside the adaptability to conditions of low pH and high ethanol concentration, *D. bruxellensis* exhibits a broader spectrum of consumable carbon and nitrogen sources in comparison to *S. cerevisiae*, and this contributes to its ecological distribution. The effect of acetic acid has been widely studied in *S. cerevisiae* and several food spoilage yeasts, but not yet studied in *D. bruxellensis.* We observed that the presence of acetic acid deeply affected its growth in glucose–based media. At metabolomics level, the effect of acetic acid was found mainly in relation to some cellular components, like carbohydrates and amides. *D. bruxellensis* is known to exhibit the Crabtree-positive effect on glucose. We found that in galactose-based media this yeast utilized a respiratory metabolism, in contrast to *S. cerevisiae* which can ferment also this sugar. Interestingly, the respiratory metabolism was shifted towards fermentative after addition of glucose, as well as when nitrate was the only nitrogen source. The expression of genes involved in different metabolic pathways was also analyzed in *D. bruxellensis*. We observed that genes involved in galactose utilization, respiratory metabolism, TCA cycle, glyoxylate cycle and gluconeogenesis were repressed in glucose-based media. These results indicate that in *D. bruxellensis* glucose repression operates similarly to what occurs in *S. cerevisiae*.

GENOTYPING AND PHENOTYPING OF HUNDREDS OF *Dekkera bruxellensis* ISOLATES

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The non-conventional yeast *Dekkera bruxellensis* is worldwide known as a spoilage organism in the wine and ethanol industry and as an "indispensable" contributor to the flavour profile of Belgium lambic and gueuze beers, some red wines and sourdough. Furthermore, it became a model organism to investigate yeast evolutionary history. Hundreds of native *D. bruxellensis* isolates have been collected from must, wine and soft drinks all over the world (Italy, Spain, England, Mozambique, Belgium, Chile, Portugal, Australia and South Africa). To get a better understanding of the genotype-phenotype-paradigm, these isolates have been characterized in view of their phenotypic traits and genome structure. Microtiter plate screenings for several different traits allow the identification of strains that exhibit high sensitivity or resistance towards ethanol and acetate, pH, osmotolerance or the ability to grow under relatively high or low temperatures. In addition, the genomes of fifty strains are now sequenced and allow us a closer look at the genomic level to highlight differences due to the strain origin and to get an insight into the genotype-phenotype-relationship.

MALDI-TOF AS AN IDENTIFICATION TOOL FOR *Saccharomyces sensu stricto* SPECIES AND FOOD-RELATED YEASTS

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Currently, the most common method for the identification of yeasts is the sequence analysis of the internal transcribed spacers (ITS1 and 2) and the D1/D2 domains of the large subunit of the ribosomal DNA. This method provides reliable identifications, but can also be time-consuming and tedious. Here we present the Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) as a quick, accurate and easy to use method for identification of industrial yeasts. An in-house database was created of a high quality Main Spectra (MSP) from type and reference strains of representing species belonging to 25 genera, economically relevant in the food industry. These include species from the *Saccharomyces sensu stricto* complex, spoilage yeast such as *Zygosaccharomyces* species and additional food-related yeasts such as *Debaryomyces hansenii*. A blind set of strains was tested to evaluate the accuracy and precision of the MALDI-TOF MS identifications. Here we represent data obtained with this rapid and cost-effective method. Within this set, the majority of strains were correctly identified indicating that MALDI-TOF MS is a reliable mean for the identification of food related yeast isolates.

NONCONVENTIONAL YEASTS CAN BE EVOLVED TO BE EFFICIENT ETHANOL PRODUCERS UNDER BACTERIAL SELECTION PRESSURES

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Saccharomyces yeasts have evolved a fermentative life style even under fully aerobic conditions. However, the ability to metabolise sugars into ethanol is poor in pre-WGD yeasts when compared to post-WGD yeasts, such as *Saccharomyces cerevisiae*. Though, the events that have shaped the evolutionary history of budding yeasts have been widely explored, our understanding of the genetic factors responsible for variability in inter-species fermentative ability remain poorly understood. We performed an evolution experiment with *Lachancea kluyveri*, a pre-WGD yeast and a relatively poor ethanol producer, in an attempt to transform them into better fermenters and identify the mechanism underlying this transformation. A modified serial dilution transfer method was used to mimic the natural environment, where *L. kluyveri* had to compete for the available carbon source with co-habiting microbes such as bacteria. *L. kluyveri* was co-cultivated with six species of bacteria with increasing ethanol resistance, which served as a selection pressure and had to compete with bacteria to defend their niche. After 720 generations, the evolved strains of *L. kluyveri* could out-compete the co-cultured bacteria within 21 hours, while in the ancestral strain; the competing bacteria successfully thrived until its natural death. The evolved strains demonstrated a 55.8% increase in ethanol production, 30% increase in glucose consumption and quadrupled its glycerol production. This was coupled by a complete decline in acetate production and lowered dependency (27%) on oxygen in its cellular economics, suggesting *L. kluyveri's* transformation to fermentative metabolism yeast. Whole genome and RNA sequencing results has led us to speculate homozygotisation of chromosome III in evolved strains as a plausible cause for this transformation. We are currently performing experimental and computational analysis to confirm this speculation and further dissect the changes in genetic factors responsible for the evolution of *L. kluyveri* into an efficient ethanol producer.

BIOFLAVOR PRODUCTION OF NON-*Saccharomyces* YEASTS IN FERMENTED BEVERAGES

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Flavors produced by biological methods (bioflavors) are now more and more requested by consumers, increasingly concerned with health and environmental problems caused by synthetic chemicals. Currently, the use of different microorganisms for natural flavor synthesis is a fast growing sector due to interest in the development of new products, such as alcohol-free beers, reduced alcohol wines and fruity aroma drinks. Non-*Saccharomyces* yeasts constitute an untapped reservoir with a huge industrial potential in this sector. In this work, 60 non-*Saccharomyces* yeasts were screened, covering 40 different genera, for their capacity to produce pleasant novel aromas. Apart from the choice of yeast, several factors such as medium composition, contribute to flavor production. For this reason, three important beverage sectors were studied (wine, beer, cider) and their effect on fermentation activity and volatile/non-volatile production. We will present results that show that different combinations of strains and media can be used to produce new products with divergent aroma profiles.

MOLECULAR BASIS OF SUPERIOR STRESS TOLERANCE IN NONCONVENTIONAL YEASTS

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Stress resistance is a desirable characteristic of industrial yeast strains since they encounter a plethora of sequential and simultaneous stress conditions, such as high osmolarity and temperature fluctuations, in the course of their production and usage. The availability of highly stress resistant baker's and brewer's yeast strains would be beneficial in this respect. A thorough exploration of the largely untapped resource of highly stress resistant nonconventional yeast species could reveal a promising road to further improve industrial yeast strains. This research focuses on two species, *Zygosaccharomyces rouxii* and *Kluyveromyces marxianus,* which were selected based on previous reports for their high osmo- and thermotolerance, respectively. In this study, phenotypic evaluation of the CBS yeast strain collection was performed to select the most sugar tolerant and temperature tolerant strains. *Zygosaccharomyces rouxii* (CBS 741) displayed tolerance to high glucose concentrations (up to 90%) while *Kluyveromyces marxianus* (CBS 2080) managed to grow at high temperature (up to 50°C). Identification of individual unknown genes from those nonconventional yeast species responsible for their extreme phenotype is the first step to further improve traits of interest in industrially used *Saccharomyces cerevisiae.* In order to do so, a cDNA library of both species was constructed in a customized vector. The libraries were screened in the *S. cerevisiae* S288c background, aiming to identify genes that are responsible for the extreme phenotype of unconventional yeast species and capable of improving the performance of closely related species such as *S. cerevisiae.*

THE EFFECT OF THE NITROGEN SOURCES ON THE FORMATION OF VOLATILE AROMA COMPOUNDS BY NON-CONVENTIONAL *Saccharomyces* SPECIES

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Nitrogen is one of the most important yeast nutrient which significatnly influences fermentation. The use of different nitrogen sources to avoid its deficiency in the fermentation process has become a common practice in the wine industry. Another common practice in winemaking of the recent years is introducing of some non-conventional species from *Saccharomyces* genus with unusual abilities. The species *S. kudriavzevii* and *S. uvarum* have shown very interesting properties leading to higher production of certain aromatic compounds. Since ammonium and amino acids are commonly used as a nitrogen supplement, we aimed to determine how *S. kudriavzevii* and *S. uvarum* manage these nitrogen sources and how they affect the production of volatile aroma compounds. The results show differences especially in the total volatile compounds composition giving *S. uvarum* as a great producer of acetate esters while *S. kudriavzevii* dominates in the production of higher alcohols. Based on this information we decided to compare the homologous genes from *S. cerevisiae*, *S. kudriavzevii* and *S. uvarum* involved in the flavour compounds synthesis. The *in silico* analysis identified the most radical substitutions in *ARO10*, *ATF1* and *ATF2* genes. When expressed in winery *S. cerevisiae* T73 strain, the increased amount of several aromatic compounds was observed. We detected increase of the production of banana and fruity flavours, such as isoamyl alcohol, isobutanol and their esters. Enzymatic assay also revealed interesting differences among the Aro10 proteins where Aro10p from *S. kudriavzevii* shows no preference to measured substrates while for *S. cerevisiae* Aro10p phenylpyruvate is the preffered substrate. Our results and the fact of close phylogenetic relationship among the three species suggest that the modification of overall wine aroma could be reached only by substitution of a few nucleotides.

MECHANISMS BEHIND IMMUNOREGULATORY EFFECTS OF PROBIOTIC YEASTS

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The concept of individual microorganisms influencing the makeup of T cell subsets via interactions with intestinal dendritic cells (DCs) appears to constitute the foundation for immunoregulatory effects of probiotics, and several studies have reported probiotic strains resulting in reduction of intestinal inflammation through modulation of DC function. Consequent to a focus on *Saccharomyces boulardii* as the fundamental probiotic yeast, very little is known about non-*Saccharomyces* yeasts in terms of their interaction with the human gastrointestinal immune system.

