Program

Metabolic Engineering VII: Health and Sustainability

September 14-19, 2008

CasaMagna Marriott Puerto Vallarta Resort
Puerto Vallarta, Mexico
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Sunday, September 14, 2008

15.00 – 18.00	Registration (Acapulco Room)
	Setup Posters
16.50 – 17.50	Welcome Reception (El Patio) with Mariachis
17.50 – 18.05	Opening remarks: Conference Chairs
18.05 – 18.10	Introduction of **Plenary 1**
18.10 – 19.00	**Plenary 1**
	An outsider perspective on metabolic engineering from a former insider
	Doug Cameron, Piper Jaffray & Co.
19.00 – 20.30	Dinner
20.30 – 23.00	**Poster Session A** including social hour
	Chairs: Maciek Antoniewicz, University of Delaware
	Michael Dauner, DuPont
	Mervyn De Souza, Cargill
	Kristala Jones Prather, Massachusetts Institute of Technology

ROOM ASSIGNMENTS

- Plenary sessions: Vallarta Ballroom
- Breakfasts: Casitas Garden (Rain back-up El Patio Tent)
- Coffee Breaks: Vallarta Foyer
- Lunches: Patio Tent
- Dinners: Sunday through Tuesday – El Patio Tent;
  Wednesday – on your own;
  Thursday – Vallarta Ballroom
- Poster Sessions/Social Hours – Vallarta Ballroom
- Thursday Reception: Vallarta Foyer
- Business Center: Computers for participant use

NOTES

- Audiotaping, videotaping and photography of presentations are strictly prohibited.
- Speakers – Please leave at least 5 minutes for questions and discussion.
- Please do not smoke at any conference functions.
- Turn your cellular telephones to vibrate or off during technical sessions.
- Be sure to make any corrections to your name/contact information on the Master Participant List or confirm that the listing is correct. A corrected copy will be sent to all participants after the conference.

Monday, September 15, 2008

07:00 – 08:30	Breakfast
08.30 – 08.35	Introduction of **Plenary 2**
08.35 – 09.15	**Plenary 2** **Metabolic determinism, selected complexity, or forced evolution of biobased chemicals?** Eleftherios (Terry) Papoutsakis, University of Delaware
	Session 1: Metabolic Engineering for Biofuels
	Chairs: David Anton, Codexis Philippe Soucaille, Metabolic Explorer
09:15 – 09:45	**Yeast as platform for biomass-to-bioethanol** Laura Ruohonen, VTT
09:45 – 10:15	**Production of renewable hydrocarbons** Lisa Friedman, LS9, Inc.
10:15 – 10:45	Coffee Break
10:45 – 11:15	**Engineering microorganisms for biobutanol production** Leonie Raamsdonk, DSM Anti-Infectives
11:15 – 11:45	**Rational and evolutionary approaches for developing efficient biofuels strains** Friedrich Srienc, University of Minnesota
11:45 – 12:15	Discussion
12.30 – 13.00	Lunch & free time
13:00 – 16:00	*Ad hoc* sessions / free time
16.15 – 16.20	Introduction of **Plenary 3**
16.20 – 17.00	**Plenary 3** **Rational or combinatorial? Real metabolic engineers do both** Gregory Stephanopoulos, Massachusetts Institute of Technology
	Session 2: Metabolic Engineering for Nutrition and Agriculture
	Chairs: Jacqueline V. Shanks, Iowa State University; Harin Kanani, DuPont-Pioneer Hi-Bred International
17:00 – 17:30	**Quantifying phenotype in photoautotrophic systems using isotopically nonstationary ^{13}C metabolic flux analysis** John Morgan, Purdue University

Monday, September 15, 2008 (continued)

17:30 – 18:00	**Time-series integrated metabolomic and transcriptomic analysis for identifying metabolic engineering targets in plant systems**
	Maria Klapa, University of Maryland, College Park
18:00 – 18:30	Coffee Break
18:30 – 19:00	**Mathematical modeling and metabolic engineering of mint essential oil biosynthesis**
	Mark Lange, Washington State University
19:00 - 19:30	**Omega-3 fatty acid production by fermentation**
	Quinn Z. Zhu, DuPont
19:30 – 20:00	Discussion
20:00 – 21:30	Dinner
21:30 – 23:30	**Poster Session B** and Social Hour

Tuesday, September 16, 2008

07:00 – 08:30	Breakfast
08.30 – 08.35	Introduction of **Plenary 4**
08.35 – 09.15	**Plenary 4** **Metabolic engineering of mammalian and insect cell culture: past successes and future prospects** Michael J. Betenbaugh, Johns Hopkins University
	Session 3: **Metabolic Engineering for Cell Culture**
	Chairs: Ashraf Amanullah, Genentech Michael J. Betenbaugh, Johns Hopkins University
09:15 – 09:45	**Precision genome editing in mammalian cells using engineered zinc finger proteins** Greg Cost, Sangamo BioSciences
09:45 – 10:15	**Macroscopic control of intracellular regulation: Application to mammalian cell cultures** Ana Teixeira, IBET-FCT/UNL
10:15 – 10:45	Coffee Break
10:45 – 11:15	**Glycoprotein sialylation engineering by targeted gene silencing strategy in CHO cells to improve product quality** Min Zhang, SAFC Biosciences
11:15 – 11:45	**Metabolic flux maps comparing carbon partitioning in soybean isolines** Jacqueline V. Shanks, Iowa State University
11:45 – 12:15	Discussion
12.30 – 14:00	Lunch
	Session 4: Metabolic Engineering for Chemicals and Materials
	Chairs: Peter Meinhold, Gevo Ka-Yiu San, Rice University
14:15 – 14:45	**Metabolic engineering and metabolic modeling for higher alcohol production as biofuels** James C. Liao, University of California, Los Angeles
14:45 – 15:15	**Selection of microbial production host for converting lignocellulose into bioproducts** Peter J. Punt, TNO
15:15 – 15:45	Coffee Break

Tuesday, September 16, 2008 (continued)

15:45 – 16:15	**Rapid optimization of microorganisms for the cost superior production of chemicals and fuels**
	Michael D. Lynch, OPX Biotechnologies
16:15 – 16:45	**Metabolic engineering and metabolic modeling of *Escherichia coli* for the production of chemicals from renewable resources (MEMORE)**
	Jo Maertens, Delft University of Technology
16:45 – 17:15	**Microbially-derived semi-synthetic artemisinin: strain and process development for the production of artemisinin, a component of potent antimalarial combination therapies**
	Chris Paddon, Amyris Biotechnologies
17:15 – 17:45	Discussion
17:45 – 18:00	Stretch break
18:00 – 20:30	**Workshop I**:
	Rational and Evolutionary Approaches for Metabolic Engineering
	Organizer: Ryan Gill, University of Colorado
20:30 – 21:30	Dinner
21:30 – 23:30	**Poster Session A** and Social Hour

Wednesday, September 17, 2008

07.00 – 08.30	Breakfast
08:30 – 08:35	Introduction of **Plenary 5**
08.35 – 09.15	**Plenary 5**
	Human antibodies made in yeast
	Barry Buckland, Merck and Co., Inc.
	Session 5: Metabolic & Infectious Diseases
	Chair: Christina Chan (Michigan State University)
09:15 – 09:45	**Adapting to life in the lung: in vivo metabolism of *Mycobacterium tuberculosis***
	John McKinney, EPFL
09:45 – 10:15	**Analysis of the metabolic impact of (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone on *Bacillus anthracis***
	Ranjan Srivastava, University of Connecticut
10:15 – 10:45	Coffee Break
10:45 – 11:15	**Interspecies signal indole and archetypal signal uracil control of *Pseudomonas aeruginosa* virulence**
	Thomas K. Wood, Texas A & M University
11:15 – 11:45	***In vivo* metabolic flux analysis: Hepatic metabolism after severe trauma**
	Francois Berthiaume, Massachusetts General Hospital/Shriners Burns Hospital
11:45 – 12:15	Discussion
12:30 – 14:00	Lunch
14:00 – 16:30	**Workshop 2**
	Contributions and Prioritization of Omics
	Organizers: Christophe Schilling, Genomatica
	Costas D. Maranas, Pennsylvania State University
16:30 - 16:45	Stretch Break
16:45 - 19:15	**Selected student poster presentations**
19:15 – 21:30	Dinner (on your own)
21:30 – 23:30	**Poster Session B** and Social Hour

Thursday September 18, 2008

07:00 – 08:30	Breakfast
08:30 – 08:35	Introduction of **Plenary 6**
08.35 – 09.15	**Plenary 6** **The use of genome scale models for metabolic engineering** Bernhard O. Palsson, University of California, San Diego
	Session 6: Globalization and the Impact of Metabolic Engineering **Chair:** Juan Asenjo, University of Chile Octavio Ramirez, Universidad Nacional Autonoma De Mexico
09:15 – 09:45	**Yeast as a platform for production of nutraceutical ingredients** Jochen Förster, Fluxome Sciences A/S
09:45 – 10:15	**New insights on the role of the sigma factor RpoS as revealed in *Escherichia coli* strains lacking the phosphoenolpyruvate:carbohydrate phosphotransferase system** Francisco Bolivar, Instituto de Biotecnologia/UNAM
10:15 – 10:45	Coffee Break
10:45 – 11:15	**Microbial high molecular weight hyaluronic acid produced through metabolic engineering** Lars Keld Nielsen, Australian Institute of Bioengineering & Nanotechnology (AIBN), University of Queensland
11:15 – 11:45	**Comprehensive phenotypic analysis for identification of genes affecting growth under stress conditions in bioprocesses** Hiroshi Shimizu, Osaka University
11:45 – 12:15	**Metabolomics of recombinant yeast: Gene expression, flux analysis and a mathematical model for gene regulation of metabolism** Juan A. Asenjo, University of Chile
12:15 – 12.20	Introduction of **Closing Plenary**
12.20 – 13:00	**Closing Plenary** **A quantitative understanding of dynamic cellular processes during detoxification in the human liver** Matthias Reuss, University of Stuttgart
13:00 – 14:00	Lunch
14:00	*ad hoc* sessions / Free time

Thursday September 18, 2008 (continued)

17:30 – 18:00	**Merck Award Reception**
18:00 – 19:30	**Merck Award Lecture**
	Systems metabolic engineering
	Sang Yup Lee, KAIST
19:30 – 20:20	Break
20:20 – 22:20	Banquet

- Presentation of the **Merck Award for Metabolic Engineering** and the **Merck Poster Awards**
- Presentation of the **Jay Bailey Young Investigator Best Paper Award** in Metabolic Engineering (sponsored by **Promethegen**)
- Announcement of the Metabolic Engineering VIII Conference
- Final comments by conference chairs Vassily Hatzimanikatis and Lisa Laffend

Friday September 19, 2008

07:00	Breakfast and Departures

AN OUTSIDER PERSPECTIVE ON METABOLIC ENGINEERING FROM A FORMER INSIDER

Doug Cameron, Piper Jaffray & Co.
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METABOLIC DETERMINISM, SELECTED COMPLEXITY, OR FORCED EVOLUTION FOR BIOBASED CHEMICALS?

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Production of biofuels, and biorefining more generally, will have to utilize more complex carbohydrate substrates, produce more complex and toxic chemicals, and do so under stressful bioprocessing environments that have never been encountered before by the producing organisms. Call it what you may, complex phenotypes, "babies" of synthetic biology (and what on earth is synthetic biology?) and "systems biotechnology", creations of the new superchefs (the Venter kind or other…),…where do we stand now and where could we go to move this forward? What is the fastest and best way to generate the "tough" new microbes? "Go fishing" again from nature, redesign pathways and programs (and how does one re-design something one does not know?), mix and match, do the *in silico* thing (and how do you do it *in silico* if you do not know how they do it *in vivo*?), scrabble, force…or start from a primordial soup? So, my plan is to give some examples and dream some more examples to highlight the key points (of the dream), and suggest some (un)realistic vision for the future. The whole point is to avoid showing any data, so that there is no danger of tripping myself in the IP wars…

YEAST AS PLATFORM FOR BIOMASS-TO-BIOETHANOL

Laura Ruohonen, VTT

PRODUCTION OF RENEWABLE HYDROCARBONS

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Current transportation fuels are predominantly derived from petroleum feedstocks and are a complex mixture of refined hydrocarbons. Petroleum, on which modern day society was built and is now dependent, is a diminishing resource with increasing environmental, political, and economic disadvantages. An ideal alternative would be chemically similar to petroleum, allowing broad and rapid adoption, derived from renewable resources, scalable to support current and future demands, domestically derived, and cost competitive. To meet this need, LS9 has developed a molecular tool box and a suite of industrial microorganisms that enable the selective production of a diverse portfolio of hydrocarbon products and derivatives. This talk will cover an introduction to the technology and several case studies of its application to the production of relevant products.

ENGINEERING MICROORGANISMS FOR BIOBUTANOL PRODUCTION

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Richard Kerkman, DSM Anti-Infectives
Ulrike Mueller, DSM Anti-Infectives

DSM has focused for many years on the fermentative production of various pharmaceuticals and other chemicals by genetic modified organisms. Since 2000, (semi)synthetic pharmaceuticals are produced successfully on commercial scale by engineered fungi.

At the moment more molecules are under investigation, one of them being biobutanol. Butanol fermentation is one of the oldest fermentation processes together with the ethanol fermentation process. In the 1930's and 1940's, DSM produced biobutanol on commercial scale by using the natural producer, *Clostridium acetobutylicum*, in the so-called ABE process (acetone, butanol, ethanol). Right after the Second World War, those same fermentors were used to produce the necessary penicillin and the production of biobutanol was discontinued.