We evaluated the immune stimulating capabilities of a diverse selection of non-*Saccharomyces* yeasts by incubation with human monocyte-derived DCs followed by DC incubation with autologous naive T cells. Quantification of secreted cytokine levels led to the identification of yeasts with highly reproducible and distinct DC and T cell cytokine induction profiles, as compared to the established probiotic *S. boulardii*. The observed differences in induced cytokine profiles indicate that certain yeasts are capable of inducing an immune response dominated by T_{reg} cells, whereas others appear to induce a more complex adaptive immune response involving T_H1, T_H17, and T_{reg} cells.

To explore the mechanisms behind the observed cytokine induction, we blocked relevant DC pattern recognition receptors and investigated the cytokine inducing properties of yeast cell wall extracts. Our data identify the β-glucan receptor Dectin-1 as key for DC recognition of *S boulardii* as well as non-*Saccharomyces* yeasts, initiating downstream signaling pathways leading to the observed DC cytokine profiles. In contrast, TLR2 and DC-SIGN do not appear involved in the recognition. As expected based on the identification of Dectin-1 as involved in yeast recognition, β-glucan containing yeast cell wall extracts induced robust DC cytokine secretion, an observation that parallels recent *in vivo* findings and appears to support a hypothesis that yeast cell wall components are responsible for the observed immune cell stimulation.

HIGH THROUGHPUT SCREENING OF NON-CONVENTIONAL YEASTS IN FERMENTATION PERFORMANCE AND FLAVOUR PRODUCTION

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The yeast biodiversity harbours many thousand species. This represents a yet untapped natural resource of strains that could contribute with novel flavors to industrial fermentations. Our approach to cover this biodiversity was based on the previous identification of strains in fermentations, for example of beverages, fruits or meat. Our list of several dozen strains encompasses a wide variety of species within the *Saccharomyces* clade. These strains were tested via plate assays for various responses to stresses, e.g. ethanol, osmolarity and temperature. Then all strains were subjected to defined fermentation conditions and their flavour profiles were analyzed by GC/MS. As could have been expected the list of aroma compounds we detected covered a wide variety of substances and compound groups. Data mining and statistical analysis software were employed to cluster and highlight species related flavors. Interestingly, significant differences in the production of volatile compounds were observed even within the same species. Esters were the major volatile compounds detected. Strong producers of sweet and fruity fatty acid ethylesters such as ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate were found within *Schwanniomyces* sp., *Torulaspora* sp. and *Wickerhamomyces* sp. Strains of the genus *Wickerhamomyces* also produced significant amounts of acetic acid and ethyl acetate also in comparison to other species. In addition to the traditional volatiles, e.g. isoamyl alcohol, we also discovered strains rich in specific flavors. *Candida* sp. and *Pichia* sp., for example, were able to produce small amounts of terpenes (isogeraniol and geraniol), flavors that are usually derived from grapes. This work provides further insight into the flavour characteristics of non-*Saccharomyces* yeasts and their suitability e.g. for mixed fermentations.

DEVELOPMENT OF A NEW FERMENTED BEVERAGE EMPLOYING CASHEW APPLE JUICE AND NON-CONVENTIONAL YEASTS

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Cashew apples are considered low cost products or a waste stream in the cashew nut processing industry. Around 90% of the cashew apple production is left on the field after removing the nuts. However, such an amount of biomass presents a high potential as fermentation substrate, since it is rich in vitamins (especially vitamin C), carbohydrates and minerals. The objective of this study, as part of Cornucopia project, was to investigate the use of cashew apple juice as a low cost substrate for developing a process for the production of a novel beverage fermented by non-conventional yeasts. A number of modifications were done to improve the juice quality, decreasing its typical sourness and astringency due to the high tannin contents. Yeast strains were selected and subsequently tested for their growth potential at various conditions of temperature and oxygen and the aroma profiles of the fermented juices were determined using GC-MS. Statistical analysis was applied to evaluate the impact of oxygen, temperature and strains on volatile aroma production. The final product was slightly yellowish, acidic in taste, low in alcohol and maintained a high level ascorbic acid and total polyphenols.

NOVEL CYSTEINE-CENTERED SULFURE METABOLIC PATHWAY AND ITS REGULATION IN THE METHYLOTROPHIC YEAST *Hansenula polymorpha*

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Sulfur, an essential element for all living organisms, is present in two amino acids, cysteine and methionine. In yeast and filamentous fungi, sulfide can be condensed either with *O*-acetylhomoserine to generate homocysteine, the precursor of methionine, or with *O*-acetylserine to directly generate cysteine. We systematically analyzed the sulfur metabolic pathway of the thermotolerant methylotrophic yeast *Hansenula polymorpha*, which has attracted much attention as an industrial yeast strain for various biotechnological applications. Quite interestingly, the detailed sulfur metabolic pathway of *H. polymorpha,* which was reconstructed based on combined analyses of the genome sequences and validation by systematic gene deletion experiments, revealed the absence of *de novo* synthesis of homocysteine from inorganic sulfur in this yeast. Thus, the direct biosynthesis of cysteine from sulfide is the only pathway of synthesizing sulfur amino acids from inorganic sulfur in *H. polymorpha,* despite the presence of both directions of transsulfuration pathway between cysteine-homocysteine-methionine. Moreover, only cysteine, but no other sulfur amino acid, was able to repress the expression of a subset of sulfur genes, suggesting its central and exclusive role in the control of *H. polymorpha* sulfur metabolism. Our results revealed the unique features of *H. polymorpha* sulfur metabolic pathway and its regulation, which are noticeably distinct from those of other yeast and filamentous fungal species.

COMBINING GENETIC AND FUNCTIONAL APPROACHES TO DECIPHER THE MOLECULAR BASES OF LOW SULFITE PRODUCTION BY WINE YEASTS

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Enological yeasts contribute greatly to the final aromatic balance of wines through the production of volatile compounds of interest; nevertheless, they can also be responsible for the production of negative off-flavours, such as sulfur compounds. Sulfite and sulfide are two of those compounds whose production has to be controlled. The sulfate assimilation pathway has been widely studied, however, little is known about the molecular basis responsible for the differences in sulfite and/or sulfide production between yeasts strains. In order to address the genetic determinism of such properties, we implemented a QTL mapping approach along with phenotypic and transcriptomic characterization of a couple of wine yeast strains highly differing in their sulfite production. This global study resulted in the identification of two new allelic variants of the *MET2* and *SKP2* genes. Functional validations demonstrated their involvement in the control of the production of sulfite, sulfide and acetaldehyde. The combination of both alleles of the low sulfite producer strain is responsible for a strong control of the entire sulfur assimilation pathway and we show that it is strong enough to control the production levels of sulfur compounds in other wine yeast. Transfer of those alleles in a high sulfite producer strain has already been performed through backcrossing cycles using marker-assisted selection and allowed us to construct an optimized strain low SO_2, low H_2S and low acetaldehyde producer.

A COMPARATIVE APPROACH TO STUDY NITROGEN CATABOLITE REPRESSION IN YEASTS

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Fungi are ubiquitous free living cells and are able to use a wide range of compounds as carbon and nitrogen sources. The yeast *Saccharomyces cerevisiae*, in a mechanism known as Nitrogen Catabolite Repression (NCR), is able to preferably utilize good nitrogen sources (e. g. glutamine) instead of bad nitrogen sources (e. g. proline). When good nitrogen sources are available, NCR-sensitive genes are repressed. In *S. cerevisiae*, NCR is regulated by the Ure2 protein and by four GATA family proteins: two activators (Gln3 and Gat1) and two repressors (Dal80 and Gzf3). Our interest is to study, using a comparative approach, how different yeasts use diverse nitrogen sources and how this process is regulated. *Candida glabrata* is a human pathogen yeast that is more phylogenetic related to *S. cerevisiae* than to the *Candida* species. *Lachancea kluyveri* is a budding yeast that was first isolated within the yeast flora from the intestinal canal of *Drosophila*. In none of these yeasts a nitrogen repression mechanism has been described yet. For *C. glabrata* and *L. kluyveri*, we have generated a collection of single and double mutant strains in the orthologous GATA factor genes (*GLN3* and *GAT1*) and in the *URE2* gene in order to evaluate its role in nitrogen assimilation when cells were grown on glutamine, ammonia or proline as sole nitrogen sources. In *C. glabrata*, Gln3 but not Gat1, is involved in the uptake of ammonium and proline. In contrast for *L. kluyveri*, Gat1 has a principal role in ammonium and proline utilization. To elucidate if there is a NCR-like mechanism in *L. kluyveri* and in *C. glabrata*, we are evaluating, by qRT-PCR, the transcription of some of the genes involved in the transport and catabolism of the nitrogen sources tested.

TRANSCEPTOR SIGNALING AND ENDOCYTOSIS ARE INDEPENDENT EVENTS WHICH MAY BE TRIGGERED IN PARALLEL DEPENDING ON SPECIFIC SUBSTRATE-TRANSCEPTOR INTERACTIONS

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In the last few years, work in our laboratory has shown the existence of plasma membrane nutrient transporters with signal transduction activity, which have been designated as transceptors. These transceptors are usually present in a high concentration at the plasma membrane under conditions in which cells are starved for the nutrient they transport. Upon re-addition of the missing nutrient to the medium, transceptors transport the nutrient and simultaneously transduce a signal that results in protein kinase A activation and resumption of cell growth. When the transceptors are exposed to sufficient amounts of the nutrient they transport, they are usually endocytosed and sorted to the vacuole for degradation and then replaced by another set of specific plasma membrane transporters without signaling function, which remain present during normal growth. This is for example the case for the amino acid permease, Gap1, and the sulfate transporters, Sul1,2. We wanted to ascertain whether transceptor signaling triggers endocytosis of the transceptor. Through work with the transceptors Gap1 and Sul1,2, we have found that different substrates can cause signaling without triggering endocytosis and vice versa. Moreover, we have found conditions in which oligo-ubiquitination (a primary signal until now considered sufficient to target a plasma membrane protein for endocytosis) is triggered by interaction of the transceptor with a non-transported competitive inhibitor for signaling, without triggering endocytosis. On the other hand, we have found specific mutants blocking transport and endocytosis but not signaling which indicate that the substrate interaction sites/trajectory for signaling and for triggering endocytosis may be partially overlapping but not always coincident. We are also gathering increasing evidence that signaling and endocytosis occurring during interaction and transport of extracellular substrates with the transceptor do not require previous intervention of intracellular metabolic pathways in order to take place. Our results rather support the concept that different substrates bind to partially-overlapping binding sites in the same general substrate-binding pocket of Gap1, triggering divergent conformations, resulting in different conformation-induced downstream processes.

RAS/PKA SIGNAL TRANSDUCTION PATHWAY PARTICIPATES IN THE REGULATION OF *Saccharomyces cerevisiae* CELL APOPTOSIS IN AN ACIDIC ENVIRONMENT

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The acidification of the medium is observed during yeast cell growth. This process contributes to the emission of organic acids, mainly acetic acid. Acetic acid is known as the inducer of apoptosis in the yeast Saccharomyces cerevisiae. In this study, we showed that hydrochloric acid can also induce apoptosis in yeast cells, and the apoptotic phenotype triggered by treating yeast cells with hydrochloric acid is modulated by the Ras/PKA pathway. The Ras/PKA pathway is highly conserved between all eukaryotic organisms, as well as cell processes that are related to apoptosis and aging. Apoptosis of the yeast cell is the source of molecules such as amino acids and peptides. In this research, we demonstrated that the activation of the Ras/PKA pathway by insertion of Ras2Val19 allele or deletion of PDE2 gene increases cell death, displaying the markers of apoptosis
in an acidic environment. Downregulation of the pathway by deletion of RAS2, RAS1, PDE1, and insertion of the Ha-ras gene increases the cell viability and diminishes cell death with the apoptotic phenotypes. The deletion of PDE1 gene and double deletion of both phosphodiesterase genes prevent the induction of apoptosis in the cells. Modulations in the Ras/PKA pathway affect cell viability and apoptosis during natural gradual acidification of the medium as well as in acid stress conditions.

NUTRIENT SENSING PATHWAYS CONTROL WINE YEAST LIFESPAN

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Nutrient sensing pathways promote growth and protein translation when nutrients are abundant, while repressing stress response. When food is scarce (dietary restriction), growth stops and stress response mechanisms are activated leading to an increased longevity on stationary phase, or an expanded chronological lifespan (CLS). We have previously studied the genetic determinants of CLS in wine yeast on grape juice fermentation, when nitrogen is scarce and in laboratory media, where glucose if the limiting factor. We noticed that some mechanism that control longevity are sensitive to growth condition, as the transcriptional regulator member of the SAGA complex *GCN5*, whose deletion shortens CLS in laboratory conditions, while extending it in winemaking conditions. Others components of the SAGA complex were studied. The most important pathway devoted to the response to nitrogen source is TOR/Sch9. Sch9 is a kinase that acts below the TOR kinases controlling protein translation and metabolism. Its deletion in wine yeast leads to an extended CLS in laboratory synthetic medium SC. This effect is partially dependent of *GCN5*, as it deletion blocks the lifespan extension of *SCH9* mutation. Surprisingly *SCH9* deletion shortens maximum CLS during grape juice fermentation. This effect is not related to respiration, but to the response to low nitrogen concentration. Surprisingly the amount of glycerol at the end of fermentation of this mutant is highly increased. Therefore, modification of longevity genes is a way to modify metabolite production during winemaking. The relationship between *GCN5* and *SCH9* and the retrograde response and the mitochondria is studied. Another interesting nitrogen-sensing pathway is the general amino acid control ruled by the Gcn2 kinase and the transcription factor Gcn4. The effect of deleting those genes is quite different. *GCN4* deletion leads to a shortened CLS, while deletion of *GCN2* promotes a mild extension.

INFLUENCE OF THE PROPAGATION STRATEGY IN AN ETHANOL PRODUCTION PROCESS FORM GLUCOSE AND XYLOSE: PROTEOMICS AND GENE EXPRESSION ANALYSIS

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The replacement of fossil fuels with renewable fuels, like bioethanol, is decisive for diminishing CO_2 emissions and reducing the oil dependency. The current industrial bioethanol production relies on sugar- and starch-based raw materials and it is crucial to shift to more sustainable resources like lignocellulosic biomass (e.g. wheat straw, corn stover, etc.). Yeast strains used in lignocellulosic bioethanol production have to cope with challenging conditions, such as high concentration of inhibitors produced during pretreatment and simultaneous use of different carbon sources. A very important step in every fermentation process is the yeast propagation, in which it is crucial to obtain high number of cells with the best fermentative efficiency. In the last years, the possibility of exposing the cells to lignocellulosic hydrolysate with inhibitors during the propagation step has given good results in terms of cell viability and high ethanol concentrations in the following fermentation. The yeast molecular adaptation to different growth conditions during the propagation is, however, poorly characterized. In this work we used the xylose-fermenting strain *Saccharomyces cerevisiae* KE6-12 to study the impact of the propagation strategy in the ethanol production process. We proved that the propagation strategy influences significantly the ethanol production. In fermentation tests with wheat straw hydrolysate, cells propagated in minimal media with 15% (v/v) hydrolysate produced 25% more ethanol and consumed 50% more xylose than cells propagated without hydrolysate (0% v/v). Furthermore, cells fermentation performance varies when the hydrolysate is added at early, mid or late exponential phase during propagation. The different hydrolysate addition points implied changes in gene expression of *ADH6, ZWF1, GSY2, TPS1, ERG2* and *ALD6*. The whole proteome of *S. cerevisiae* KE6-12 after hydrolysate addition at mid exponential phase was analyzed and compared with the proteome of cells subjected to heat shock to unravel the beneficial effect of the hydrolysate addition.

INDUCING PSEUDO-WARBURG EFFECT IN YEAST *Saccharomyces cerevisiae*

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Human cancer cells are characterised by rapid glucose consumption, with most of the glucose-derived carbon being secreted as lactate despite abundant oxygen availability. This deviant energetic metabolism known as the »Warburg effect« has been designated as one of the hallmarks of the cancer. We were first to show that 6-phosphofructo-1-kinase (PFK1), the key regulatory enzyme of glycolysis might be posttranslationally modified in cancers. After proteolytic cleavage of the C-terminal portion of the enzyme, an active, shorter fragment was formed that was insensitive to citrate and ATP inhibition and could cause un-restricted metabolic flow over glycolysis leading to lactate excretion. Excretion of a primary metabolite – ethanol under the aerobic conditions is characteristic also for the yeast cells during the growth at high specific growth rates. To study the role of human shorter PFK1 fragments on the deregulated glycolytic flow, yeast cells might be the most appropriate model organism. For this purpose HD114-8D *pfk* nul strain has been chosen as a recipient for the modified truncated *pfk*M gene encoding 47 kDa fragment of human muscle type PFK-M isoenzyme. Transformants enabling different levels of gene expression were designed and tested under different growth conditions. Initially no growth of transfromants could be detected in the supplemented minimal media (SSM) with different fermentative sugars as a sole C-source. Detailed analyses revealed that deregulated glycolytic flux caused by highly active shorter fragments led to unbalanced NADH/NADPH ratio in transformants growing on fermentative sugars. By optimising the growth conditions a medium was selected that enabled faster growth of the transformant with the truncated *pfk*M gene in respect to those with the native PFK-M enzyme. Recombinant yeast cells could be eventually used for high throughput screening of drugs that might inhibit the activity of shorter fragments in cancer cells and restrict their proliferation.

PHENOTYPIC ANALYSIS OF MULTIPLE *ADH* DELETION MUTANTS OF *Saccharomyces cerevisiae*

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Five alcohol dehydrogenase (*ADH*) genes are associated with ethanol metabolism in *Saccharomyces cerevisiae*. Five single deletion mutants, five quadruple deletion mutants and a null mutant were engineered to investigate the physiological role of each of these alcohol dehydrogenase (Adh) isozymes. . A complement of alcohol dehydrogenase (Adh) isozymes are required for normal metabolic function in *S. cerevisiae*. However, even with multiple *ADH* deletions the mutant strains were still able to grow, albeit with different degrees of impaired growth.
The objective of this report was to elucidate the metabolic effects of these multiple *ADH* gene deletions, using a high-throughput analytical approach. The metabolic profiles were determined using an OmniLog® Phenotype MicroArray™ system, which is based on redox chemistry and employs cell respiration as a universal reporter. The assay facilitates the rapid and precise quantitation of phenotypes. Phenotypic analysis provided a deeper understanding of the possible functional substitution of these five Adh isozymes by comparing the lost or gained ability of the mutant strains to utilise substrates compared to the reference strain.

UNRAVELLING THE MOLECULAR BASIS OF FRUCTOPHILY IN YEASTS: A COMPARATIVE GENOMICS APPROACH

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Saccharomyces cerevisiae consumes glucose slightly faster than fructose when both sugars are present, which may lead to problems during wine production due to slow or incomplete fermentations. Other yeasts, like many *Zygosaccharomyces* species, exhibit instead a fructophilic behaviour at high glucose and fructose concentrations. This phenotype seems to be linked to the presence of a particular type of high capacity, low affinity fructose transporters related to Ffz1, which was first identified in *Z. bailii*[1]. Outside the *Zygosacharomyces* genus, Ffz1-like transporters have been found only in the distantly related *Candida magnolia* clade[2], where it also seems to play a role in fructophily.

We adopted a comparative genomics approach to uncover other genetic requirements underlying fructophily and are presently focusing on the yeast *Hanseniaspora guilliermondii,* which exhibits fructophilic behaviour under some conditions, but lacks Ffz1-like transporters. We identified 10 putative hexose transporters in the draft genome sequence of *H. guilliermondii* and determined which were expressed under conditions that trigger fructophilic behaviour. Some of these transporters were expressed in a *S. cerevisiae* strain devoid of hexose transporters and their kinetic properties and substrate specificity were determined.

We are also exploring the possibility that the kinetic properties and specificity of hexokinases, that normally accept both glucose and fructose as substrates, may play a role in fructophily. To that end, hexokinase genes were identified in the genomes of fructophilic yeasts and the kinetics of hexokinase activity was compared between fructophilic and non-fructophilic yeasts.

Novel genetic determinants of fructophily will be expressed in *S. cerevisiae* and the ability of resulting strains to ameliorate imbalances in the consumption of fructose during wine fermentation will be evaluated.