Currently, butanol production is oil based and due to rising oil prices and sustainability issues, fermentative butanol is back in the spotlight. Unlike the early 1900's, the availability of genetic tools has made it possible to switch butanol production to other, more attractive organisms.
We are focusing on biobutanol production in various microorganisms. Proof of principle was established by introducing codon optimised *Clostridium* butanol biosynthesis genes.
Subsequently, the butanol productivity was improved by debottlenecking the butanol route, decreasing by product formation and improving the supply of precursors.
Both classical strain improvement and directed metabolic engineering approaches were used, leading to significant improvements in the butanol yield and productivity. In addition, hight throughput butanol analysis was developed.

During this presentation, the efforts to build, develop and improve new butanol producing organisms for commercial purposes will be discussed in detail.

RATIONAL AND EVOLUTIONARY APPROACHES FOR DEVELOPING EFFICIENT BIOFUELS STRAINS

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Alan Gilbert, University of Minnesota

Microorganisms that are specialized to convert sugars into biofuels in the most efficient way are expected to be cells with minimal metabolic functionality. Such cells should be equipped only with the specialized catalytic capability for the conversion of the feedstock into the desired product and for the replication and renewal of this catalytic function. We have rationally designed *E. coli* cells with minimal metabolic functionality that efficiently convert hexoses and pentoses into ethanol. The performance of the constructed strains closely match the theoretical predictions (1). In order to obtain technologically robust strains we developed the cytostat cultivation technology that enables rapid evolution of organisms based on precisely defined selection pressure (2). The approach resulted in the introduction of resistance to several inhibitors into *Saccharomyces cerevisiae* cell lines. The responsible genetic changes in the adapted yeast strains have been identified using whole genome microarray analysis. The combination of rational and evolutionary metabolic engineering represents a powerful tool to rapidly develop desired strains.

(1) Trinh et al. “Construction of a Minimal Cell for the Most Efficient Production of Ethanol from Hexoses and Pentoses” Applied and Environmental Microbiology, in press (2008)

(2) Kacmar at al. “The cytostat: A new way to study cell physiology in a precisely defined environment.” J. Biotechnol. 126:163-72 (2006)

RATIONAL OR COMBINATORIAL? REAL METABOLIC ENGINEERS DO BOTH

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Metabolic engineering emerged, just over 15 years ago, as the field aiming at engineering microbes for the overproduction of fuels and chemicals using modern genetic tools. During the interim period, it developed a well-defined methodology and effective tools for integrated pathway manipulation of particular relevance to biotechnology and biological engineering. Its methodological approach and rich intellectual content yielded many successes but also made the field almost synonymous with *rational approaches* for pathway engineering. While this is certainly true when it comes to pathways with well-described kinetics and regulation, it leaves out a vast space of other modulations that could yield significant improvements to both pathway performance and cellular properties. These modulations are predominantly combinatorial in nature as they explore a space of genetic and biochemical changes that lies beyond what has been explored by science to-date. The potential of such combinatorial methods is truly immense and they should be included as an integral part of the toolkit of modern metabolic engineering. Although the focus (e.g. improving cells and assessing cell physiology) of metabolic engineering remains the same, new tools are required in an era where instead of too few genes lots and lots of genes are available and instead of a handful of measurements, researchers need to cope with avalanches of data.

In this talk I will review how metabolic engineering, as a field, is evolving by combining its rational origin with a combinatorial approach and mind-frame. The latter are critical in eliciting complex multigenic cellular properties, a task difficult to achieve following the usual single-gene paradigm. Combinatorial methods are embodied in the field of *Inverse Metabolic Engineering,* which is poised to exploit *man-made diversity* for the purpose of identifying genetic variants that are portable, well-described and quite powerful in eliciting new cellular traits. Of particular importance in the deployment of combinatorial methods are, the order in which they are combined with rational approaches, quantitative methods of assessing the power of libraries, and efficient screening methods. These ideas will be illustrated with examples from applications of Metabolic Engineering to the production of chemical products and biofuels from renewable resources.

QUANTIFYING PHENOTYPE IN PHOTOAUTOTROPHIC SYSTEMS USING ISOTOPICALLY NONSTATIONARY ^{13}C METABOLIC FLUX ANALYSIS

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Photoautotrophic metabolism is the major process by which plants and other photosynthetic organisms use solar energy to fix available CO_2 into complex organic molecules. This represents the primary source of all food on earth as well as raw materials for bio-based production of fuels and chemicals. The ability to perform quantitative studies using isotope tracers and metabolic flux analysis (MFA) is critical for detecting pathway bottlenecks and deciphering flux regulation in these systems. Although ^{13}C is the preferred isotope tracer for studying central carbon metabolism in heterotrophic systems, photoautotrophic organisms assimilate carbon solely from CO_2 and therefore produce a uniform *steady-state* ^{13}C-labeling pattern that is insensitive to fluxes. However, *transient* measurements of isotope incorporation following a step change from unlabeled to labeled CO_2 can be used to estimate fluxes successfully with newly developed techniques of isotopically nonstationary MFA (INST-MFA). We have developed a package of computational routines that achieves more than 5000-fold speedup relative to previous INST-MFA approaches. These computational tools now permit comprehensive flux analysis of photoautotrophic metabolism, complementing previous studies that have been limited to heterotrophic or mixotrophic conditions.

We have applied the INST-MFA approach to study the metabolism of *Synechocystis* sp. PCC 6803, a model photosynthetic organism, under autotrophic conditions using both GC/MS and LC/MS/MS to quantify changes in metabolite labeling over time. The INST-MFA flux map was compared to values predicted by a linear programming (LP) method that does not require experimental measurements but instead assumes that carbon and light utilization are regulated to provide optimal growth. Although the LP predicts that there should be no flux through the oxidative pentose phosphate pathway, the experimental results indicate that around 10% of the fixed carbon is lost via this pathway. Due in part to these losses, 142 ± 12 moles of CO_2 must be fixed to yield a net gain of 100 C-moles of biomass. This is significantly more than the 111 moles of CO_2 predicted by the LP model, indicating that growth is suboptimal with respect to carbon utilization. Another notable result of the flux analysis is that *Synechocystis* appears to use transaldolase rather than aldolase as its primary route to regenerate S7P in the Calvin cycle. This is an interesting result, since plants are known to use aldolase exclusively for this purpose. Lastly, ^{13}C flux analysis confirms that the oxygenation side reaction of RuBisCO is insignificant, as expected due to the potent CO_2-concentrating effect of carboxysomes in this organism.

TIME-SERIES INTEGRATED METABOLOMIC AND TRANSCRIPTOMIC ANALYSIS FOR IDENTIFYING METABOLIC ENGINEERING TARGETS IN PLANT SYSTEM

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The ability of plants to fix light, carbon dioxide and nitrogen in a renewable manner, make them an attractive biological system for the production of bio-fuels, bio-polymers and even industrial chemicals. In order to use plants for such industrial applications, however, it is important to engineer their primary metabolism and to improve (a) carbon and inorganic nitrogen fixation (b) stress tolerance (c) nutritional content and composition (d) growth rate. The difficulties in using Metabolic Flux Analysis (MFA), an important tool for metabolic engineering of biological systems, in a high-throughput manner to whole plants systems due to (i) difficulty in achieving a (pseudo) steady state, (ii) Incomplete information about plant's biochemical-network and compartmentalization of reactions, and (iii) High cost of labeled substrates for whole plant systems. Hence an alternate technique and experiment design strategy is needed to obtain the metabolic fingerprint and for identifying metabolic engineering targets in a high-throughput manner in whole plant system.

In this context, we carried out a time-series metabolomic analysis of a systematically perturbed *Arabidopsis thaliana* liquid culture system to study regulation of its primary metabolism and response to perturbation. The biological system was studied under conditions of elevated CO_2 stress, salt (NaCl) stress, sugar (trehalose) signal, and hormone (ethylene) signal, applied individually; the latter three stresses also applied in combination with the CO_2 stress. Transcript profiles were obtained using full genome cDNA microarrays for *A. thaliana*. Accurate polar metabolomic profiles were obtained using gas chromatography-mass spectrometry (GC-MS) and a novel data correction, validation and normalization strategy which significantly increased accuracy.

The metabolomic and transcriptomic analysis of salt (NaCl) stress and the combined elevated CO_2 - salt (NaCl) stress identified important new regulatory information about salt stress response, resulting in new metabolic engineering targets. Comparison of individual and combined stresses allowed identification of interaction between these perturbations at the system level. Comparison of the dynamic multiple perturbations enhanced understanding about dynamics of system response and also allowed identification of new biochemical/co-regulatory elements. In addition to identifying this important biological information, the results also demonstrate the advantages of dynamic, multiple-stress metabolomic and transcriptional profiling analysis for increasing understanding of regulation in complex eukaryotic systems.

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MATHEMATICAL MODELING AND METABOLIC ENGINEERING OF MINT ESSENTIAL OIL BIOSYNTHESIS

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OMEGA-3 FATTY ACID PRODUCTION BY FERMENTATION

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Fish oils containing the omega-3 fatty acids, eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), have been shown to confer wide-ranging health benefits. However, the fish oil supply is not sustainable and consistent, and it contains contaminants such as PCBs and dioxins. We have developed Yarrowia lipolytica strains to produce EPA and DHA by introducing omega-3 fatty acid biosynthesis pathways into this oleaginous yeast. Yarrowia lipolytica is Generally-Recognized-As-Safe (GRAS) for production of food-grade citric acid, and has been demonstrated as a robust fermentation performer. Yarrowia normally accumulates triglycerides as an energy reserve when starved for nitrogen in the presence of excess glucose, and it doesn't synthesize omega-3 fatty acids. To permit their synthesis, we have introduced various desaturase and elongase genes under the control of strong Yarrowia promoters. The desaturases are integral membrane, multiple-subunit enzyme complexes that introduce a double bond into an acyl-chain esterified to a phospholipid backbone. The elongases are integral membrane, multiple-subunit enzyme complexes that catalyze the condensation of a malonyl group and acyl-chain esterified to Coenzyme- A. By introducing these genes into Yarrowia lipolytica, we have demonstrated the synthesis of oil containing EPA free of DHA or both EPA and DHA.

METABOLIC ENGINEERING OF MAMMALIAN AND INSECT CELL CULTURE: PAST SUCCESSES AND FUTURE PROSPECTS

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In many ways, we are in the midst of a golden age of cell culture biotechnology. Mammalian cell lines are workhorses of the biopharceutical industry with revenues exceeding tens of billions of dollars. However, as the technology has matured, there are increasing demands to make these systems even more efficient in the future. Up until this now, the principal methodology to achieve this goal has been through the optimization of media and bioreactor operating conditions. However, if we would like to continue to improve the efficiency of cell culture systems, we must optimize the cell itself in order to continue to gain improvements in productivity. While there have been some successes in the past, we have even more opportunities for engineering cell culture systems in the future. In this presentation and the following session, we will give examples of how researchers are manipulating cell culture pathways for improved growth and quality. In one example from our group, the mammalian's cells programmed cell death or apoptosis pathways are being modified in order to inhibit cell death and increase the robustness for cell lines under different bioreactor operating conditions. In addition, we are using genomic tools to identify genes from natural diversity that can imbue host mammalian cells with desirable cell culture properties. However, it is equally important to generate these products in a form that meets regulatory guidelines for product quality. One area of product quality that is critical is N-linked glycosylation involving the generation of a preformed oligosaccharide, its tranfer onto Asn acceptor sites, and the subsequent processing of these N-glycans. Unfortunately, the N-glycosylation acceptor sites in the lumen of the endoplasmic reticulum (ER) may not always be fully occupied and this process can lead to site occupancy heterogeneity, lower yields, and poor host cell viability. In yet another study, we are using mathematical models to examine glycosylational processing in host mammalian cells in order to design strategies that optimize glycoforms generated in host cells of interest. Our laboratory and others are also evaluating glycosylation processing limitations in mammalian and insect cells in order to demonstrate that oligosaccharides processing can be engineered and improved in these hosts. Metabolic engineering projects have been undertaken to manipulate sugar processing pathways such that these hosts can generate more complex and improved humanized glycoproteins. In the long term, the most effective strategy for obtaining highly productive systems will be to engineer the physiology of cell culture systems with properties that are necessary to achieve both high productivity and high quality. This metabolic engineering strategy coupled with bioreactor optimization methods will ensure continued improvements in cell culture biotechnology for the foreseeable future.

PRECISION GENOME EDITING IN MAMMALIAN CELLS USING ENGINEERED ZINC FINGER PROTEINS

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Rational genome engineering in mammalian cells has enormous potential across basic research, drug-discovery and cell-based medicines. Existing methods for targeted gene knock out or site-specific gene insertion rely on homologous recombination, which occurs at the low frequency of ~ 1 event in 10^6 in most somatic cells. The scale of screening effort and the time required for the multiple rounds of gene targeting needed to generate the knock out cell can therefore be prohibitive. Thus, there exists a strong need for new technologies that can edit the genome with high speed and efficiency, thereby greatly reducing the overall engineering effort.