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IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF VINYL PHENOL REDUCTASE FROM *Dekkera bruxellensis* CBS4481

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In enology "Brett" character refers to the wine spoilage caused by the yeast *Dekkera/Brettanomyces bruxellensis* and its production of volatile phenolic off-flavours. This peculiarity is due to the activity of the vinylphenol reductase enzyme, that catalyses the conversion from vinyl- to ethyl-phenols and it is both strain- and cultural condition dependent. Nineteen strains of *D. bruxellensis* were screened for vinyl phenol reductase (VPR) activity. Presence/absence of the precursors in the growth medium gave no significant differences in VPR activity, confirming that this enzyme is constitutively expressed. A purified protein with VPR activity was extracted from *D. bruxellensis* CBS4481 cells. The amino acidic sequence, achieved by trypsinolysis and mass spectrometry, revealed a high homology with a Cu/Zn superoxide dismutase (SOD) in the *D. bruxellensis* AWRI1499 genome. Actually, the isolated protein possessed both vinyl phenol reductase and superoxide dismutase activities. The bioinformatics analysis showed the presence of cofactor-binding sites, that are absent or severely altered in sequences of superoxide dismutases from other wine-relevant yeast species, which do not display vinyl phenol reduction. This moonlighting activity of *D. bruxellensis* SOD as a VPR is related to its capacity of catalyzing NADH-dependent reduction of vinyl phenols.

NITRIC OXIDE-MEDIATED ANTIOXIDATIVE MECHANISM OF *Saccharomyces cerevisiae* AND ITS APPLICATION TO INDUSTRIAL YEAST

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The yeast *Saccharomyces cerevisiae* possesses various defense mechanisms against environmental stresses that generate reactive oxygen species (ROS), leading to growth inhibition or cell death. Our recent study showed a novel antioxidative mechanism mediated by nitric oxide (NO) in yeast cells, but the mechanism underlying the oxidative stress tolerance remained unclear. We report here one of the downstream pathways of NO involved in stress-tolerance mechanism in yeast. Our microarray and real-time quantitative PCR analyses revealed that exogenous NO treatment induced the expression of genes responsible for copper metabolism under the control of the transcription factor Mac1, including the *CTR1* gene encoding high-affinity copper transporter. We also found that NO produced under high-temperature stress conditions increased the transcription level of the *CTR1* gene, intracellular copper content, the activity of Cu,Zn-superoxide dismutase Sod1, and cell viability after exposure to high-temperature in a manner dependent on Mac1. NO did not affect the expression of the *MAC1* gene, indicating that NO activates Mac1 through its post-translational modification. Based on the results shown here, we propose a novel NO-mediated antioxidative mechanism through the activation of Mac1. During bread-making processes, baker's yeast mostly *S. cerevisiae* cells are exposed to baking-associated stresses, such as air-drying, high-sugar concentrations and freeze-thaw stresses. These stresses in common induce protein misfolding and mitochondrial damage leading to ROS generation. To develop the commercial production and fermentation process, it is necessary to construct yeast strains with higher tolerance to these stresses. We showed that engineered baker's yeast strains with enhanced NO synthetic ability are tolerant to multiple baking-associated stresses by reducing intracellular ROS level. The increased NO level also improved the fermentation ability after air-drying and freeze-thaw stress treatment in baker's yeast. Hence, appropriate NO production could be promising for breeding novel industrial yeast strains that are tolerant to various stresses.

ENGINEERING OF *Saccharomyces cerevisiae* FOR THE PRODUCTION OF POLY-3-D-HYDROXYBUTYRATE FROM XYLOSE

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Poly-3-D-hydroxbutyrate (PHB) is a promising biopolymer produced naturally by several bacterial species. However PHB production from undetoxified lignocellulosic hydrolysate, that represent a renewable and cheap substrate, has not been demonstrated. Since baker's yeast *Saccharomyces cerevisiae*, is known to be efficient in fermenting this type of material to ethanol, it could represent an interesting alternative host for PHB production. However, S. cerevisiae needs to be engineered for the utilization of xylose, the main pentose found in lignocellulosic biomass and for PHB production.
In the present study, we evaluated the capacity of metabolically engineered *Saccharomyces cerevisiae* to produce PHB from xylose. In-house engineered *S. cerevisiae* strains, capable of pentose utilization were transformed with the PHB pathway genes from the well-characterized PHB-producer *Cupriavidus necator.* The two host strains carried genes for xylose utilization from the yeast *Scheffersomyces stipitis*, but had different NADH/NADPH preference ratio for the xylose reductase (XR) enzyme, the initial enzymatic step in the *S. stipitis* xylose pathway. We will present the results of the strain evaluation for PHB production under aerobic conditions.

OPTIMIZATION OF pAOX1 INDUCTION IN A METHANOL/SORBITOL CO-FEEDING PROCESS IN *Pichia pastoris*

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The methylotrophic yeast *Pichia pastoris* is currently one of the most interesting hosts for production of heterologous proteins thanks to its short doubling time, its strong methanol-induced p*AOX1* promoter and the availability of glycoengineered strains. In the context of heterologous protein production optimization, recent studies have highlighted the benefits of using methanol/sorbitol mixture as a feeding medium. However, very few studies have been performed to quantitatively analyze the cellular physiology during co-feeding. Most of them focused only on the final product (i.e. heterologous protein) without any insights into p*AOX1* regulation. In the present work, we report on the quantitative characterization of *P. pastoris* cell metabolism, with special emphasis on the quantification of pAOX1 induction during a methanol/sorbitol co-feeding process by the mean of a *LacZ* reporter gene and transient continuous culture. Our results demonstrated that the cell-specific oxygen consumptions (qO2) could be reduced by decreasing the methanol fraction in the feeding media and that optimal p*AOX1* induction was achieved and maintained in the range of 0.45 to 0.75 C-mol/C-mol of methanol fraction. In addition, the qO2 was reduced by 30% at most in those conditions. The effect of the feeding rate on p*AOX1* induction was also investigated in fed-batch culture for a 0.6 C-mol/C-mol of methanol fraction. Our results clearly show a significant improvement of the induction level with an optimal feeding rate of 6,6 mmolC.gDCW.h-1. The results also showed that *P. pastoris* tends to use methanol as the main carbon source in non-limiting oxygen conditions, while sorbitol seems to be the main carbon source in oxygen-limiting conditions.

FLD GENE DISRUPTION LEADS TO AN ENHANCED AOX1 PROMOTER INDUCTION IN RESPONSE TO EITHER METHANOL OR FORMATE

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The understanding of mechanisms of methanol induction in methylotrophic yeasts, targeting increase in pAOX activity and inducers variety as well, has valuable biotechnological application. Recently we showed that not only toxic methanol but non-toxic and safe formate as well can effectively induce pAOX1 in yeast *K.phaffii* (GS115) and *K.kurtzmanii* Y727 [Naumov et al. 2013]. However, the nature of intracellular inducer of pAOX1 is still unknown. The data obtained allowed us to consider two scenarios of induction: either formed of formate methanol itself can act as a direct inducer, or one of the intermediate of methanol utilization pathway can induce pAOX1. In order to check the first "methanol" hypothesis, we derived the mutant strains GS115 *fldΔ* and Y727 *fldΔ* with FLD gene coding for formaldehyde dehydrogenase deleted to subject them to formate induction. It was expected that there would be no induction in these cells in response to formate due to the reason that FLD disruption breaks the methanol utilization pathway, so no methanol can be formed of formate. But in fact, that assay revealed that FLD disruption, on the contrary, led to an even enhanced induction of pAOX1 in the above mentioned strains in response to formate as well as to methanol. In particular, the activity of beta-galactosidase under control of pAOX1 increased 2-3-fold in mutant strains with disrupted FLD compared to the one in the wild strains. Thus, these data suggest that methanol is not likely to be a direct inducer of pAOX1. Accordingly, there should be yet non-identified intermediate directly involved in the mechanism of induction or there might be an alternative, yet unknown, pathway of transformation of formate into methanol.

A WIDE VARIETY OF DEVELOPMENTAL YEAST FROM OYC

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Various bakery markets have been formed in Japan. Each yeast maker has developed various yeasts that combine various abilities to meet market needs. In Japan, Oriental Yeast Co., LTD (OYC) has been developed various yeast and supported the market.
OYC has yeasts which combine the unique abilities such as for ultra-sweet dough, cold sensitive fermentation, fungicidal property, cold dough, and so on. We will report each of the ability in this conference, and discuss about the factors to exert the abilities.

GENETIC ENGINEERING FOR ALTERING METABOLIC FLOW IN *Saccharomyces cerevisiae* TO PRODUCE HIGHER ALCOHOLS

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The production of higher alcohols including C3-C5 alcohols in engineered bacteria has received significant attention, because they can be used as fuels, fuel additives, or commodity chemicals. The budding yeast, *Saccharomyces cerevisiae*, has considerable potential as a producer of higher alcohols because of its capacity to naturally fabricate fusel alcohols, in addition to its robustness and tolerance to low pH. However, because its natural productivity is not significant, we considered a strategy of genetic engineering to increase production of higher alcohols. For example, the branched-chain higher alcohol isobutanol, which is involved in valine biosynthesis, offers higher octane values than their straight-chain counterparts with equivalent carbon numbers and can be used with current infrastructure in addition to possessing almost the same capabilities (energy density, hygroscopicity and octane number) as gasoline. To produce isobutanol, we first overexpressed two genes encoding 2-ketoacid decarboxylase (KDC) and alcohol dehydrogenase (ADH) in *S. cerevisiae* and then tried the gene deletions involved in pyruvate metabolisms. We further tested the overexpressions of a number of endogenous genes to alter the metabolic flow for improving isobutanol production. To achieve this, we constructed the autonomous replication plasmid for expressing three genes simultaneously, and prepared several sets with several selection markers. Using the constructed vectors, we overexpressed nearly 10 genes and successfully improved the isobutanol production in *S. cerevisiae*. This work was supported by Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe; iBioK), MEXT, Japan, and in part by Industrial Technology Research Grant Program in 2011 from New Energy and Industrial Technology Development Organization (NEDO) of Japan.

STUDY OF THE PHYSIOLOGICAL STATE OF *Kluyveromyces marxianus* USING NON-CONVENTIONAL METHODS

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In bioprocesses and particularly in yeast fermentation the activity and the physiological state of microorganisms are variables still difficult to assess. Most of the information is obtained from delayed off-line measurements and remains insufficient for the development of real time control strategies to optimize the potential of microorganisms and design high performance processes. On-line quantification of the physiological state of cells is of paramount importance for the understanding and improvement of cell metabolism and thus to control pathways of interest.
In the project, Spectroscopies for the evaluation and control in real time of biological cells and physiological state (SPECTRE), innovative techniques such as: optical and dielectric spectroscopy are combined to estimate and measure the instantaneous biomass and the physiological state of microorganisms during fermentation. The yeast *Kluyveromyces marxianus* was chosen as a study model due to its capacities for enzyme production (fructanase) and aromatic compounds like esters. The Fogale® dielectric spectroscopy probe allows determine the viability of cells and the Casy® cell counter permit to determine single cell size and volume. The first results obtained in batch culture with different carbon substrates, with/without induction for enzyme production using the dielectric spectroscopy and the Casy cell counter showed that additionally to the measurement of number of cells and cell viability, the selected tools permitted to unravel additional physiological data of the cells, like the evolution of cell volume, membrane capacitance and cellular conductivity reflecting the physiological state of the cell. From the strategies developed it will be possible to set optimization and automatic control strategies to guarantee high performance processes.

METABOLIC ENGINEERING OF *Saccharomyces cerevisiae* FOR PRODUCTION OF ADIPIC ACID FROM RENEWABLE SOURCES - ONE ROUTE TOWARDS A BIOECONOMY

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Adipic acid is a six carbon long dicarboxylic acid, considered to be the most important synthetic dicarboxylic acid annually produced, according to the International Energy Agency (IEA). The global production of adipic acid had in 2010 a volume of 2.8 million tonnes, for a total market price of 4.9 billion USD. The current production of adipic acid relies on non-renewable fossil raw materials, leading to emission of the greenhouse gases carbon dioxide and N_2O. In addition, the production starts from benzene, whose use has several health related negative implications. This project aims to create a greener process for production of adipic acid developing a fermentation-based process using Swedish domestic renewable raw materials, such as forest residues and/or algae. These materials will be used to establish a biorefinery, wherein the fermentation process for the biosynthesis of adipic acid will represent the core process. Our current strategy is based on the generation of genetically modified strains of the yeast *Saccharomyces cerevisiae*, harbouring heterologous enzymatic activities allowing the conversion of lysine into adipic acid. This system is our first choice and will also work as proof-of-concept for bio-based production of adipic acid. Here we present the metabolic engineering strategy we are pursuing, based on two possible metabolic pathways for conversion of lysine into adipic acid. Preliminary results on the effect of adipic acid on *S. cerevisiae* physiology, lysine uptake, the expression of the heterologous genes of choice, and the conversion of lysine into adipic acid precursors are presented.

YEASTS FOR THE SUSTAINABILITY IN VITICULTURE AND OENOLOGY: THE "YESVITE" PROJECT

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The YeSVitE project faces the ongoing process of innovation in winemaking taking into account the continuous challenge of the development of sustainable production practices and new food products. The relevance of the programme stems in the exploitation of the genetic and metabolic potential of yeasts providing answers to questions such as how to produce economically and environmentally sustainable quantities of wines of high quality, or "typical" and traditional wines, and how to produce a healthier product. The main activities of YeSVitE address to the safety and quality of wine with the aim to the development of strains characterised by genetic and phenotypic traits that can modify the final product and the investigation of natural interactions between grape and wine-related microbes to propose alternative solutions for a sustainable viticulture and oenology. YeSVitE has seen the birth of a consortium composed by seven research units with relevant expertise in the study of yeasts as model organisms and as key tools for wine and biotechnological applications. All the involved partners of the network improve the programme with novel knowledge and skills since they operate in areas of unique interest and are characterised by a strong know-how in winemaking. The project is organised in four work packages that are expected to be completed in four years of joint activity.

THE YESVITE PROGRAM: RESEARCH FOCUS AND CONTRIBUTION OF THE INSTITUTE FOR WINE BIOTECHNOLOGY @ STELLENBOSCH UNIVERSITY

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The IWBT promotes an integrated research program that covers the biology of the full wine research value chain from grape production to wine fermentation, wine chemistry and sensory properties. Research related to the YESVitE program at the IWBT focusses on broader microbiological questions related to wine making, and includes studies on yeast diversity, the fermentation properties of yeast and bacteria, the interactions between species, and the genetic and metabolic regulation of these processes as well as the production of aroma and other impact compounds. In 2014, the YESVitE program focuses on the cellular and molecular response to sulphur dioxide exposure in *Brettanomyces bruxellensis*. The potential role of specific nitrogen sources present in wine is particularly investigated, and molecular responses will be investigated using a transcriptomic approach. This work is performed in collaboration with the University of Milan. For 2015 and 2016, exchanges will focus on interactions between different wine relevant yeast and bacterial species, as well as on their impact on fermentation kinetics and aromatic properties

EXPLOITATION OF BIODIVERSITY IN VITICULTURE AND OENOLOGY TO ALLOW A SUSTAINABLE WINEMAKING

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Universally recognized as a resource of inestimable value, biodiversity is becoming a recurring topic in several roundtables. As one of the key theme of the next World Expo, biodiversity has nowadays to face several concepts, such as innovation and technology transfer, which aspire to “zero impact" on ecosystems but that inherently remain related to the productivity meaning. Thus, sustainability becomes the "chance" to maintain all the future human activities and the biodiversity preservation the "tool" to limit the environmental impact of industrial productions. Main contribution to the YeSVitE project of the Italian Partner in Milan will be to handle this topic from a qualitative point of view, focusing on the theme of biodiversity analysis and its safeguard, and from an economic/social side, through a wine optimization that promotes the use of yeasts in non-conventional ways. For example, yeast spoilage in beverages has become of increasing importance because of the use of less-severe processing, the great variety of new formulations and the tendency to reduce the use of preservatives. In this context, a better control in the sulphur dioxide manage during winemaking could avoid both the yeast spoilage and, in a sustainable perspective, to limit sulphite in bottled wines. Finally, to face the market, the wine industry should consider the development of innovative wine styles, suitable for the modern consumer needs. Wines with peculiar, distinguishable and intense varietal aroma should be projected with specific grapes varieties and yeast strain, able to amplify the varietal quality.

YEAST METABOLOMICS AND TAXONOMY IN WINE MICROBIOLOGY RESEARCH

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Most of the efforts in yeast taxonomy during the last three decades have been deployed in the application of molecular DNA techniques, particularly focused on a handful of markers such as LSU, ITS and few others. In spite of the great advancement in phylogenetic terms, this strategy has necessarily overlooked most of the phenotypic description of strains and isolates. The impact of this choice from a general microbiological point of view is questionable, whereas it has brought about a general disinterest in knowing what a strain can effectively "do", which is at the basis of the industrial and biotechnological application of these organisms.
Wine microbiology is coping with a general crisis of the sector in Europe, with an increasing interest on the microbial biodiversity potential and with the challenges launched by modern life styles, such as the alcohol reduction, the aroma characterization etc. In this scenario, yeast diversity must be necessarily studied with both the molecular and the phenotypic approaches, taking advantage of the technological opportunities available.
From the viewpoint of molecular taxonomy, an important target is to produce effective concepts and efficient algorithms to delimitate species and to allow for an objective definition of the biodiversity present in various geographic areas, wines and winemaking operations.
FTIR (Fourier Transform InfraRed spectroscopy) offers a great tool for a general, rapid and insespensive way to characterize strains not only for the species they belong to but especially to seek correlations between FTIR bands and relevant phenotypic characters.

QUANTITATIVE PHENOTYPIC MEASUREMENTS FOR POLYGENIC TRAIT ANALYSIS

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The laboratory and natural *Saccharomyces cerevisiae* and other yeast strains are phenotypically very diverse. Some of their phenotypes have biotechnologically and industrially important characteristics. In our laboratory we have been developing growth rate based and fluorescent marker based methods for phenotyping of yeast strains. An example of the latter is being used to determine the lipid content of different strains with flow cytometer or plate reader, where this trait is determined on a single cell or on the population level. The growth rate based method can be performed either in liquid or on agar based media. For the latter, we have developed a designated robotic manipulator that enables quantitative measurements of fitness of a very large number of different strains, based on images acquired using transmission scanner and R package Gitter automated analysis pipeline. As an example, we have been using this approach to identify traits that are potentially interesting for the wine industry from a new collection of natural yeast isolates. For instance, we determined growth under different chemical or physical stresses, such as different carbon and nitrogen sources, or growth inhibitory compounds (acetate, ethanol, sulfites etc.). Such quantitative phenotypic measurements are especially useful for polygenic trait analysis, as they provide important information for inference of causal genetic elements.

BIO-PROTECTION OF GRAPE QUALITY IN VINEYARD AND POST-HARVEST WITHERING

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The control of fungal diseases and mycotoxins contamination during grape maturation and post-harvesting is currently based on treatments with chemical fungicides. However the environmental dispersion, the progressive loss of effectiveness, the emergence of resistant strains and the increasing level of residues in wine, overall straw wines, have led the European Union to the restrict the use of these compounds (UE Directive 128/2009), addressing the researchers towards innovative and eco-friendly protocols to face the problem. A sustainable approach consists of exploiting the natural antagonistic potential of various yeasts against molds that occurs by different mechanisms such as nutrient competition, killer toxins character, iron depletion, ethyl-esters production or inducing host-plant resistance. Inhibition capabilities on mycelial growth or conidia germination of *Botrytis* and *Penicillium* spp. have been reported by some strains of species living in vineyard and cellar ecosystem, like *Criptococcus laurentii*, *Metschnikowia fructicola*, *Pichia guilliermondii*, *P. membranifaciens*, *P. ohmeri, Rhodotorula glutinis.* In this background, the Georgian Partner of the YeSVitE project will carried out a deepen investigation on antagonism patterns that can constitute a promising source of new knowledge to set biological control strategies in order to prevent or reduce harvested commodity damages.

SEQUENCING NATURAL YEAST POPULATIONS FOR FUNCTIONAL INSIGHTS

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Much is still unknown about the relative phenotypic importance of different kinds of genomic variation, and about the evolution of the genotype-phenotype relationship. We address these and related questions by population level sequencing in baker's yeast. By Illumina sequencing of 42 natural yeast genomes, we found that while genetic divergence in terms of SNPs is much lower in *Saccharomyces cerevisiae* than its sister species *S. paradoxus*, copy number and genome content variation is higher. We hypothesize that this might explain the observation that the domesticated *S. cerevisiae* displays much higher phenotypic diversity than its wild relative. The subtelomeres were highly enriched for functionally relevant variation, highlighting their importance to phenotypic evolution. We are now expanding to sequence 1002 *S. cerevisiae* genomes from a wide range of locations and habitats, greatly widening the population genomics repertoire for the species. Additionally, we are sequencing 10 strains on the long-read PacBio platform to overcome some of the technical limitations of short read sequencing, which particularly affect the repetitive and variable subtelomeres.