We have developed a novel technology that enables high-frequency genome editing via the application of designed zinc finger protein nucleases (ZFNs). Within these chimeric proteins the DNA binding specificity of the engineered zinc finger protein moiety determines the site of nuclease action. Such ZFNs are able to recognize and bind to a specified locus and evoke a double-strand break (DSB) in the targeted DNA with high efficiency and base-pair precision. The cell then employs either of two natural DNA repair processes to heal the targeted break: (i) Homology-directed repair (HDR). The induced DSB causes the frequency of homologous recombination to increase ~1000-fold. By simultaneously providing the cell with a homologous donor DNA that contains within it the genetic change(s) that we wish to make, we can achieve precise genomic editing at efficiencies greater than1% - without the need for selection strategies; (ii) Non-homologous end joining (NHEJ). This imperfect repair mechanism typically results in the loss or gain of DNA at the site of the DSB, producing frameshifts or deletions of critical codons that eliminate target gene function. The frequency of biallelic gene disruption is normally 1-10% of all clones analyzed, thus eliminating the need for selection-based approaches once again.

These two pathways provide the investigator with the ability to provoke three distinct outcomes in targeted genome editing: (i) gene correction (specifically changing one or more bases); (ii) gene deletion; and (iii) targeted gene addition. Furthermore, the speed and efficiency of this process enables us to knockout multiple genes in the same cell. Drawing from our work with transformed cell lines, primary human cells, and multi-potent stem cells, we will present several examples of single, double and triple gene knockout, as well as targeted gene insertion into native chromosomal loci.

MACROSCOPIC CONTROL OF INTRACELLULAR REGULATION: APPLICATION TO MAMMALIAN CELL CULTURES

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Objective: Elementary modes (EMs) of a metabolic network are unique and nondecomposable sets of reactions that are able to operate coherently in steady-state. These pathways define a solution space, circumscribing the functional capabilities of the biochemical network. In general, the number of EMs within metabolic networks is very high, denoting their innate adaptability and robustness properties. However, for a given environment, many of the EMs may not be used by the cells. In this work, we developed an algorithm that is able to identify the set of active EMs which best describe the dynamics of the envirome. The algorithm aims at the maximisation of the covariance between the EM fluxes and the environmental factors through the combination of the projection to latent structures (PLS) method with the elementary modes (EMs) analysis. Results: The PLSEM algorithm was applied to identify, from a total of 251 EMs, the active subset during different fed-batch cultures of recombinant Baby Hamster Kidney (BHK) cells. The selected EMs were further used to reconstruct a reduced metabolic network, and the well-known metabolic flux analysis (MFA) technique was used to compare the intracellular fluxes of both reduced and original networks. All the reactions excluded by the PLSEM had nearly zero fluxes in the original network. It was also possible to obtain consistency between experimental data and the stoichiometry of the reduced networks with less than 18 EMs (explaining over 90 % of the variance of the envirome data). Conclusions: These results illustrate the ability of the proposed methodology for reducing large metabolic networks to a set of meaningful EMs, thereby facilitating the quantification of intracellular flux distributions in large, underdetermined networks. Furthermore, the results obtained demonstrate that the PLSEM is a powerful tool to design macroscopic control strategies of intracellular regulation processes, with the respective regression coefficients providing specific information of how the envirome impacts on EM fluxes.

GLYCOPROTEIN SIALYLATION ENGINEERING BY TARGETED GENE SILENCING STRATEGY IN CHO CELLS TO IMPROVE PRODUCT QUALITY

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Glycoproteins produced by mammalian cell culture using recombinant DNA technology represent an important category of therapeutic pharmaceuticals for human health care. The biological functions of glycoproteins are often highly dependent upon their carbohydrate structures. Of the numerous sugars found in glycoproteins, the terminal Sialic Acid is considered particularly important. When terminal sialic acid was removed from the glycans, desialylated serum glycoproteins have significantly lower circulatory half-lives as compared to the sialylated counterparts. During production of recombinant glycoproteins, variability in sialylation would result in glycoform heterogeneity of the product introducing significant batch-to-batch variation in the production process. Such changes are unacceptable in a bioprocess used for large-scale production of protein therapeutic agents. Therefore, in mammalian cell culture it is often desirable to maximize the final sialic acid content of a glycoprotein product to ensure its quality and consistency as an effective pharmaceutical.

Sialylation engineering strategies that are currently used to improve product quality include the manipulation of glycoenzymes involved in opposing aspects of the sialylation processe, i.e. addition and removal of sialic acid residues from the glycoproteins. In an effort to achieve this comprehensive goal, we developed an effective gene silencing strategy using short interfering (siRNA) and short hairpin RNA (shRNA) technologies to enhance glycoprotein sialylation and improve protein quality. Prior to this study, only one gene encoding sialidase was reported in CHO cells. To broaden the study targets, we identified and characterized two novel sialidase genes in CHO cells, through a portfolio of state-of-the-art technologies, including molecular gene cloning, protein functional analysis with biochemical enzymatic analysis and inhibitory study, and subcellular localization study using Confocal Fluorescence Scanning Microscopy. Short interfering RNAs (siRNAs) were designed against the novel sialidase genes along with the previously reported sialidase gene encoding a cytosolic sialidase in CHO cells. These siRNAs were transfected into recombinant CHO cells stably expressing human Interferon Gamma (hIFNg). Sialidase gene expression and sialidase activities were measured by quantitative RT-PCR (QPCR) and biochemical activity assay, to evaluate the gene silencing effects with those siRNAs. Based on siRNA results, short-hairpin RNAs (shRNAs) (MissionRNAi, Sigma-Aldrich) were employed to construct new CHO-hIFNg cell lines that stably express interfering RNA to knockdown sialidase expression permanently. Enhanced sialylation of hIFNg was observed in the selected shRNA-CHO clones. The targeted gene silencing by RNAi was found to provide an effective way to improve product quality in the CHO expression system. It also provides useful information for next-step gene targeting study (gene knockout/knockin) in CHO cells.

METABOLIC FLUX MAPS COMPARING CARBON PARTITIONING IN SOYBEAN ISOLINES

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The biomass composition of soybeans is fundamental in establishing its market price. Soy proteins have a huge market as raw material for animal feed, other food products and industrial non-food applications. Soybean oil is used in cooking, in making margarine and in industrial applications, particularly biodiesel and lubricants. Additionally, an inverse relationship has been observed between the protein and oil content in soybeans. However, the underlying physiology causing this inverse relationship remains unclear.

This study involved comparison of intracellular fluxes of three different soybean genotypes with considerable difference in protein contents to investigate the changes in the pathway interactions. BC3-128 genotype, a back crossed line created from High PI and Evans, produced more protein than Evans and less than High PI. Further, Evans had the maximum lipid content, followed by BC3-128 and then High PI. The starch content was highest in Evans, then High PI, followed by BC3 128. BC3-128 was also nearly isogenic (94%) to Evans and as a plant, grew as well as Evans in the fields.

13-C MFA was performed on soybeans of the above three genotypes during early stages of development (21 days after flowering) to analyze the central carbon metabolism. The 13-C labeling pattern, which is reflected in the proteinogenic amino acids and starch, was analyzed using Nuclear Magnetic Resonance (NMR) spectroscopy using 2-dimensional (2D) HSQC (1H, 13C Heteronuclear single quantum correlated spectroscopy) to evaluate the extent of carbon-carbon bond coupling. Extracellular measurements coupled with NMR analysis were employed to quantify the fluxes in the pathways of the three genotypes, using a generic mathematical framework developed in our lab, NMR2Flux.

Comparisons of the intracellular fluxes between the three genotypes enable the development of hypotheses of important metabolic nodes in protein/oil partitioning. Approximately 68 % of BC3-128 metabolic fluxes lie within or are the average of High PI and Evans flux range. The oxPPP total/glycolysis total for BC3-128 and High PI (~2.2) is greater than Evans (~1.6), indicating the influence by higher protein and lipid production in High PI and BC3-128 than in Evans. Anaplerotic reactions (plastidic malic enzyme, cytosolic pep carboxylase and malate transporters) are flexible, with higher plastidic malic enzyme flux associated with higher protein production.

Previous studies with variation in temperature (Iyer et al, Plant Cell Environment (2008) 31: 506-417) also demonstrated the flexibility of anaplerotic reactions with temperature, directly influencing the carbon partitioning into protein and oil from the plastidic pyruvate pool. These results indicate plastidic malic enzyme and cytosolic PEP carboxylase are interesting genetic targets that may enhance protein/oil production in soy.

METABOLIC ENGINEERING AND METABOLIC MODELING FOR HIGHER ALCOHOL PRODUCTION AS BIOFUELS

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Global energy and environmental problems have stimulated increased efforts in synthesizing biofuels from renewable resources. Compared to the traditional biofuel, ethanol, higher alcohols offer advantages as gasoline substitutes because of their higher energy density and lower hygroscopicity. In addition, branched-chain alcohols have higher octane numbers compared to their straight-chain counterparts. However, these alcohols cannot be synthesized economically using native organisms. Here we present a metabolic engineering approach using Escherichia coli to produce higher alcohols including isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol from a renewable carbon source, glucose. This strategy leverages the host¡¦s highly active amino acid biosynthetic pathway and diverts its 2-keto acid intermediates for alcohol synthesis. In particular, we have achieved high yield, high specificity production of isobutanol from glucose. The strategy enables the exploration of biofuels beyond those naturally accumulated to high quantities in microbial fermentation.

The above example and others demonstrate the power of metabolic engineering in production of biofuels. To improve the production further, a proper kinetic modeling technique is needed. Complete modeling of metabolic networks is desirable but difficult for the lack of kinetics. As a step towards this goal, we develop an approach to build an ensemble of dynamic models which reach the same steady state. The models in the ensemble are based on the same mechanistic framework at the elementary reaction level, including known regulations, and span the space of kinetics allowable by thermodynamics. This ensemble allows for the examination of possible phenotypes of the network upon perturbations, such as changes in enzyme expression levels. The size of the ensemble is reduced by acquiring data for such perturbation phenotypes. If the mechanistic framework is approximately accurate, the ensemble converges to a smaller set of models and becomes more predictive. This approach bypasses the need for detailed characterization of kinetic parameters and arrives at a set of models that describes relevant phenotypes upon enzyme perturbations.

SELECTION OF MICROBIAL PRODUCTION HOST FOR CONVERTING LIGNOCELLULOSE INTO BIOPRODUCTS

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Industrial biotechnology is increasingly applied in the production of a large number of chemicals (i.e. bioethanol, citric acid, lysine, 1,3-propanediol). As the cost-price of large scale industrial fermentations is primarily determined by the substrate costs, increasingly cheap lignocellulosic biomass streams are used in order to make these production processes more cost-effective and, at the same time, more environmentally friendly. Currently, the selection of the microbial production host is primarily based on its potential to produce the product of interest or based on prior experience with the micro-organism.

However, lignocellulosic feedstocks consists out of a mixture of different fermentable sugars (i.e. glucose, xylose, arabinose, galactose, mannose, etc). Moreover, depending on the pretreatment and hydrolysis processes applied to convert the lignocellulose into the fermentable sugars, high salt concentrations as well as inhibitors are present in these feedstock hydrolysates. A substrate oriented instead of a product oriented approach towards production host selection could therefore avoid extensive metabolic engineering as several substrate utilization routes are required and only one biosynthesis route. Moreover, feedstock hydrolysate-related growth inhibition can be minimized in this way.

We have compared the performance of six industrially relevant microorganisms i.e. two bacteria (*Escherichia coli* and *Corynebacterium glutamicum*) two yeast (*Saccharomyces cerevisiae* and *Pichia pastoris*) and two fungi (*Aspergillus niger* and *Trichoderma reesei*), for their (i) ability to utilize monosaccharides present in lignocellulosic hydrolysates, (ii) resistance against inhibitors present in lignocellulosic feedstocks, (iii) their ability to utilize and grow on different renewable feedstock hydrolysates (corn stover, wheat straw, bagasse and willow wood). The feedstock hydrolysates were generated in two manners: (i) thermal pretreatment under mild acid conditions followed by enzymatic hydrolysis and (ii) using TNO's Biosulfurol process (non-enzymatic method in which the lignocellulose is pretreated and hydrolyzed by concentrated sulfuric acid in combination with recycling of >99% of the sulfuric acid). Moreover, their ability to utilize waste glycerol from the biodiesel industry was evaluated. Large differences in the performance of these micro-organisms were observed, with *Pichia pastoris* and *Aspergillus niger* performing the best.

RAPID OPTIMIZATION OF MICROORGANISMS FOR THE COST SUPERIOR PRODUCTION OF CHEMICALS AND FUELS.

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As the pressure for renewable sources of both fuels and chemicals increases world-wide, biologically produced products from renewable feedstocks have become a means of supplementing traditional petroleum based products. The growing field of bio-refining relies upon the use of microorganisms to convert renewable carbon sources such as sugar into higher value products. Traditional bio-refining processes have taken advantage of the unique abilities of specific micro-organisms to produce desired product, or the genetic engineering of micro-organisms to produce non-natural products. Bio-processes have then been designed around these organisms. These bioprocesses are often very costly in large part due to the complex requirements of the microorganisms themselves, which can necessitate expensive growth conditions as well as separations and processing steps both before and after the micro-organisms' conversion step. OPX has developed several new high-resolution and comprehensive genomics tools that can be used to optimize industrial organisms. We have employed these generalizable methods to very rapidly construct and optimize commercially relevant microorganisms. We are able to optimize micro-organisms that enable both variable and capital cost savings across the entire bioprocess. In particular, in a matter of months OPX has been able to construct and optimize micro-organisms for the production for several bioprocesses including the biorefining of 3-hydroxypropionic acid. 3-hydroxypropionic acid is a bio-product with several market applications. The most notable being the $7 Billion acrylic acid market, as 3-hydroxypropionic acid is readily converted by conventional methodologies to acrylic acid. Our platform technology has enabled the construction of microbial strains capable of producing commercially relevant titers of 3-hydroxypropionic acid at commercial productivities in inexpensive growth conditions, a strain that will enable a cost competitive bio-processing route to acrylic acid.

METABOLIC ENGINEERING AND METABOLIC MODELLING OF *ESCHERICHIA COLI* FOR THE PRODUCTION OF CHEMICALS FROM RENEWABLE RESOURCES (MEMORE)

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Ad hoc identification of metabolic engineering targets is a difficult task due to the complexity of the metabolic network and its regulation. For this reason, a systematic approach is pursued in the MEMORE project, using analytics, mathematical and genetic toolboxes.

By taking a steady state modelling approach as a starting point, in combination with multivariate statistics, a mathematical model was developed for identification of metabolic engineering targets. Based on this model a set of mutant strains were constructed and analysed. The genetic modification toolbox for *Escherichia coli* involved the development of strategies for multiple gene knock-outs and knock-ins, a strategy for the directed modification of enzyme properties through chromosomal point mutations, and of a promoter library covering a wide range of promoter strength, useful for tuning gene expression. Using a quantitative sequence activity model the promoter strength was correlated to its activity which allows the rational design of promoters. These tools have successfully been used to create the mutants of interest. This holistic approach has led to the design of an *E. coli* phenotype that overproduces succinate aerobically with a biomass specific productivity of 0.4 $g/g_{dw}/h$, which is significantly higher than values reported in literature under anaerobic production conditions. In aerobic batch fermentation studies a volumetric productivity of 2 g/L/h was achieved which is close to economical feasibility (2.5 g/L/h).

However, the yield of the best producing strain is still too low (0.3 c-mole/c-mole) as a result of the production of several by-products like pyruvate, oxaloacetate and acetate. This undesired co-production is a result of regulatory constraints that are currently being identified. A metabolomics platform was established for *E. coli*, which allows the accurate determination of intracellular metabolites. Subsequently, labelled and unlabelled metabolome data and genome-scale transcriptome data were gathered from wild type and several constructed mutants in steady state and dynamic metabolomics experiments using a dedicated Bioscope plug-flow mini-bioreactor and several perturbation agents. The gathered dynamic metabolome data serves as input for a dynamic metabolic model that is in an advanced stage of development and uses evolutionary algorithms for model structure identification and optimal experimental design for parameter estimation and model discrimination in order to reduce the uncertainty on the model's prediction.

MICROBIALLY-DERIVED SEMI-SYNTHETIC ARTEMISININ: STRAIN AND PROCESS DEVELOPMENT FOR THE PRODUCTION OF ARTEMISININ, A COMPONENT OF POTENT ANTIMALARIAL COMBINATION THERAPIES

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There are estimated to be 350-500 million clinical episodes of malaria annually, caused by the *Plasmodium* parasite, with more than 1 million deaths, the highest mortality occurring in children under 5 years of age. Traditional drug treatments have become ineffective as *P. falciparum* has become resistant to almost all currently used drug therapies. Exceptions to the pattern of clinical drug resistance are artemisinin-based combination therapies (ACTs), which are potent anti-malarials. Artemisinin is currently extracted from the plant *Artemisia annua*, but an additional semi-synthetic source would be advantageous to stabilize the price and availability of ACTs. We are developing a process to produce artemisinic acid in microbes, with subsequent chemical conversion to artemisinin.

Two microbes, *Escherichia coli* and *Saccharomyces cerevisiae*, were engineered to produce amorpha-4,11-diene, a precursor of artemisinic acid (Martin et al., 2003; Ro et al., 2006). Initial production of amorpha-4,11-diene in flasks was 24 mg/L from *E. coli* and 153 mg/L from *S. cerevisiae*. Further genetic manipulation and development of a fed-batch fermentation process to produce over 10 g/L amorpha-4,11-diene from *E. coli* will be described.

The cDNA encoding the cytochrome P450 responsible for the oxidation of amorpha-4,11-diene to artemisinic acid (CYP71AV1) was isolated from *A. annua* along with its cognate P450 reductase (CPR). CYP71AV1 and CPR were expressed in amorpha-4,11-diene producing *E. coli* (Chang et al., 2007) and *S. cerevisiae* (Ro et al., 2006). Initial production of artemisinic acid in flasks was 105 mg/L in *E. coli* and 115 mg/L in *S. cerevisiae*. A purification procedure was developed to isolate artemisinic acid from *S. cerevisiae* at high purity. Further genetic manipulation of both organisms and development of fermentation processes to produce greater than 1g/L artemisinic acid will be described. Chemical conversion of artemisinic acid derived from *S. cerevisiae* to artemisinin demonstrated comparable product purity to commercial artemisinin derived from *A. annua*.

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THE PRODUCTION OF HUMAN ANTIBODIES BY YEAST

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It has been known for some time that yeast can be used to express antibodies but the resulting glycosylation means that the product made is not suitable as a human therapeutic. The GlycoFi team led by Tillman Gerngross recognized this issue and began to construct different strains of Pichia pastoris in which the yeast glycosylation pathway was systematically replaced by human glycosylation. This not only allows for the expression of antibody with human glycosylation but also gives us the capability to control glycosylation in order to be able to obtain specific glycoforms.

Excellent progress has been made and results will be presented which illustrate how controlling glycosylation is a powerful tool for improving potency. In addition, a summary will be given of the various techniques used to improve expression levels of antibody by Pichia.

ADAPTING TO LIFE IN THE LUNG: IN VIVO METABOLISM OF *MYCOBACTERIUM TUBERCULOSIS*

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The term "parasite" is derived from the Greek παρασιτοσ, meaning "one who eats at someone else's table". This definition underscores the central role that assimilation of host-derived nutrients plays in the lifestyle of pathogenic microorganisms, yet this aspect of microbial pathogenesis has received surprisingly little attention until recently. Our laboratory studies the mechanisms of carbon substrate acquisition and metabolism in the human pathogen *Mycobacterium tuberculosis*. Using a combination of genetics, biochemistry, and animal infection models, our group and others have adduced evidence that fatty acids are the dominant substrate utilized by *M. tuberculosis* during "life in the lungs" of the mammalian host. In particular, we have identified a key role for the glyoxylate cycle, an anaplerotic pathway that is essential for bacterial growth on fatty acid substrates but dispensable for growth on carbohydrates. Bacteria lacking the glyoxylate cycle enzyme isocitrate lyase (ICL) are unable to replicate or persist in the lungs of infected mice or in macrophages cultivated and infected *ex vivo*. As expected, the mutant (ICL-deficient) bacteria are also unable to grow *in vitro* with fatty acids as the sole source of carbon and energy. Unexpectedly, however, fatty acids also kill ICL-deficient bacteria, even in the presence of otherwise metabolizable substrates such as glucose. Our current studies are focused on elucidating the mechanistic basis of fatty acid toxicity towards ICL-deficient bacteria, in conjunction with ongoing drug discovery efforts focused on the glyoxylate cycle enzymes in *M. tuberculosis*. The absence of these enzymes in human cells makes them particularly attractive targets for antibacterial drug development.

ANALYSIS OF THE METABOLIC IMPACT OF (*5Z*)-4-BROMO-5-(BROMOMETHYLENE)-3-BUTYL-2(*5H*)-FURANONE ON *BACILLUS ANTHRACIS*

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Bacillus anthracis is the etiological agent of anthrax. Due to its potential as a weapon for bioterrorism, it has been classified by the CDC as a Category A pathogen, the highest possible priority rating. Recent studies have shown that (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (furanone) is capable of inhibiting *B. anthracis* virulence [1]. However, the mechanism by which furanone accomplishes this result, as well as its impact on *B. anthracis* metabolism, are unknown. A genome-scale metabolic model of *B. anthracis* was created to generate and investigate hypotheses regarding the distribution of metabolic resources in the presence and absence of furanone. We experimentally monitored the affect of furanone on growth rate, glucose uptake rate, and oxygen uptake rate on the Sterne strain of *B. anthracis* when grown in R media. In the presence of furanone *B. anthracis* showed a 36-fold increase in glucose uptake rate and an 18-fold increase in the oxygen uptake rate as compared to cultures without furanone. Remarkably the growth rate appeared to be unaffected by the presence of furanone, maintaining a value of approximately 0.65 hr^{-1} in both experimental and control cultures. Results on the distribution of metabolic resources based on a flux balance analysis approach under the observed experimental conditions will be presented.

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INTERSPECIES SIGNAL INDOLE AND ARCHETYPAL SIGNAL URACIL CONTROL PSEUDOMONAS AERUGINOSA VIRULENCE

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Bacteria regulate their behavior using secreted compounds known as quorum-sensing (QS) signals; hence, one effective strategy to control bacteria is to focus on disrupting QS pathways. By screening 5,850 of Pseudomonas aeruginosa transposon mutants for altered biofilm formation, we identified seven uracil-related mutations that abolished biofilm formation. Whole-transcriptome studies showed uracil mutations (e.g., pyrF) alter the regulation of all three QS pathways (LasR-, RhlR-, and PQS-related regulons) and that the addition of extracellular uracil restored wild-type regulation. Phenotypic studies corroborated the microarray results since the pyrF mutant was unable to form compounds related to the regulons controlled by LasR (elastase), RhlR (pyocyanin, rhamnolipids), and PQS (2-heptyl-3-hydroxy-4-quinolone). Addition of extracellular uracil restored all of these QS phenotypes to wild-type levels. Our results demonstrate that uracil is a signal that controls QS and that it directly affects virulence (cells with the pyrF mutation were less virulent to barley). These results also predict that the well-studied, anti-cancer uracil analogs would also be effective in antagonizing QS signaling for bacteria, and we show 5-fluorouracil effectively represses biofilm formation as well as RhlR-regulated phenotypes (pyocyanin, rhamnolipids, and swarming) and virulence.

P. aeruginosa and Escherichia coli are found together both in the intestinal tract and in the environment where indole secreted by E. coli is a biofilm signal for E. coli and pseudomonads and where oxygenases convert indole to various oxidized compounds including 7-hydroxyindole (7HI). We investigated the impact of indole and 7HI on P. aeruginosa PAO1 virulence and quorum sensing (QS)-regulated phenotypes; this strain does not synthesize these compounds. Indole and 7HI both altered extensively gene expression in a manner opposite that of acylhomoserine lactones; the most-repressed genes encode the mexGHI-opmD multidrug efflux pump and genes involved in the synthesis of QS-regulated virulence factors including phenazine (phz operon), 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) signal (pqs operon), pyochelin (pch operon), and pyoverdine (pvd operon). Corroborating these microarray results, indole and 7HI decreased production of pyocyanin, rhamnolipid, PQS, and pyoverdine and enhanced antibiotic resistance. Furthermore, 7HI reduced pulmonary colonization of P. aeruginosa in guinea pigs and increased clearance in lungs. Screening 5850 transposon mutants showed that the indole response requires the PQS transcriptional regulator MvfR. Our results suggest P. aeruginosa detects indole through the PQS system and represses its QS-controlled virulence factors while degrading indole, enhancing biofilm formation, and increasing antibiotic resistance. Hence, both indole-related and uracil-related compounds constitute novel anti-virulence approaches for the recalcitrant pathogen P. aeruginosa.

IN VIVO METABOLIC FLUX ANALYSIS: HEPATIC METABOLISM AFTER SEVERE TRAUMA

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Metabolic derangements in disease are poorly understood because of the complex, highly connected nature of the metabolic networks. Metabolic engineering techniques, such as Metabolic Flux Analysis (MFA), which uses an integrative systems-level analysis, could tackle the problem of complexity. However, prior in vivo studies commonly lack a thorough analysis of metabolic pathways, whereas ex vivo studies, such as isolated organ perfusions, introduce experimental artifacts. The purpose of this study was to implement MFA to characterize the metabolic changes in liver following severe trauma. As a means to develop this approach, we used a rat model of thermal injury. Three groups of Sprague Dawley rats received a sham, 20% or 40% TBSA (Total Body Surface Area) burn. On the fourth day post-injury, following an 18-24 hour fast, flow rates of the portal vein, hepatic artery and suprahepatic vena cava were measured using an ultrasound probe. Blood was sampled from each vessel, and processed for blood gases and biochemically analyzed for multiple metabolites. Flow rates, and input/output concentrations in each blood vessel were used as raw data to feed into a simplified metabolic model of liver metabolism and yield intracellular fluxes via MFA. The results show significant metabolic alterations in liver as a consequence of injury and are consistent with the notion that the liver plays a key role in the metabolic derangements and hypercatabolic response following severe burns and trauma. MFA showed an increase in pyruvate synthesis, intracellular glutamate synthesis from histidine and increased tyrosine and phenylalanine uptake and metabolism for the 20% TBSA injury as compared to the sham control condition. For the 40% TBSA condition, a significant increase in urea cycle, gluconeogenesis, pentose phsophate pathway (PPP), tricarboxylic acid (TCA) cycle, acetyl-CoA synthesis, oxygen uptake and mitochondrial metabolism were observed as compared to the sham controls. In addition, we compared these in vivo results to prior results obtained from ex vivo perfusions of livers isolated similar animals. As expected, ex vivo perfusion introduced striking changes in liver metabolic fluxes; however, some of the major effects of thermal injury were observed both in vivo and ex vivo, such as the flux increases in PPP, TCA cycle, and gluconeogenesis.