LINKED QTLs OF MIXED EFFECTS IN COMPLEX TRAITS OF YEAST

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The use of backcrosses and multigenerational crosses in linkage analysis of complex traits in yeast has revealed that many QTLs identified in F1 progeny are actually composed of several linked QTLs. The linked sets generally are comprised of loci with QTLs with effects in the same phenotypic direction as the original, as well as antagonistic QTLs with opposite effects. Initially thought to be a surprising result, this observation fits expectations of how yeast populations adapt and evolve. A re-examination of the high resolution mapping of over 20 QTLs involved in heat tolerance from a 12-generation cross reveals such a linked set with very rare haplotypes of 3 linked segregating variants found in the heat tolerant population. Other non-additive associations are found between unlinked loci as well. We are now generating large numbers of arrayed individuals from multigenerational mapping populations to determine how much of the complex phenotypic variation has this underlying genetic architecture. This has implications as to how QTL analysis should be done in the future.

HIGH-THROUGHPUT PHENOTYPING AND SELECTIVE BREEDING AS AN EFFECTIVE TOOL TO GENERATE NOVEL BEER YEASTS

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Fermented foods and beverages have been consumed by humans for over 8000 years. The shift from spontaneous to inoculated fermentations, which was started in the late 19th century, greatly increased the quality and reproducibility of the fermentations. However, the main difficulty was to select a yeast strain with all beneficial characteristics necessary for an efficient and high-quality fermentation. Until recently, the appropriate tools and knowledge were lacking to make a well-considered and scientifically found choice. In some industrial foodstuff fermentations, yeast strains are used as starter cultures because of historical rather than scientific reasons. In this study, we screened a large collection of feral and industrial *Saccharomyces* yeast strains (>600), the majority being particularly suited for industrial purposes, such as the production of beer and wine. Our high throughput screening and subsequent data analysis showed an enormous geno- and phenotypic diversity among different *Saccharomyces* strains. Next, hybrids with superior beneficial characteristics were constructed using several different breeding approaches. For example, new hybrid strains with an increased production of fruity aromatic compounds (producing up to 45% more isoamyl acetate) or a higher tolerance to ethanol (producing up to 18,5% ethanol in rich growth medium) were developed. This research provides a unique insight in the variability of industrial and wild yeast strains. It enables us to identify yeast strains for specific production tasks and allows the development of custom-made hybrid strains with specific characteristics. Furthermore, it paves the way for fundamental insight in complex, polygenic phenotypes such as aroma production and ethanol tolerance, which can lead to the identification of target genes for metabolic engineering.

DEVELOPMENT OF INTERSPECIES YEAST HYBRIDS TO INTRODUCE AROMATIC DIVERSITY IN LAGER BEERS

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Despite the enormous diversity in ale yeasts, the diversity of yeasts used in the lager beer industry (*Saccharomyces pastorianus*) is very limited. In fact, only two archetypes of lager yeasts (Saaz and Frohberg-type) are currently used in breweries. These two types are the result of two independent interspecies hybridization events, presumably originating from the 15th or 16th century, between the traditional brewer's yeast Saccharomyces cerevisiae and the Saccharomyces eubayanus. This confined genetic (and as a consequence phenotypic) diversity of lager yeasts is reflected in the relatively limited influence of the yeast strain on the aroma profile of lager beers. Because product innovation has become more and more important to breweries, new lager yeasts with a diverse aroma production opens the window for the production of new lager beers with different flavors. In this study, we generated a large set of new lager yeasts by crossing carefully selected S. cerevisiae and cold tolerant *Saccharomyces* species like *S. eubayanus, Kudriavzevii* and *mikatae*. . We obtained more than 250 new and unique lager strains with a diversified aroma production profile. Next, these newly developed hybrids were genetically stabilized and screened for their fermentation capacity and aroma production in lager beer fermentations and their temperature tolerance. The applied approach resulted in a large set of new lager yeasts that display an enormous phenotypic diversity, with different lineages able to produce a whole new spectrum of lager beers with diverse aroma profiles. Moreover, these strains are directly applicable in industry, since the experimental procedure only relies on natural crossing and not on genetic modification.

REPLICATION ELEMENTS IN THE WINE YEAST *Dekkera bruxellensis*

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The wine yeast *Dekkera bruxellensis* is adapted to environmental conditions that are particularly harsh and limiting. It is asexual, and its different strains' chromosomes greatly vary in number and organization. In the present study we isolated and described the replication elements of *D. bruxellensis*. For this purpose we first created auxotrophic strains, prepared genomic libraries, and developed a transformation protocol. Surprisingly, from two different strains, only three different loci (named *CIGO1, CIGO2* and *CIGO3*) were sub-cloned and supported autonomous replication. The *CIGO* loci differ from the known yeast *CEN* elements, and their biological activity was retained within only the 600-900 bp segments. For the three loci, the plasmid loss in the non-selective medium was between 20-75%. The reporter gene expression indicated that the copy number exceeded ten per cell. In the tested strains having very different karyotypes, each *CIGO* loci hybridized to at least one chromosome, but each chromosome hybridized to only one of the three CIGO loci. In the recently sequenced genome of Y879, two *CIGO* loci are over-represented in the genome sequence and thereby present in several copies. These properties suggest that the *CIGO* loci could function as a base for a new replication unit, for example a new chromosome, which thereby could increase genome dynamics. An enhanced ability to rearrange the genome could be an efficient tool to exploit new and demanding niches.

YEAST CELL EVOLUTION THROUGH POST-TRANSLOCATIONAL ADAPTATION (PTA)

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We developed a system named BIT (Bridge-Induced Translocations) that allows to link together two different chromosomes of *S. cerevisiae* exploiting the homologous recombination system of the yeast cells [1, 2]. The BIT mechanism is strongly Rad52, and Rad54 dependent, while Pol32-independent [3] and its efficiency is related to the length and the features of the chosen DNA homologies. The main consequence of BIT is an increase of gene expression that spreads up to 160 Kb from the translocation breakpoints [2]. The same BIT event may lead to different ploidy of the translocants and therefore generates a heterogeneous population by the genotypic and phenotypic point of view [4]. In this process, yeast cells may undergo an adaptation phase during which they acquire not only different gene expression patterns, but also complex genomic re-arrangements [4,5].

As cellular stress we imposed the high temperature (37°C) reasoning that, since BIT might de-regulate up to 30% of all gene expression, PTA could result in temperature-resistance phenocopies. We verified that the adapted translocants able to grow at 37°C, over-expressed some of the genes coding for heat shock proteins and glycogen metabolism.

In conclusion, PTA produces a phenotypic variability that can be selected upon by particular environmental conditions, contributing to cellular evolution.

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ADAPTIVE EVOLUTION OF *Saccharomyces cerevisiae* TO EARLY STAGE OF AN ALCOHOLIC FERMENTATION

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Experimental evolution was used to identify genes involved in the adaptation to the early stages of wine fermentation. Evolution experiments were performed in continuous culture for 150-250 generations, in conditions emulating the initial stages of alcoholic fermentation. We performed three independent experimental evolution experiments of a haploid laboratory strain BY4741 and one evolution with a haploid strain (not adapted to winemaking growth conditions) obtained by meiotic segregation of the wine yeast EC1118. By the end of the experiments, colonies were phenotypically characterized, and strains that showed improved initial growth rates were selected. We used next generation sequencing techniques in order to find the genetic changes. Four strains were selected from the evolution of BY4741. The mutations found, pointed to the Rsp5p-Bul1/2p ubiquitin ligase complex as the preferred evolutionary target under these experimental conditions. Rsp5p is a multifunctional enzyme able to ubiquitinate target proteins participating in different cellular processes, while Bul1p is an Rsp5p substrate adaptor involved in the ubiquitin-dependent internalization of Gap1p and other plasma membrane permeases. Our results might be related to increased halftime of plasma membrane amino acid permeases. Apparently the genetic background of the laboratory strain is conditioning the result, however, we have shown the strength of this approach. The evolution of the haploid segregant of EC1118 was run for 250 generations, three adpated strains were selected. Preliminary bioinformatic analysis highlighted changes in chromosome numbers in mutant strains. We are currently testing this results by karyotyping, qPCR, flow cytometry and other techniques.

GENETIC BASIS OF PHENOTYPIC VARIATION WITHIN *S. cerevisiae* STRAINS DURING SECOND FERMENTATION

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The production of sparkling wine by the traditional method (champagne, cava) implies a second fermentation that takes place inside the bottle. Prior the inoculation into the base wine, yeasts need to go through an adaptation process called "*pied-de-cuve*", which will allow yeast cells to maintain their metabolic activity in the restrictive conditions of the second fermentation (high ethanol, low oxygen availability, increasing CO_2 pressure, low temperature). With the aim of understanding the genetic basis causing the phenotypic diversity of *Saccharomyces cerevisiae* strains during the second fermentation, we performed a QTL mapping analysis. Different QTL mapping strategies can be applied to identify natural variations explaining complexes traits. The classical F1-hybrid design allows the QTL mapping on several different traits for one subset of progeny but requires the genetic map of hundreds of individuals to be actually efficient. The development of NGS technics allow the construction of the genetic map for hundreds of progenies by sequencing their genome at low coverage with a reduced cost. In this work we carried out this strategy by genotyping 190 progenies using Illumina technology. A genetic map of more than 5000 reliable markers was obtained for each progeny. We evaluated the phenotypic landscape of sparkling wine production by measuring 47 phenotypes involved in both the "pied-de-cuve" and second fermentation, including single cell, population and fermentation traits. Linkage analysis on the phenotypes of 117 segregants resulted in the mapping of over 60 QTLs, including the population size at the final point of the "pied-de-cuve", the fermentative performance, or the formation of pseudo-hypha-growing cells. To decipher the molecular basis of the phenotypic variation observed, we are currently testing the candidate genes by reciprocal hemizygosity. Overall, the molecular dissection of the QTLs can provide useful information about the adaptation mechanisms of the yeast during sparkling wine production.