THE USE OF GENOME SCALE MODELS FOR METABOLIC ENGINEERING

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Since the formulation of the first genome-scale models in 1999/2000, their use and scope has growth rapidly (NBT, 26:659, 2008). Their practical utility for metabolic engineering applications is being realized and impacting strain designs and process development. This talk will review the history of this field, assess its current status, and speculate on the potential future uses of genome-scale metabolic models.

YEAST AS PLATFORM FOR PRODUCTION OF NUTRACEUTICAL INGREDIENTS

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Today, the major part of nutraceutical ingredients is extracted from plants and animals or chemically synthesized. However, the production of nutraceutical ingredients by fermentation will play an increasingly important role in supplying high quality nutraceutical ingredients. The present presentation will focus on the production of two different nutraceutical ingredients in *Saccharomyces cerevisiae*: the polyphenol trans-resveratrol and long chain polyunsaturated fatty acids (PUFA's)

Trans-resveratrol has been reported to have anti aging properties, cancer preventive activity, and cardiovascular protective activity. Today, it is available as a dietary supplement containing extracts from plants including grape and Japanese knotweed. However, there exist demands for supply of resveratrol at reproducible quality and at lower cost.

The long chain omega-3 PUFA's eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) are well established as nutraceutical ingredients mainly because of their beneficial effects in preventing cardiovascular disease. DHA in combination with the omega-6 PUFA arachidonic acid (ARA) is required for proper development of brain and neural systems of infants, and intake of these fatty acids is therefore vital during the first years of life. Additionally, adequate intake of omega-3 PUFAs can promote cognitive development in children, and reduce inflammation.

S. cerevisiae does not naturally produce trans-resveratrol or long chain PUFA's. It is possible to achieve trans-resveratrol production from the aromatic amino acids such as L-phenylalanine or L-tyrosine by expression of 3 – 4 heterologous genes, encoding phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumaroyl-ligase (4CL) and resveratrol synthase (RES). Alternatively, the first two steps (PAL, C4H) can be replaced by tyrosine ammonia lyase (TAL). Through various rounds of metabolic engineering and pathway optimization a strain was constructed that is currently used to scale up the production process from laboratory to industrial scale.

Synthesis of the omega-3 PUFA eicosapentaenoic acid (EPA) by *S. cerevisiae* was achieved by expression of heterologous genes encoding a delta-12 desaturase, a delta-6 desaturase, a delta-6 elongase, a delta-5 desaturase and an omega-3 desaturase. We found that additional expression of a heterologous delta-9 desaturase with high specificity towards C18:0 fatty acids improved the EPA content of the recombinant yeast. Furthermore, the EPA yield has been improved by targeted modifications to the fatty acid and lipid metabolism, for example by manipulation of the endogenous cytochrome b5/cytochrome b5 reductase complex which is involved in fatty acid desaturation. In summary, EPA volumetric productivity was improved more than 100-fold from initial results through pathway optimization, metabolic engineering, and fermentation optimization.

NEW INSIGHTS ON THE ROLE OF THE SIGMA FACTOR RPOS AS REVEALED IN ESCHERICHIA COLI STRAINS LACKING THE PHOSPHOENOLPYRUVATE:CARBOHYDRATE PHOSPHOTRANSFERASE SYSTEM

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It has been demonstrated that about 10% of the E. coli genes are under direct or indirect control of RpoS. Therefore, Weber et al. [2005] proposed that this sigma subunit should be considered a second vegetative sigma factor under non-optimal growth conditions. We have demonstrated that in the phosphoenolpyruvate: carbohydrate phosphotransferase system deficient (PTS-) derivatives PB11 and PB12 of strain JM101 that grow permanently slowly on glucose, the inactivation of RpoS resulted in decreased growth rates of 50 and 10%, respectively. Real-time PCR (RT-PCR) analysis confirmed the important role of this sigma factor in the PTS- strains and allowed the identification of 19 genes including almost all the glycolytic genes, not previously reported, to be at least partially dependent on RpoS. The transcription level of gpp, spoT, ppa and ndk whose products are involved in ppGpp metabolism was upregulated in strain PB12 as compared to the parental strains PB11 and JM101. In the PTS- strains, at least three of these genes (gpp, spoT and ppa) were mainly or partially transcribed by RpoS which is known to require ppGpp for activation, while only gpp was highly RpoS-dependent in the parental PTS+ strain JM101. The role of RpoS in the transcription of these and other genes (poxB and acs), involved in slow growth on glucose, will be discussed.

MICROBIAL HIGH MOLECULAR WEIGHT HYALURONIC ACID PRODUCED THROUGH METABOLIC ENGINEERING

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Hyaluronic acid synthase (HAS) is similar to other membrane-associated glycosyl transferases producing abundant beta-linked polysaccharides such cellulose, chitin and beta-glucan. While the mechanisms of elongation are well understood, the mechanism of termination and hence molecular weight control is not. Intrinsic features of HAS such as conserved cysteine residues are believed to dictate the maximum chain length that can be retained against the torque exerted by the expanding polymer. The molecular weight realized in microbial culture, however, is much less than this maximum and – significantly – much less than the molecular weight that can be realized through extraction from rooster comb.

The molecular weight realized in streptococcal cultures is greatly affected by culture parameters such as sugar source and oxygen availability indicating that resource availability (energy and carbon) is a major factor. Over the past decade, we have explored with limited success various process and strain engineering strategies to release more resources for HA production. Through systematic overexpression of each gene in the HA pathway and comprehensive omics analysis of the resultant strains, we have now successfully broken the 4 MDa barrier, up from 1-2 MDa for wildtype strains.

The study highlights the power of systems biotechnology to tackle quality traits such as molecular weight. The key to the success in this case was to achieve a proper balance between the two activated HA precursors, UDP-N-acetyl glucosamine and UDP-glucuronic acid. Presumably, HAS terminates polymerization if a precursor enters the enzyme out of turn. Through the understanding gained through the systems approach, it was possible to identify a number of strain and bioprocess engineering strategies that led to high Mw.

COMPREHENSIVE PHENOTYPIC ANALYSIS FOR IDENTIFICATION OF GENES AFFECTING GROWTH UNDER STRESS CONDITION IN BIOPROCESSES

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Studies of the genome-wide response of industrial microorganisms to stress conditions using microarrays and comprehensive expression data have resulted in the construction of some stress tolerant strains. However, the effect of gene manipulation such as deletion and overexpression on the acquisition of stress tolerance in the cells remains obscure. Therefore, analysis of phenotypic changes such as sensitivity and tolerance to stress conferred by genetic modification is important to improve cellular properties. We quantified the growth behavior of all available single gene deletion strains of budding yeast under ethanol stress as a model case. Genome-wide analyses enabled extraction of the genes and determination of the functional categories required for growth under this condition. Statistical analyses revealed that the growth of more than hundred deletion strains under stress induced by 8% ethanol was defective. We classified these genes into known functional categories, and found that many were important for growth under ethanol stress including several that have not been characterized, such as peroxisome. We also performed genome-wide screening under osmotic stress and identified the specific genes and functional categories that are important for growth under ethanol stress. The genes and functional categories identified in the analysis might provide clues to improving ethanol stress tolerance among yeast cells.

METABOLOMICS OF RECOMBINANT YEAST: GENE EXPRESSION, FLUX ANALYSIS AND A MATHEMATICAL MODEL FOR GENE REGULATION OF METABOLISM

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Metabolomics provides the tools for detailed analysis of cell metabolism using extensive gene expression data and flux calculations. As a model system we are using a recombinant *Saccharomyces cerevisiae* that accumulates a very high level of intracellular Superoxide Dismutase (SOD) (ca. 30% of total protein). The results of this study show, for the first time, that it is possible to separate two strains of the same species, the native and the recombinant ones, only on the basis of gene expression data. Regarding global gene expression when the 2 strains change from one growth phase to another, a large part of the genetic machinery of both strains is activated when changing from growth on glucose to growth on ethanol. The situation is reversed when passing from growth on ethanol to the stationary phase. The global analysis of the transcription levels of those genes which participate in the central energy metabolic pathways shows a strong metabolic burden in the recombinant strain.

A stoichiometric model was built, which included 78 reactions. It allowed calculation of the distribution of metabolic fluxes during diauxic growth on glucose and ethanol. Fermentation profiles and metabolic fluxes were analyzed at different phases of growth for the recombinant strain (P+) and for its wild type (P-). The synthesis of SOD by the strain P+ resulted in a decrease in specific growth rate of 34 and 54 % (on glucose and ethanol respectively) in comparison to the wild type. Both strains exhibited similar flux of glucose consumption and ethanol synthesis but important differences in carbon distribution with biomass/substrate yields and ATP production 50 % lower in P+. When comparing gene expression for both P- and P+ during growth on ethanol to that on glucose (Eth/Gluc) 98% of genes (80 genes) of the central metabolic pathways are overexpressed which is totally different from what is observed for Metabolic Flux Analysis, where the specific growth rate is lowered to ca. 40% similar to the reduction observed in fluxes of the TCA cycle and protein synthesis.

We have developed a conyinuous mathematical model that simulates gene regulation of the metabolism of both strains. Gene activation predicted by the model, microarray data and flux calculation will be discussed. The model was evcaluated using 1000 simulations with different initial concentrations, and numerical convergence was obtained in all of them, obtaining the stationary state fluxes. They gave 75% accordance compared with real values (admitted error of 20%). The behaviour of the recombinant strain was also modelled and 95% accordance was found in this case.

A QUANTITATIVE UNDERSTANDING OF DYNAMIC CELLULAR PROCESSES DURING DETOXIFICATION IN THE HUMAN LIVER

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A quantitative understanding of dynamic cellular processes during detoxification in the human liver Matthias Reuss University of Stuttgart, Institute of Biochemical Engineering and Centre Systems Biology, University of Stuttgart, Allmandring 31, D – 70569 Stuttgart, Germany The first part of the contribution focus at discussing results on dynamic modelling of the detoxification system of the liver. The detoxification metabolism shows a high inter-individual variability of the enzyme expression level, especially in the phase I catalyzing cytochrome P450 monooxygenases (CYP). This is caused by individual food and drug treatment, sex, age, diseases, or due to the polymorphism resulting in phenotype plasticity. However, the detoxification functionality has to be maintained against these external and internal perturbations, characterizing its robustness. Based on different mathematical models for structure and dynamics of the detoxification system the important issues of structural and dynamic robustness are discussed. The dynamic modelling of the detoxification process is illustrated for two examples: (1) Drug-drug interaction during simultaneous degradation of Dextromethorphane and Propafenone and (2) degradation of the statin Atorvastatin including modelling of membrane transport. Superimposed to the metabolism responsible for the detoxification we also tackle the complex phenomena related to the genetic regulation of the expression of the various enzymes. Based upon time series of transcript data a Boolean/Probabilistic Boolean framework is presented to reconstruct the regulatory networks governing the activity of a specific CYP in response to a specific drug. The network reconstructed from the time series micro array data is further reduced to a quasi-autonomous subnetwork through application of Markov chain simulations. The results clearly indicate the tight link between gene regulation network and bile acid metabolism. The modelling of these phenomena therefore needs to be based on a strong coupling of the different networks. In addition to the dynamic modelling of the regulatory network a careful analysis of the dynamics of central metabolism of the hepatocytes is therefore required. This issue is also important for one of the model drugs investigated within the project – the statins. The final part of the lecture introduces a new approach of instationary 13C flux analysis for quantitatively describing the metabolic traffic within the central metabolism under different physiological conditions. This flux analysis proved a convenient basis for quantifying cell physiology in terms of engagement of metabolic pathways in overall cellular processes and also in context with detoxification. Also first results of modelling the dynamics of the metabolism based on measurements of intra – and extracellular metabolites are presented.

SYSTEMS METABOLIC ENGINEERING FOR CHEMICALS

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Metabolic engineering became much more powerful thanks to the development in the field of systems biology. Systems-level engineering of metabolic and regulatory networks allows much more global scale engineering of cellular metabolism towards a desired goal. In this lecture, I will present the recent results on systems metabolic engineering of E. coli for the production of chemicals. In particular, I will describe detailed strategy for developing engineered strains producing amino acids, which are metabolites under robust regulation. In the case of threonine (Thr), feedback inhibitions of aspartokinase I and III and transcriptional attenuation regulations were removed. Next, the pathways for Thr degradation were removed. The metA and lysA genes were deleted to make more precursors available for Thr biosynthesis. Transcriptome profiling was performed to identify further targets to be engineered. Then,in silico flux response analysis was performed to identify target genes to be modulated, and their expression levels were manipulated. The final engineered E. coli strain, which is 100% genetically defined, was able to produce Thr with a high yield of 0.393 g Thr per g glucose, and 82.4 g/l Thr by fed-batch culture. Similarly, the development of an engineered E. coli strain overproducing a branched-chain amino acid will be described. To make a bioprocess economically competitive, it is now essential to take a systems metabolic engineering approaches, which take into account the upstream to midstream to downstream processes all together. The general systems metabolic engineering strategy for developing genetically-defined organisms for the efficient production of various bioproducts will be discussed. [This work was supported by the Korean Systems Biology Research Project of the Ministry of Education, Science and Technology. Further support by LG Chem Chair Professorship is appreciated.] [References] Lee, S.Y., Lee, D.-Y., and Kim, T.Y., 'Systems biotechnology for strain improvement', Trends Biotechnol., 23: 349-358 (2005). Park, J.H., Lee, K.H., Kim, T.Y., and Lee, S.Y., 'Metabolic engineering of Escherichia coli for the production of L-valine based on transcriptome analysis and in silico gene knockout simulation', PNAS, 104: 7797-7802 (2007). Lee, K.H., Park, J.H., Kim, T.Y., Kim, H.U., and Lee, S.Y., ' Systems metabolic engineering of Escherichia coli for L-threonine production', Molecular Systems Biology (2007.12). Kim, H.U., Kim, T.Y., and Lee, S.Y., 'Metabolic flux analysis and metabolic engineering of microorganisms', Mol. Biosyst. 4: 113-120 (2008).

Poster Presentations

SESSION A

Subject category: Metabolic Engineering for Biofuels

1. **A yeast biocatalyst for fermenting acid hydrolysate to ethanol**
   Brian Rush and Holly Jessen, Cargill, Inc.

2. **Recombinant expression of resolvase proteins for inducing genome plasticity and generating superior, complex *Clostridium* phenotypes**
   Bryan P. Tracy, Northwestern University and Delaware Biotechnology Institute

3. **Succinic acid production by *Actinobacillus succinogenes* 130Z: Growth on hemicellulosic sugars and elucidation of metabolic pathways for analysis and engineering**
   Bryan Schindler, Michigan State University

4. **Engineering n-butanol production in bacteria**
   David R. Nielsen, Massachusetts Institute of Technology

5. **Nitrate respiration and butanediol production in *Bacillus subtilis*, *Klebsiella oxytoca* and *Paenibacillus polymyxa***
   Espinosa de los Monteros F. Joel, UNIDA-Instituto Tecnologíco de Veracruz

6. **More ethanol in recombinant yeast from modeling: towards pathway modifications using hybrid cybernetic models**
   Hyun-Seob Song, Purdue University

7. **^{13}C-metabolic flux analysis suggests that suppression of carbon dioxide fixation is an important component of hydrogen production by *Rhodopseudomonas palustris***
   James 'Jake' B. McKinlay, University of Washington

8. **Solvent tolerant *Pseudomonas*: towards engineering of an improved biocatalyst for biobutanol production**
   Jana Rühl, Laboratory of Chemical Biotechnology, Faculty of Biochemical and Chemical Engineering, TU Dortmund

9. **Changes in metabolic fluxes of xylose-fermenting *Saccharomyces cerevisiae* strains by overexpression of NADH- or NADPH-dependent 5-hydroxymethylfurfural (HMF) reductase**
   João R. M. Almeida, Lund University

10. **Dynamic modeling and metabolic analysis of ethanol production network in *Saccharomyces cerevisiae***
    Jinwon Lee, Department of Chemical and Biomolecular Engineering, Sogang University

11. ***In silico* simulation for fine-tuning metabolic engineering: application to the improvement of ethanol production yield during *Saccharomyces cerevisiae* alcoholic fermentation.**
    Julien Pagliardini, Ingénierie des Systèmes Biologiques et des Procédés, CNRS, INRA, INSA

12. **Predicting proton flux with the genome-scale model of *Clostridium acetobutylicum*: the model organism for butanol production**
    Ryan S. Senger, University of Delaware

13. **Metabolic and protein engineering for fermentative hydrogen production**
    Thomas K. Wood, Texas A & M University

14. **Culture characterization of an *E. coli* mutant strain metabolically engineered for improved performance under oscillating DOT conditions**
    Ramsés García-Cabrera, Instituto de Biotecnología, Universidad Nacional Atónoma de México (UNAM)

15. **Simulation of dissolved CO2 gradients in recombinant *Escherichia coli* cultures: metabolic and transcriptional response**
    Antonino Baez, Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM)

Subject category: Metabolic Engineering for Nutrition & Agriculture

16. **Metabolic engineering of vanillin production in *S. cerevisiae***
    Ana Rita G Brochado, CMB, BioSys, Technical University of Denmark

17. **An integrated flux analysis and metabolic profiling study to identify pathways causing hepatic lipoapoptosis**
    Jamey D. Young, Massachusetts Institute of Technology

18. **Biotechnological methionine production – potential, pitfalls, prospects**
    Jens O Krömer, Australian Institute of Bioengineering and Nanotechnology (AIBN), University of Queensland

19. **Metabolic flux analysis of maize (*Zea mays*, GS3 X Gaspe) cell suspension culture by using ^{13}C labeling experiment and 2-dimensional nuclear magnetic resonance (NMR) spectroscopy**
    Jong Moon Yoon, Iowa State University

20. **Rational engineering of NADPH metabolism in *Corynebacterium glutamicum* for improved lysine production**
    Judith Becker, TU Braunschweig, Institute of Biochemical Engineering

21. **Improving sesquiterpene production in *Saccharomyces cerevisiae* through integration of the MEP pathway**
    Luca R. Formenti, BioSys-Denmark Technical University

22. **Target selection by top-down systems biology**
    Mariët J. van der Werf, TNO Quality of Life

23. **Anaerobic growth and potential for amino acid production by nitrate respiration in *Corynebacterium glutamicum***
    Seiki Takeno, Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University

Subject category: Metabolic Engineering for Cell Culture

24. **Metabolic flux analysis of differentiating mouse embryonic stem cells (mES cells)**
    Barbara Andrews, University of Chile

25. **Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of ORCA3 along with jasmonic acid elicitation of *Catharanthus roseus* hairy roots over time**
    Christie A. M. Peebles, Rice University

26. **Application of metabolic flux analysis to identify the mechanisms of palmitate toxicity in human hepatoma cell line**
    Christina Chan, Michigan State University

27. **Using the "OMICS" technologies as complementary tools to study the molecular mechanisms involved with the adaptation of myeloma cell line to protein-free medium**
    K. R. de la Luz-Hernández, Center of Molecular Immunology

28. **Rapid generation of FUT8 knockout CHO cell lines using engineered zinc finger nucleases**
    Dr Andrew Snowden, Genentech Inc

29. **Development of large scale kinetic models for metabolic networks: challenges, pitfalls, practical solutions**
    I. Emrah Nikerel, Department of Biotechnology, Delft University of Technology

30. **Tandem mass spectrometry method for metabolic flux analysis**
    Jungik Choi, University of Delaware

31. **Relationship between energetic metabolism and sialic acid levels of r-tPA: flux balance analysis under different hexose concentration in continuous culture of CHO cells**
    Ramón González, Department of Chemical and Biomolecular Engineering, Rice University

32. **A systematic method for modeling the dynamics and heterogeneity of cellular metabolism in batch culture**
    Ryan Nolan, Tufts University / Wyeth BioPharma

33. **Overcoming Lactate Accumulation in Mammalian Cell Culture**
    Zhaohui Geng, Pfizer

34. **Metabolomics as molecular analysis tool in cell culture engineering**
    Maria Klapa, Foundation for Research and Technology-Hellas

Subject category: Miscellaneous

35. **Metabolomic analysis of sex-specific pathways in adult zebrafish**
    Maria I. Klapa, Foundation for Research and Technology-Hellas

36. **Metabolic profiling of a recombinant *E. coli* in fermentation process**
    Shun Luo, Amgen Inc.

37. **Getting the right numbers: how to avoid some (common) mistakes in metabolomics-based research in *S. cerevisiae***
    André B. Canelas, Department of Biotechnology, TU Delft

38. **Microfluidic droplets as nanobioreactors for screening metabolic engineering libraries**
    Benjamin L. Wang, Massachusetts Institute of Technology

39. **Understanding of *E. coli in vivo* evolution under NADPH accumulation stress**
    Isabelle Meynial-Salles, LISBP, INSA

40. **Modeling, rational design and in vivo evolution of a 1, 2 propanediol producer**
    Philippe Soucaille, Metabolic Explorer

41. **Engineering complex phenotypes to enable biofuels and biochemical production**
    Ryan T. Gill, University of Colorado, Colorado Center for Biorefining and Biofuels

42. **An integrated "low volume high throughput cultivation platform" for industrial systems biology: *Streptomyces coelicolor* a case study**
    Prashant Madhusudan Bapat, Technical University of Denmark

43. **Metabolic flux analysis of *Shewanella* spp. reveals evolutionary robustness in central carbon metabolism**
    Hector Garcia Martin, Lawrence Berkeley National Lab

44. **Improved thermostability and acetic acid tolerance of *Escherichia coli* by directed evolution of homoserine o-succinyltransferase**
    Jae-Gu Pan, KRIBB

45. **Uracil-excision based cloning: a fast and efficient method for the creation of DNA-constructs**
    Bjarne Gram Hansen, Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark

46. **Dynamic metabolic flux analysis with linear flux functionality**
    Robert W. Leighty, University of Delaware

47. **Evolutionary multiobjective algorithms for *in silico* metabolic engineering**
    Isabel Rocha, IBB - Institute for Biotechnology and Bioengineering - Center of Biological Engineering - University of Minho

48. **^{13}C-Metabolic flux analysis for the transient in the batch culture using CE-TOF/MS**
    Yoshihiro Toya, Institute for Advanced Biosciences, Keio University
    Systems Biology Program, Graduate School of Media and Governance, Keio University

49. **Simple local flux quantification using ^{13}C-tracer substrate in isotopic non-stationary experiments**
    Zheng Zhao, Delft University of Technology, Department of Biotechnology

50. **Recombineering with Red®/ET® - modification of the bacterial chromosome**
    Tim Zeppenfeld, Gene Bridges GmbH

51. **Enzyme states allow identification of rate-limiting steps**
    Ljubisa Miskovic, Ecole Polytechnique Federale de Lausanne (EPFL)

52. **Identification of the design principles of signaling pathways for metabolic engineering**
    Andrijana Radivojevic, Ecole Polytechnique Fédérale de Lausanne (EPFL)

53. **Systematic reduction of models of template polymerization processes**
    Luis Mier-y-Teran, Ecole Polytechnique Federale de Lausanne (EPFL)

54. **An elementary metabolite units (EMU) method for rational design of labeling experiments for metabolic flux analysis**
    Maciek R. Antoniewicz, University of Delaware

55. **Identification and evaluation of approximative kinetic model structures**
    Jo Maertens, Ghent University

56. **Approximate flux functions**
    Sergio Rossell, Delft University of Technology

57. **Dynamic linlog modeling of the central metabolism of *E. coli*: estimation of elasticities from pulse-response data using gPROMS**
    K. Bernaerts, Kluyver Laboratory of Biotechnology, Department of Biotechnology, Delft University of Technology

58. **^{13}C -EMU FLUX: A simple application for ^{13}C-based steady-state metabolic flux analysis**
    Lake-Ee Quek, Australian Institute for Bioengineering and Nanotechnology (AIBN), University of Queensland

59. **Development of an accurate method for intracellular metabolome analysis in *Escherichia coli* for *in vivo* kinetic analysis**
    Hilal Taymaz Nikerel, Department of Biotechnology, Delft University of Technology

60. **Ranking of most influential kinetic parameters in metabolic networks through Global Sensitivity Analysis**
    J. Di Maggio, Planta Piloto de Ingenieria Quimica PLAPIQUI - Universidad Nacional del Sur

61. **Pairwise selection assembly for metabolic pathway construction and engineering**
    William J. Blake, Codon Devices, Inc.

62. **Mutagenesis of the bacterial RNA polymerase core enzyme for engineering complex phenotypes**
    Daniel Klein-Marcuschamer, Massachusetts Institute of Technology

SESSION B

Subject category: Metabolic Engineering for Chemicals and Materials

1. **Production of shimikic acid in an *Escherichia coli* strain lacking the phosphoenolpyruvate:carbohydrate phosphotransferase system.**
   Adelfo Escalante, Instituto de Biotecnología, Universidad Nacional Atónoma de México (UNAM)

2. **Metabolic modeling of the n-alkane bioconversion: functional modeling package development**
   Alistair P. Hughes, University of Cape Town

3. **High cell density accumulation of engineered *Escherichia coli* without external glucose feeding for the production of biopharmaceuticals: overcoming overflow metabolism in batch cultures**
   Alvaro R. Lara, Departamento de Procesos y Tecnología, Universidad Autónoma Metropolitana-Cuajimalpa

4. **Genomic and proteomic analysis of lycopene-overproducing *Escherichia coli* strains**
   Brian E. Mickus, Massachusetts Institute of Technology

5. **Enhancement of anti-HIV peptide T-20 production in recombinant *Escherichia coli* by analysis of metabolic load**
   Byoung Hoon Yoon, Korea Advanced Institute of Science and Technology (KAIST)

6. **Combinatorial engineering of *Escherichia coli* for optimizing L-tyrosine production**
   Christine Nicole S. Santos, Massachusetts Institute of Technology

7. **Modeling of batch fermentation kinetics for succinic acid production by *Mannheimia succiniciproducens***
   Yong Jae Jeon, Korea Advanced Institute of Science and Technology (KAIST)

8. **Towards novel biopolyamides - metabolic engineering of *Corynebacterium glutamicum* for production of 1,5-diaminopentane**
   Christoph Wittmann, Biochemical Engineering Institute, Technical University Braunschweig

9. ***In-silico* design of multiple mutations for amino acid production in *Corynebacterium glutamicum***
   Elmar Heinzle, Biochemical Engineering Institute, Saarland University, Germany

10. **Proteome analysis of hyaluronic acid producing bacteria for strain optimisation**
    Esteban Marcellin, Australian Institute of Bioengineering and Nanotechnology (AIBN), University of Queensland

11. **Effect of the pyruvate kinase activity on the phenylalanine yield of *E coli* strains that lack phosphotransferase system**
    Eugenio Arturo Meza Mora, Instituto de Biotecnología, Universidad Nacional Atónoma de México (UNAM)

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    Goutham Vemuri, Chalmers University of Technology

A YEAST BIOCATALYST FOR FERMENTING ACID HYDROLYSATE TO ETHANOL

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A Yeast Biocatalyst for Fermenting Acid Hydrolysate to Ethanol Biotechnology Development Center Cargill, Incorporated Several characteristics are required for an economically viable biocatalyst for cellulosic ethanol production, including high yield, ethanol productivity and tolerance, robust growth with simple media, and resistance to inhibitors such as acetate in acid hydrolyzate. While we have previously demonstrated a technology that enables efficient fermentation of xylose to ethanol in yeast (US Patent App 10/554887), the utilization of hydrolyzate sugars in an acid hydrolyzate presents a greater challenge. With funding assistance from the US DOE, Cargill is attempting to meet this challenge by applying our xylose fermentation technology to an acetate-tolerant proprietary yeast. To enable simultaneous saccharification and fermentation (SSF), project goals have been set for ethanol production from mixed sugars (dextrose, mannose, xylose, and arabinose) in the presence of 10 g/L acetate at 40oC and at a pH less than 5.0. Under these conditions, and before improvement of acetate tolerance, the Cargill host strain can utilize 80 g/l of dextrose and 80 g/l of mannose in less than 36 hours, producing ~ 70 g/l ethanol. Under the same conditions, the *Saccharomyces cerevisiae* strain CEN.PK 113-7D is unable to consume either sugar. A xylose utilization pathway has been engineered into this strain and the fermentation of this sugar to ethanol has been demonstrated.