ISOLATION AND EVOLUTION OF A NOVEL NON-*SACCHAROMYCES* XYLOSE-FERMENTING STRAIN FOR LIGNOCELLULOSIC BIOETHANOL PRODUCTION

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The economic success of lignocellulosic bioethanol requires the fermentation of all available sugars from biomass. Being the major pentose sugar in lignocellulose, the fermentation of xylose is, therefore, considered essential. The fermentative yeast *Saccharomyces cerevisiae* is the most promising candidate for lignocellulosic bioethanol production due to its very effective glucose fermentation, high ethanol tolerance and resistance to inhibitors present in lignocellulosic streams. Nevertheless, wild type *S. cerevisiae* is not able to ferment xylose and all of the genetically modified *Saccharomyces* strains (GMO) still need further improvements to fulfill all requirements for an economically viable lignocellulosic ethanol production. We have discovered a non-*Saccharomyces* xylose-fermenting yeast (here called C5-yeast), which shows a great potential to be used for bioethanol production from lignocellulosic streams. Unlike xylose-fermenting *Saccharomyces* strains, the C5-yeast is not genetically modified and its application in the industry would find less legislative problems when reaching the market. In the present work, the C5-yeast was isolated from a xylose-fermenting population and evolutionary engineered to enhance its fermentation abilities and robustness. During the isolation process, three different morphologies (smooth, flat and wrinkled) of the C5-yeast were found when growing the xylose-fermenting population in plates with minimal media and xylose as a sole carbon source. Among all morphologies, flat-C5-yeast showed the highest xylose consumption rates (>90% after 72 h) and the highest ethanol conversion yields (≈50% of the theoretical considering glucose and xylose) during the fermentation of wheat straw hydrolysates. The isolated flat-C5-yeast was selected for evolutionary engineering in order to enhance its sugar conversion yields and the tolerance towards the inhibitory compounds that are present in the hydrolysate. Although further characterization is needed, an evolved C5-yeast could be considered a suitable fermentative strain for lignocellulosic bioethanol production.

Sunday, October 12, Nova Gorica – Plenary Sessions

Novo Nordisk session on Yeast Evolution

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10:00 - 10:20 Gabor Boross: The genomic landscape of compensatory evolution 195

10:20 – 10:40 Coffee Break

Part II *(chair: Daniela Delneri)*

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11:10 - 11:35 Alicia Gonzalez: Functional duplication of paralogous genes in *S. cerevisiae*: Role of transcriptional regulation and isozyme oligomeric organization 197

11:35-11:55 Matthew Goddard: Niche construction initiates the evolution of a mutualistic interaction between *S. cerevisiae* and Drosophilid flies 198

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EVOLUTION OF YEAST ALCOHOLIC FERMENTATION

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One of the most prominent and unique features of *Saccharomyces cerevisiae* is its ability to rapidly convert sugars to ethanol at both anaerobic and aerobic conditions. This characteristic is called the "Crabtree effect" and represents the background for the "make-accumulate-consume" strategy, which in natural habitats rich of sugars helps this yeast to out-compete other microorganisms. Why, when and how did yeasts remodel their carbon metabolism to be able to accumulate ethanol under aerobic conditions and at the expense of decreasing biomass production? Recent data on the carbon metabolism in Saccharomycetaceae species, allowed us to reconstruct the ancient environment, which could promote the evolution of alcoholic fermentation. The coming out scenario suggests that the first step towards the so-called "fermentative lifestyle" was the exploration of anaerobic niches, resulting in an increased metabolic capacity to degrade sugar to ethanol. The strengthened glycolytic flow produced in parallel a beneficial effect on the microbial competition outcome, and later evolved as a "new" tool promoting the yeast competition under aerobic conditions. The basic aerobic alcoholic fermentation ability was subsequently "upgraded" in several lineages by evolving additional regulatory steps, like glucose repression in the *S. cerevisiae* clade, in order to achieve a stricter metabolic control.

GENOMIC ANALYSIS OF COLD TOLERANT TRAITS IN YEAST SPECIES

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Temperature is one of the leading factors that drive adaptation of organisms and ecosystems. Remarkably, many sister species share the same habitat because of their different temporal or micro-spatial thermal adaptation. In this study, we seek to find the underlying molecular mechanisms of the cold-tolerant phenotype of closely related yeast species adapted to grow at different temperatures, namely *S. kudriavzevii* CA111 (cryo-tolerant) and *S. cerevisiae* 96.2 (thermo-tolerant). Using two different systems approaches, *i.* thermodynamic-based analysis of a genome-scale metabolic model of *S. cerevisiae* and *ii.* large-scale competition experiment of the yeast heterozygote mutant collection, genes and pathways important for the growth at low temperature were identified. In particular, defects in lipid metabolism, oxidoreductase and vitamin pathways affected yeast fitness at cold. Combining the data from both studies a list of candidate genes was generated and mutants for two predicted cold favoring genes, *GUT2* and *ADH3*, were created in two natural isolates. Compared to the parental strains, these mutants showed lower fitness at cold temperatures, with *S. kudriavzevii* displaying the strongest defect. Strikingly, in *S. kudriavzevii* these mutations also significantly improve the growth at warm temperatures. In addition, overexpression of *ADH3* in *S. cerevisiae* increased its fitness at cold. These results suggest that temperature-induced redox imbalances could be compensated by increased glycerol accumulation or production of cytosolic acetaldehyde through the deletion of *GUT2* or *ADH3*, respectively.

THE GENOMIC LANDSCAPE OF COMPESATORY EVOLUTION

Bela Szamecz[1], Gabor Boross[1], Dorottya Kalapis[1], Karoly Kovacs[1], Gergely Fekete[1], Zoltan Farkas[1], Viktoria Lazar[1], Monika Hrtyan[1], Patrick Kemmeren[2], Marian J.A. Groot Koerkamp[2], Edit Rutkai[3] , Frank C. P. Holstege[2], Balazs Papp[1], Csaba Pal[1]

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Adaptive evolution is generally assumed to progress through the accumulation of beneficial mutations. However, as deleterious mutations are common in natural populations, they generate a strong selection pressure to mitigate their detrimental effects through compensatory genetic changes. This process can potentially influence directions of adaptive evolution by enabling evolutionary routes which are otherwise inaccessible. Therefore, the extent by which compensatory mutations shape genomic evolution is of central importance. Here, we studied the capacity of the baker's yeast genome to compensate the complete loss of genes during evolution, and explored the long-term consequences of this process. We initiated laboratory evolutionary experiments with over 180 haploid yeast genotypes, all of which initially displayed slow growth due to the deletion of a single gene. Compensatory evolution following gene loss was rapid and pervasive: 68% of the genotypes reached near wild-type fitness through accumulation of adaptive mutations elsewhere in the genome. Genomic analysis revealed that as compensatory mutations were generally specific to the functional defect incurred, convergent evolution at the molecular level was extremely rare. Moreover, the majority of the gene expression changes due to gene deletion remained unrestored. Accordingly, compensatory evolution promoted genomic divergence of parallel evolving populations. However, these different evolutionary outcomes are not phenotypically equivalent, as they generated diverse growth phenotypes across environments. Taken together, these results indicate that gene loss initiates adaptive genomic changes that rapidly restore fitness, but this process has substantial pleiotropic effects on cellular physiology and viability upon environmental change. Our work also implies that gene content variation across species could be partly due to the action of compensatory evolution rather than the passive loss of genes.

EVOLUTION OF MATING-TYPE SWITCHING SYSTEMS IN NONCONVENTIONAL YEASTS

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The yeast *Saccharomyces cerevisiae* has a well-known system that allows haploid cells to switch mating-types between MATa and MATalpha, but how this complex system originated is unknown. Switching is a programmed chromosomal rearrangement event in which a segment of DNA is removed from the active (expressed) MAT locus, and replaced by an unrelated sequence copied from one of two silent (unexpressed) loci, HMLalpha or HMRa. Switching is probably evolutionarily adaptive because it enables isolated haploid cells to form spores if growth conditions are poor. The *S. cerevisiae* system requires a lot of components: 3 MAT-like loci in the genome (MAT, HML, HMR); triplication of two sequences (X and Z) that flank these 3 loci to guide the DNA exchanges; an endonuclease (HO) to make the dsDNA break that initiates the process; and a mechanism (Sir proteins) to prevent transcription and HO cleavage at HML and HMR. We have characterized the switching systems of *Hansenula polymorpha* and *Pichia pastoris*, two methylotrophic yeasts that are very distantly related to *S. cerevisiae*. The MAT loci of these species are orthologous and syntenic with the *S. cerevisiae* MAT locus, but they switch mating-types by a mechanism that is simpler than that of *S. cerevisiae* and which shows how the *S. cerevisiae* system may have evolved from simpler components.

FUNCTIONAL DUPLICATION OF PARALOGOUS GENES IN *S. cerevisiae*: ROLE OF TRANSCRIPTIONAL REGULATION AND ISOZYME OLIGOMERIC ORGANIZATION

Geovani López, James Gonzalez, Mijail Lezama, Maritrini Colon, Ximena Martinez de la Escalera and Alicia Gonzalez

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The genome of the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) contains duplicated regions. This redundancy phenomenon is the result of single whole genome duplication, as well as the occurrence of sporadic independent short-segment repeats. Approximately 8% of the genes in the pre-duplication *S. cerevisiae* genome have been retained in duplicate, so that duplicate pairs formed by polyploidy account for approximately 16% of the current gene set. The products of most of the duplicated genes seem to have conserved their original cellular role, although diversification has resulted in sub-fuctionalization; so that retention of the paralogous pair is need to completely fulfill the original function. We have previously proposed that diversification the of *GDH1* and *GDH3*-encoded NADP-dependent glutamate dehydrogenases and the *LYS20-LYS21*-encoded homocitrate synthases could result in the formation of hetero-oligomeric isozymes showing peculiar biochemical properties, which could play an important role under certain environmental conditions. Our results indicate that Leu4 and Leu9 mitochondrial monomers, can form a Leu4-Leu9 α-isopropilmalate synthase hetero-oligomeric isozymes "in vivo", displaying leucine sensitivity which is intermediate to that present in the resistant Leu9-9 and the sensitive Leu4-4 homo-dimers, indicating *LEU4* and *LEU9* retention allows the existence of three isoforms with varying degrees of leucine-sensitivity. Our results also show that the "ancestral type" yeast *Kluyveromyces lactis* has retained two paralogous genes encoding α-isopropilmalate synthases.