RECOMBINANT EXPRESSION OF RESOLVASE PROTEINS FOR INDUCING GENOME PLASTICITY AND GENERATING SUPERIOR, COMPLEX *CLOSTRIDIUM* PHENOTYPES

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Genome manipulation and recombinant protein expression are common approaches to generate desirable microbial phenotypes for commercial production of valuable chemicals. Of considerable interest are species of the genus *Clostridium* due to their natural ability to convert cellulosic feedstocks into valuable transportation fuels like butanol. Unfortunately the genetic tool-box for manipulating *Clostridium* is lagging behind the commercial desire to accelerate the development of novel, superior cellulose degrading strains. Specifically, both industry and academia need to be able to knockout genes, knock-in large synthetic operons, accelerate horizontal gene transfer, and induce random mutagenesis in all *Clostridium*.

In response, we developed a completely novel approach to tackle all the aforementioned needs. From literature and our own comparative genomic study, we recognized that all sequenced *Clostridium* genomes are void of any annotated resolvase enzyme. Resolvase enzymes are the essential proteins for catalyzing the resolution of Holliday-junctions during homologous recombination and are shown to impair homologous recombination and DNA repair in deletion mutants. In addition to the genome study, the lack of resolvase activity in *Clostridium* is suggested by the difficulty in achieving gene disruptions by homologous recombination. Subsequently, we recombinantly expressed the resolvase, RecU, from *Bacillus subtilis* (*B. subtilis*) in *C. acetobutylicum* while attempting to disrupt the *spo0A* gene via a replicating plasmid approach. We isolated and characterized numerous perfect Campbell-like double crossover mutants, which were spo0A knockouts. We employed this same approach to generate many more gene disruption mutants that exhibit desirable commercial characteristics for generating butanol. We also demonstrate that resolvase over-expression can rapidly generate random genome mutations when grown under strong selection, can site specifically knock-in large synthetic operons into genome, and can sufficiently increase homologous recombination frequency for the generation of gene disruption libraries in *C. acetobutylicum*. Lastly, the fact that resolvase enzymes are absent from all completely sequenced *Clostridium* genomes, suggests this approach is applicable to all *Clostridium* species and will be a substantial contribution to all fields of *Clostridium* research.

SUCCINIC ACID PRODUCTION BY *A. SUCCINOGENES* 130Z: GROWTH ON HEMICELLULOSIC SUGARS AND ELUCIDATION OF METABOLIC PATHWAYS FOR ANALYSIS AND ENGINEERING

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The rumen bacterium *Actinobacillus succinogenes* 130Z (130Z) naturally produces among the highest titers of succinate in mixed acid fermentations, and is therefore attractive for industrial bio-based succinate production. For production to be economically viable, the organism must produce succinate rapidly and to high titers on inexpensive carbon sources (i.e., hemicellulosic hydrolysates or glycerol). It should also excrete little to none of its current fermentation byproducts, acetate, formate, and ethanol. To optimize 130Z's growth on lignocellulosic hydrolysates, it is useful for the organism to grow rapidly in a defined minimal growth medium (AM3) on each of the lignocellulosic sugars. Currently, in AM3, 130Z ferments glucose markedly faster than other hemicellulosic sugars. Adaptation for rapid growth on these other sugars was achieved using a serial propagation approach. After sixty passages in AM3 supplemented with D-xylose, 130Z's generation time (T_G) decreased from 6.5 h to 1.8–2.8 h. The same strain was then evolved for fast growth on other sugars. After fifty passages on L-arabinose, 130Z's T_G on L-arabinose decreased from 16.6 h to 2.1–2.7 h. These T_G's on pentoses are close to that on D-glucose, 1.2 h. Work is in progress to engineer 130Z for growth on glycerol. AM3 contains ten vitamins and cofactors, but we have shown that 130Z requires only four of them, biotin, nicotinic acid, pantothenic acid, and pyridoxine HCl, leading to the creation of a new medium, AM7. Manual annotation of 130Z's recently completed genome sequence confirmed the absence of genes involved in *de novo* biosynthesis of these compounds. The potential use of these auxotrophies in 130Z genetic engineering is discussed. To eliminate fermentation byproducts, work is in progress to clone and express additional candidate marker genes in 130Z for later use to create knockout mutants by allele exchange.

ENGINEERING N-BUTANOL PRODUCTION IN BACTERIA

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Amid rising fuel costs, emergent environmental issues, and mounting international security concerns, considerable attention has recently returned to reviving biologically derived n-butanol as a renewable transportation fuel alternative. n-Butanol boasts physical and thermodynamic properties which are akin to gasoline and are in many ways superior to those of biologically derived ethanol. The natural n-butanol fermentation pathway in *Clostridium* competes with several other native pathways for metabolite fluxes which can thereby reduce its final yield. Furthermore, the cytotoxic effects of n-butanol contribute to low titers in natural organisms due to feedback inhibition. Accordingly, there is much interest in designing robust, alternative microorganisms capable of producing n-butanol. Through metabolic engineering efforts we have explored the potential for n-butanol production in a variety of bacterial hosts, including *E. coli, P. putida,* and *B. subtilis*. Within these hosts, we have explored both the reconstruction of the natural *Clostridium* pathway as well as the use of homologous genes from a variety of source organisms. The development of alternative hosts for the heterologous n-butanol pathway reveals important implications for both production and product tolerance.

NITRATE RESPIRATION AND BUTANEDIOL PRODUCTION IN BACILLUS SUBTILIS, KLEBSIELLA OXYTOCA AND PAENIBACILLUS POLYMYXA

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When microorganisms aerobic growth is oxygen limited, some of them have the possibility of anaerobic growth by nitrate respiration. It is a biological oxidative process used to recycle reduced coenzymes and for energy generation, the final product is nitrite, which can follow two routes, one is denitrification and the other is a short circuit in the nitrogen cycle because nitrite is converted to ammonia. The catabolism of carbohydrates under these circumstances can follow a fermentation pathway, for example butanediol accumulation, so respiration and a fermentation can function simultaneously. Butanediol pathway is a buffer for transition between aerobic and anaerobic condition, acetoin is accumulated if nitrate concentration is limited and butanediol is produced under anaerobic nitrate sufficiency.

Butanediol is a fermentation product with singular chemical properties and can be used for different purposes, these include dehydration to methyl ethyl ketone (industrial solvent), reaction with acetone to produce a 'tetramethyl' compound(a possible gasoline blending agent), formation of butene, butadiene and finally as a biofuel. There are several microorganisms which have been used for butanediol studies, mainly *Paenibacillus polymyxa, Bacillus subtilis and Klebsiell oxytoca*. We have studied fermentation kinetics of butanediol fermentation under nitrate respiration conditions with them and metabolic flux of the catabolic pathways will be presented.
Furthermore product other tan butanediol can be obtained, this is exemplified by *aprE*, subtilisin gene expression which was evaluated with a wild and a genetic improved strain of *B. subtilis*, it is an example of how a this process could be used for practical purposes other than butanediol fermentation.

MORE ETHANOL IN RECOMBINANT YEAST FROM MODELING: TOWARDS PATHWAY MODIFICATIONS USING HYBRID CYBERNETIC MODELS

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In silico analysis based on cybernetic modeling was used to investigate the potential of further increasing bioethanol productivity from lignocellulose-derived sugars by modifying metabolic pathways of fermenting yeast strains. A hybrid cybernetic model was formulated for the Ho-Purdue yeast strain 1400 (pLNH33) by incorporating elementary mode analysis into the cybernetic modeling framework. The hybrid model (Kim et al., 2008; Song and Ramkrishna, 2008) features the dynamical description of metabolic processes as a combination of elementary mode fluxes, the modulation of which is made by cybernetic control laws towards maximizing a predetermined metabolic fitness function (e.g., carbon uptake rate). In a comparative study, the hybrid model has outperformed other dynamic metabolic models such as macrokinetic models and dynamic flux balance analysis, as validated by several different sets of fermentation data. Through comprehensive dynamic simulations together with sensitivity analysis, the hybrid model could identify the most promising target pathway among many elementary modes for metabolic modification. The implications of the target mode are not only in tune with previous metabolic engineering efforts, but also suggest the importance of a few genes which have received little attention. Computer simulations show that overexpression of the target pathway can result in substantial increase of bioethanol productivity. The continuing study is being conducted to single out a specific set of target genes among the reactions included in the identified target mode using a more elaborate cybernetic model.

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^{13}C-METABOLIC FLUX ANALYSIS SUGGESTS THAT SUPPRESSION OF CARBON DIOXIDE FIXATION IS AN IMPORTANT COMPONENT OF HYDROGEN PRODUCTION BY *RHODOPSEUDOMONAS PALUSTRIS*

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Rising energy demands and the imperative to reduce CO_2 emissions are stimulating research and development of bio-based fuels. H_2 is one of the most promising biofuels, having about three times the energy content of gasoline. *Rhodopseudomonas palustris* naturally uses energy from sunlight and electrons from organic waste to produce H_2 and ammonia using any of its three nitrogenases. To improve *R. palustris* H_2 production through metabolic engineering we need to first understand the pathways and proteins that transfer electrons from substrates to nitrogenase; the site of H_2 production. Using different ^{13}C-acetate substrate isotopomers, we compared metabolic fluxes in wild-type *R. palustris*, where H_2 production is repressed by NH_3, versus a mutant strain that produces H_2 in the presence of NH_3. The results highlight the importance of Calvin cycle flux for maintaining redox balance during photoheterotrophic growth. The wild-type strain used ribulose bisphosphate carboxylase to fix 74% of the CO_2 that it produced from oxidizing acetate. The H_2-producing mutant was less reliant on Calvin cycle flux to maintain redox balance as H_2 production via nitrogenase served as an alternative electron dump. Calvin cycle flux in the mutant was only 15% of that of the wild type. The decreased Calvin cycle flux accounted for 53% of the H_2 produced by the mutant, with the rest of the electrons for H_2 production being diverted away from biomass production. We also compared the wild type and an H_2-producing mutant, grown under the same conditions, by microarray analysis. Interestingly, genes encoding Calvin cycle enzymes were down-regulated 4-16 fold in the mutant, compared to the wild type. Thus, H_2 production does not simply out-compete CO_2 fixation for electrons but rather the *R. palustris* mutant senses the availability of H_2 production as an alternative electron dump and down-regulates the Calvin cycle accordingly.

SOLVENT TOLERANT PSEUDOMONAS: TOWARDS ENGINEERING OF AN IMPROVED BIOCATALYST FOR BIOBUTANOL PRODUCTION

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Within the last years, fuels from renewable feedstocks have attracted increasing interest as alternatives to fuels from petrochemical sources. Thereby, not only economical but also ecological aspects like resource efficiency of biofuels have been considered. As the biofuels ethanol and biodiesel perform badly in these regards, we considered it worth to investigate fundamental aspects of the production of biobutanol, a 2nd generation biofuel. Existing fermentation processes for butanol production, already developed in the 1920s, are limited mainly by the low achievable product titers. The purification of highly diluted alcohols from fermentation broths is expensive and only energetically feasible at concentrations above 3 % (v/v). At these concentrations, butanol is however highly toxic to microorganisms, resulting in the low product titers achieved by bacteria of the genus Clostridia.

With the objective to overcome this major limitation, we investigated the butanol resistance of solvent tolerant *Pseudomonas putida* strains during growth and resting cell culture conditions. Indeed, *P. putida* strains as potential candidates for the engineering of novel butanol production strains tolerated (grew at) up to 130 % higher butanol concentrations as compared to the conventionally used Clostridia strains. Using an evolutionary engineering strategy, we could further increase the butanol tolerance to more than 300 % corresponding to 5 to 8 % (v/v) of butanol in the fermentation broth. A detailed characterization of the metabolism of these hyper-butanol-tolerant mutants will be presented. In particular, the interplay of central carbon metabolism operation and butanol resistance will be highlighted and discussed. These mutants raise the limit of product tolerance to a level, which makes biobutanol production from renewable feedstocks economically feasible.