NICHE CONSTRUCTION INITIATES THE EVOLUTION OF A MUTUALISTIC INTERACTION BETWEEN *S. cerevisiae* AND DROSOPHILID FLIES

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Niche construction theory explains how organisms' niche modifications may feed back to affect their evolutionary trajectories. In theory, the evolution of other species accessing the same modified niche may also be affected. A general mechanism driving the evolution of mutualisms has not been identified. Drosophilid flies benefit from accessing yeast-infested fruits, but the consequences of this interaction for yeasts are unknown. We reveal high levels of variation among strains of *Saccharomyces cerevisiae* in their ability to modify fruits and attract *Drosophila simulans*. More attractive yeasts are dispersed more frequently, both in the lab and in the field and flies associated with more attractive yeasts have higher fecundity. These results suggest that the yeast-fly interaction is mutualistic, facilitated by the yeast's niche modification. Overall our results provide support for the idea that niche construction can initiate the evolution of mutualisms.

FROM PHENOTYPES TO PATHWAYS: GLOBAL EXPLORATION OF CELLULAR NETWORKS AND PATHWAYS USING SYSTEMATIC YEAST CELL BIOLOGY

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We have developed two powerful pipelines which combine array-based yeast genetics and automated microscopy for systematic and quantitative cell biological screens or phenomics. Our first pipeline uses the Synthetic Genetic Array (SGA) method to introduce fluorescent markers of key cellular compartments, along with sensitizing mutations, into yeast mutant collections. We then perform live cell imaging on the mutant arrays using HTP confocal microscopy to quantitatively assess the abundance and localization of our fluorescent reporters, providing cell biological readouts of specific pathways and cellular structures in response to thousands of genetic perturbations. Our second pipeline exploits the yeast GFP collection, a unique resource consisting of thousands of strains with different genes uniquely tagged with GFP. This remarkable collection has been arguably underutilized for systematic analysis of the proteome, largely due to the challenges associated with analysis of large sets of cell biological data. We addressed this challenge by adopting a high-content screening approach to measure protein abundance and localization changes in an automated fashion on a genome scale. Our general approach, in particular our network analysis and visualization methods, are readily extensible to other systems.

THE GENETIC LANDSCAPE OF A CELL

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Genetic interactions can play an important role in determining the relationship between genotype and phenotype and may underlie a significant component of the "missing heritability" in current genome-wide association (GWAS) studies. To explore the general principles of genetic networks, we've taken an unbiased and global approach to map digenic interactions in the budding yeast *Saccharomyces cerevisiae*. We developed Synthetic genetic array (SGA) analysis, which automates yeast genetics, enabling the combinatorial construction of defined mutants and the mapping of genetic interactions quantitatively. We are generating a comprehensive genetic landscape for yeast, examining networks derived from an analysis of all gene pairs, covering both nonessential and essential genes. Constructing a higher order network driven by genetic interaction profiles reveals the roles of specific genes and traces a global functional wiring diagram of the cell.

DESIGNING AND BUILDING YEAST FOR THE FUTURE

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Synthetic Biology is poised to shift yeast genetics and strain development from a 'reading DNA' to a 'writing DNA' paradigm. Some researchers are already going beyond sequencing and modifying genes; they are designing and building entire genomes from scratch. They are taking genetic and metabolic engineering to the next level – genome engineering. Following the development of the first 'synthetic' virus (poliovirus in 2002) and bacterium (*Mycoplasma mycoides* in 2010), yeast has just become the latest organism to be equipped with a fully synthetic chromosome. This breakthrough marks the first fully functional designer eukaryotic chromosome. The global *Yeast 2.0* project aims to build the ultimate yeast genome by 2017. With a genome size of ~12 Mb – distributed along 16 chromosomes that encompass ~6000 of which ~5000 are individually nonessential – *Saccharomyces cerevisiae* is poised to become the world's first eukaryote with a chemically-synthesised genome. In designing the *Sc2.0* strain, the natural yeast genome will be streamlined by building in sites that will make it possible to reshuffle the genome at will, potentially yielding more desirable properties. This will pave the way for the design of 'neo-chromosomes' – purpose-built for specific applications. But for now, advances with the *Sc2.0* project (and with constructive input from a wide range of other disciplines, including Informatics, Engineering and Bio-ethics) will be of interest to the entire emerging field of Synthetic Biology. Each innovative contribution will form part of the thousands of tiny waves that drive the tides of progress towards the goal of understanding complex lifeforms and systems that will ultimately have a positive impact on the environment, economy and all of society.

POPULATION GENOMICS OF WILD YEAST AND THE ORIGIN OF WINE STRAINS

Ping Liu[1], Sofia Dashko[2], Helena Volk[2], Lorena Butinar[2], Jure Piškur[2,3], Justin Fay[1]

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Saccharomyces are frequently found in association with trees, particularly *Quercus* species. In addition to trees, *Saccharomyces cerevisiae* is also found in vineyard soil, grape and wine must. While most wine strains of *S. cerevisiae* have a European origin, *S. cerevisiae* is not often found on trees in Europe, Portugal and Spain being exceptions. As such, the origin of wine yeast is not known. In this study, we surveyed vineyards and forests in Slovenia for Saccharomyces species. We found both *S. cerevisiae* and *S. paradoxus* within and outside of vineyards. However, *S. cerevisiae* was more rare in forest samples. Population genomic analysis of these yeast strains will be used to test whether vineyard and forest populations are genetically differentiated from one another.

ACQUIRING NOVEL PHENOTYPES OF YEAST THROUGH ADAPTIVE LABORATORY EVOLUTION

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The yeast *Saccharomyces cerevisiae* is widely used for production of fuels, chemicals, pharmaceuticals and materials. Through metabolic engineering of this yeast a number of novel new industrial processes have been developed over the last 10 years. Besides its wide industrial use, *S. cerevisiae* serves as an eukaryal model organism, and many systems biology tools have therefore been developed for this organism.

Despite our extensive knowledge of yeast metabolism and its regulation we are still facing challenges when we want to engineer complex traits, such as improved tolerance to toxic metabolites like butanol and elevated temperatures or when we want to engineer the highly complex protein secretory pathway. In this presentation it will be demonstrated how we can combine directed evolution with systems biology analysis to identify novel targets for rational design-build-test of yeast strains that have improved phenotypic properties. Examples will be on identifying targets for improving tolerance towards butanol and increased temperature and for improving secretion of heterologous proteins.

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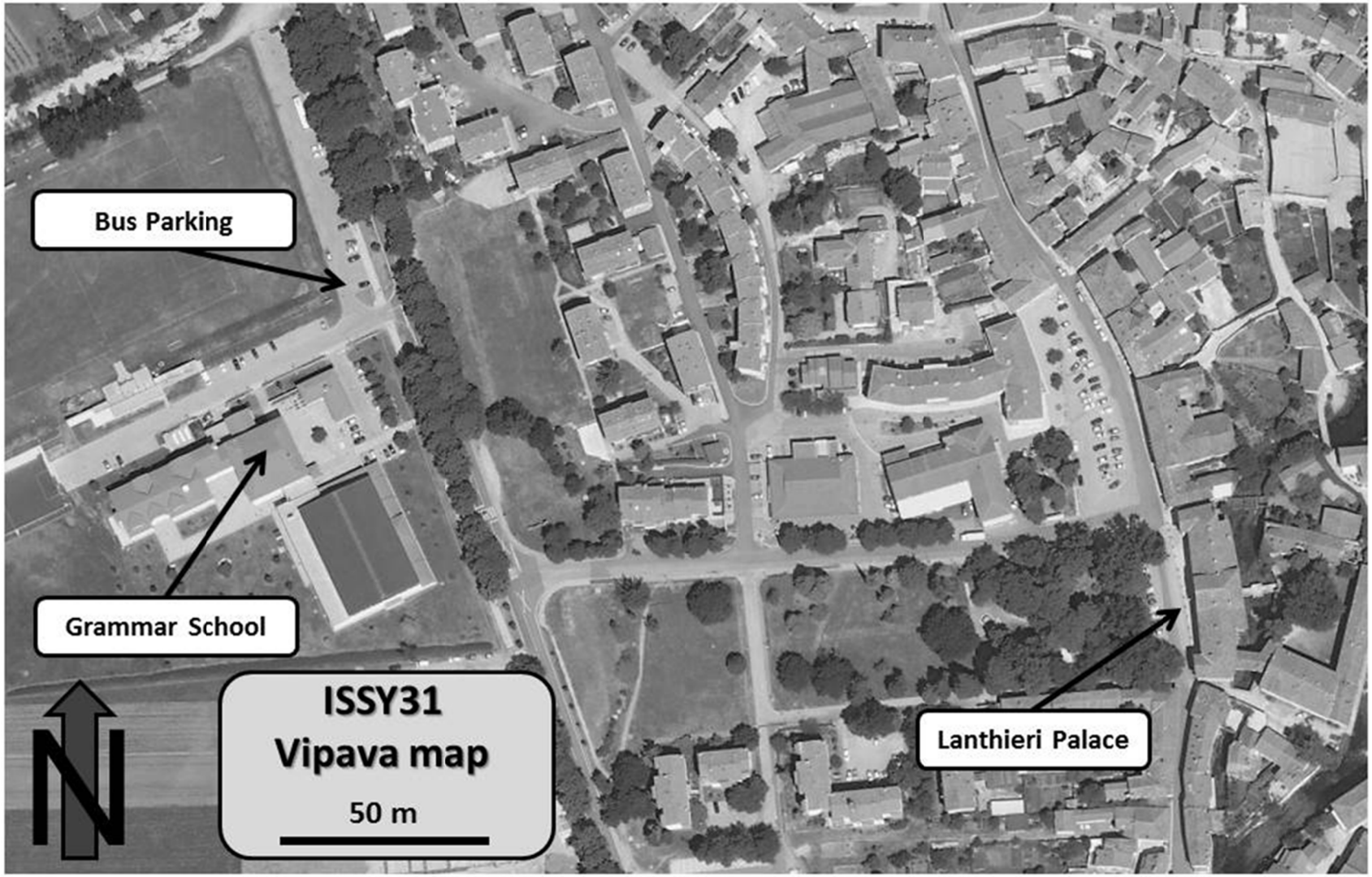
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