CHANGES IN METABOLIC FLUXES OF XYLOSE-FERMENTING *SACCHAROMYCES CEREVISIAE* STRAINS BY OVEREXPRESSION OF NADH- OR NADPH-DEPENDENT 5-HYDROXYMETHYLFURFURAL (HMF) REDUCTASE

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Baker's yeast *Saccharomyces cerevisiae* is notably considered as the organism of choice for the production of bioethanol from lignocellulosic feedstocks. Efficient ethanol production by *S. cerevisiae* requires generating yeast strains that (i) can efficiently ferment all sugars that are present in the lignocellulosic hydrolysate, i.e. both hexose and pentose sugars and (ii) can tolerate the inhibitors (furaldehydes, acids and phenolics) that are released during the hydrolysis steps.

S. cerevisiae strains capable of fermenting xylose to ethanol have been generated by introducing the fungal xylose pathway consisting of NAD(P)H-dependent xylose reductase (XR) and NAD+-dependent xylitol dehydrogenase (XDH). However, the by-product xylitol is formed due to the difference in co-factor usage between XR and XDH.

Furaldehydes, like 5-hydroxymethylfurfural (HMF) and furfural, are known to inhibit yeast growth and viability and to reduce ethanol productivity. Yeast strains are able to reduce HMF and furfural to less toxic compounds, however, the rate of inhibitor conversion and cofactor utilization are strain dependent. Recently, we identified a mutated-ADH1p (mut-ADH1p) and ADH6p as NADH- and NADPH-dependent enzymes responsible for HMF conversion in *S. cerevisiae*.

The influence of enhanced HMF conversion on xylose metabolism was investigated in xylose-fermenting *S. cerevisiae* strains in which either the mut-ADH1 or ADH6 gene was overexpressed. In presence of HMF, strains overexpressing mut-ADH1 or ADH6 showed better sugar consumption rate and ethanol yield than control strain in glucose and xylose anaerobic batch and continuous culture. Furthermore, xylitol, glycerol and acetate yields were differently affected by the preferential use of NADH or NADPH in the HMF reduction. The influence of NADH- and NADPH-dependent HMF reduction on metabolic fluxes is discussed.

DYNAMIC MODELING AND METABOLIC ANALYSIS OF ETHANOL PRODUCTION NETWORK IN SACCHAROMYCES CEREVISIAE

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Mathematical that can organize the available experimental information, and provide insight and guidance for successful metabolic engineering and computational methods are needed. In order to optimize the ethanol production in Saccharomyces cerevisiae a metabolic pathway of the ethanol production was constructed and analyzed. In this study the analysis of mechanism in metabolic system, regulation pattern, and regulations control were carried out by studying each factors in the regulation map. In order to compose the regulation map, effectors that mainly effect the enzyme reaction were especially closely studied, among the various effectors in the metabolic system of Saccharomyces cerevisiae. The studied effectors in this research are inhibitors, activating compounds, cofactors, and metal ions. All of them are related in the metabolic mechanism of the Saccharomyces cerevisiae, and has an effect on the enzyme reaction. Moreover, the mainly studied effectors were enzymes in each reaction steps. The data of enzymatic kinetic related to carbohydrate metabolic network of Saccharomyces cerevisiae are based on literature and web database. Kinetics of several specific enzymes that have particular impedance were also studied. In addition, the new enzyme kinetics from the literature, enables the experimenter to acquire more accurate design model which has higher resemblance with the actual cell metabolism. MATLAB were used as a simulator. Newton method was mainly used to derivative calculation in MATLAB, and LSDOA(Livermore Solver of Ordinary Differential Equation) was mainly used to solve ODE(Ordinary Differential Equation) in MATLAB. The merit of data provided by MATLAB is that it is easy to read kinetics information of metabolites in timescale, and also the information of MCA(metabolic control analysis). The key factor of ethanol production in Saccharomyces cerevisiae was studied by measuring the elasticity coefficient and parameter elasticity. In order to obtain the quantitative value of the parameter elasticity coefficients the euclidean norm which base on vector norm were used. MATLAB was used for calculation of the euclidean norm. Parameter sensitivities represents the fundamental aspects of flux control so they summarize the extent of systemic flux control exercised by the activity of a single enzyme in the pathway. It will be help to desigin an optimization pathway to ethanol production in Saccharomyces cerevisiae.

IN SILICO SIMULATION FOR FINE-TUNING METABOLIC ENGINEERING: APPLICATION TO THE IMPROVEMENT OF ETHANOL PRODUCTION YIELD DURING *SACCHAROMYCES CEREVISIAE* ALCOHOLIC FERMENTATION.

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The recent advances in yeast promoter engineering open a new era in the metabolic engineering for improving the robustness of engineered strains. Fine-tuning the metabolic fluxes with the help of different strength promoters gives a higher flexibility in engineering strains and allows to better consider cell's requirements such as redox balances, energetic and stress issues. Therefore quantitative information on metabolic fluxes is needed in order to help choosing the respective promoter strength for the targeted genes. This could be obtained using metabolic simulator based on hybrid metabolic flux model.

We developed here a metabolic simulator constituted of both a model of the metabolic network of *Saccharomyces cerevisiae* and a phenomenological model. The metabolic network is a MFA compartmented model with segregation between mitochondrial and cytosolic reactions. The phenomenological module includes equations modelling growth, substrate consumption, metabolite production and inhibition/stress related phenomena.

We applied this strategy at engineering *S. cerevisiae* for improving its ethanol production yield during high performance ethanolic fermentation. Numerous studies have investigated the opportunity of redirecting the carbon flow from glycerol (the main by-product of alcoholic fermentation) toward ethanol production. Though such modifications considerably alter the central metabolism of the micro-organism and severely compromised its ability to cope with the stress encountered in an industrial process. These studies have enlightened the complexity of the problem and stressed out the necessity of using a predictive tool able to anticipate the comportment of the engineered strains. Our metabolic simulator was used to determine the optimal carbon flow needed in the glycerol synthesis pathway to fulfilled growth and stress resistance requirements. The model predictive capacities were then evaluated by testing mutant strains in which fine-tuning of the glycerol synthesis was obtained in gpd2 delta CEN.PK113-7D background where the native *GPD1* promoter was replaced by weaker promoter. These strains designed to present a reduced glycerol production were tested in industrial relevant conditions both aerobically and anaerobically. The best result showed an improvement of 7% of the ethanol production yield on glucose for an identical final ethanol concentration of 114 g/L in 48 hours (compared to 115 g/L ethanol in 38 hours with the wild type). The 85% decrease in the glycerol production led to a 25% reduction of the µmax but no loss in cell's viability.

PREDICTING PROTON FLUX WITH THE GENOME-SCALE MODEL OF *CLOSTRIDIUM ACETOBUTYLICUM*: THE MODEL ORGANISM FOR BUTANOL PRODUCTION

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Genome-scale models represent the mathematical link from a cellular genotype to an expressed phenotype. These models also serve as an adaptable platform for discovering: (i) the metabolic potentials of an organism, (ii) metabolic flux distribution in response to stress or changing environment and (iii) targets for metabolic engineering. A genome-scale model consisting of more than 400 metabolites, involved in greater than 500 biochemical reactions (including over 80 membrane transport reactions), has been constructed for *Clostridium acetobutylicum* ATCC 824, a strict anaerobe producing butanol, acetone, ethanol, butyrate and acetate. Model simulations have identified the importance of specific proton flux states during vegetative growth of this bacterium. Additionally, the incorporation of a complex pH model led to correct predictions of the extracellular medium pH, and this allowed for a more in-depth flux analysis of all reactions responsible for proton secretion. Proton efflux from cation channels was found significant during acidogenic phases of batch growth; however, slight proton uptake through cation channels was calculated for the late exponential phase of batch growth, which is accompanied by highly acidic culture conditions. Further evaluation of model constraints determined the number of constrained and unconstrained metabolic reactions required to predict observed phenotypes using the genome-scale model.

METABOLIC AND PROTEIN ENGINEERING FOR FERMENTATIVE HYDROGEN PRODUCTION

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Molecular hydrogen is an environmentally-clean fuel and the reversible (bi-directional) hydrogenase of Escherichia coli hydrogenase 3 holds great promise for hydrogen generation using renewable feedstocks (e.g., glucose). Hydrogenase 3 produces hydrogen via the reaction 2H+ + 2e- „\ H2 (g); the source of the two electrons is formate. There are three hydrogenase enzymes in E. coli: hydrogenase 3 (encoded by hycABCDEFGHI) primarily produces hydrogen and hydrogenase 1 (encoded by hyaABCDEF) and hydrogenase 2 (encoded by hybOABCDEFG) only consume hydrogen. All three enzymes are [NiFe]-hydrogenases and require the auxiliary proteins HypABCDEF for maturation. E. coli produces hydrogen from formate by the formate hydrogen lyase system (FHL) that consists of hydrogenase 3 and formate dehydrogenase-H (encoded by fdhF). Cloning the (bi-directional) hydrogenase of the cyanobacterium Synechocystis sp. PCC 6803 in E. coli to increase hydrogen production 41-fold (BMC Biotechnology 7:25, 2007) taught us the importance of eliminating the activity of hydrogenase 1 (encoded by hyaABCDEF) and hydrogenase 2. Therefore, using the isogenic E. coli K-12 library containing all non-lethal deletion mutations (3985 genes) created by the Genome Analysis Project in Japan (Keio collection), we developed a rapid (2 day) technique to make unlimited metabolic deletions in the chromosome using successive P1 transductions. The result was a quintuple mutant BW25113 hyaB hybC hycA fdoG/pCA24N-FhlA) that produces 141 times more hydrogen by incorporating the best of the pathway mutations hyaB, hybC, focA, focB, fnr, narG, fdoG, and fdnG along with fhlA and hycA (Microbial Biotechnol. 1: 30-39, 2008). This strain produces the most hydrogen to date and reaches the theoretical limit of 1 mol H2/mol of formate. We also developed a septuple mutant BW25113 hyaB hybC hycA fdoG frdC ldhA aceE that increased hydrogen production 5-fold from glucose and increased the hydrogen yield 2-fold from 0.65 to 1.3 mol H2/mol glucose (maximum yield is 2 mol H2/mol glucose) (Appl. Microb. Biotechnol. 77:879-890, 2007). The rapid P1 transduction method for metabolic engineering is general and allowed us to discern that hydrogenase 3 also has significant hydrogen consumption activity (Appl. Microb. Biotechnol. 76: 1035-1042, 2007). We have also used protein engineering to create hydrogenase 3 variants with 30-fold enhanced hydrogen production; this is the first random protein engineering of a hydrogenase, provides the first structure-function information for hydrogenase 3, and showcases the direct detection of hydrogen in bacterial colonies using a tungsten oxide/palladium detector for rapid screening (Appl. Microb. Biotechnol. 79: 77-86, 2008).

CULTURE CHARACTERIZATION OF AN *E. COLI* MUTANT STRAIN METABOLICALLY ENGINEERED FOR IMPROVED PERFORMANCE UNDER OSCILLATING DOT CONDITIONS

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In large scale bioreactors under conventional operating conditions, dissolved oxygen tension (DOT) gradients may occur, affecting the metabolic and physiological behavior and in most of the cases reducing the productivity. Recently our group developed an engineering strain named VAL24, a triple mutant genetically engineering to reduce the generation of acetate, lactate, and formate, when exposed to DOT fluctuations (Lara et al., 2006). In our previous work, VAL24 was characterized under simulated DOT gradients in a two compartment scale-down systems with circulation time (t_c) of 50s. The strain was able to maintain a faster growth rate and higher recombinant protein productivity than the parental strain. In this study we evaluated the metabolic and physiological response of VAL24 subjected to DOT gradients simulated in a scale-down system consisting of two interconnected stirred tank bioreactors, one maintained at a DOT near 0% and the other at 10%. Kinetic, stoichiometric, and physiological data obtained from VAL24 cultured at control conditions (constant 10% DOT in both bioreactors), and at different t_c (50, 100 and 150s) were compared to the parental strain. Both strains expressed recombinant green fluorescent protein (rGFP). The specific growth rates for VAL24 cultures were $0.54h^{-1}$, $0.49h^{-1}$, $0.12h^{-1}$ and $0.1h^{-1}$ for the control, and the recirculated cultures at 50, 100 and 150s, respectively, whereas the specific growth rate of parental strain only reached 40 to 72% of the engineered strain. The production of rGFP was evaluated by densitometric analysis of SDS-PAGE and by fluoresce intensity. In VAL24 cultures, rGFP decreased 10% for t_c of 50s and 100s, and 30% for a t_c of 150s, compared to control conditions. In contrast, the parental strain under control conditions and a t_c of 50s and 100s produced 50% and 70% less rGFP, respectively, than the engineered strain. Moreover, the rGFP fluorescence obtained from VAL24 under all conditions was about 8-times higher than that obtained with the parental strain. Flow cytometric analysis revealed that VAL24 viability during scaled-down cultures and upon glucose depletion, was reduced around 50%, whereas during control cultures the viability was maintained above 85%. Results show that VAL24 under t_c, typical of large scale bioreactors, grew faster and produced higher rGFP concentration compared to the parental strain under the same fluctuating environmental conditions. Here, we present a novel approach for solving an old bioengineering problem through genetic engineering tools.

SIMULATION OF DISSOLVED CO2 GRADIENTS IN RECOMBINANT ESCHERICHIA COLI CULTURES: METABOLIC AND TRANSCRIPTIONAL RESPONSE

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Deficient mixing in industrial-scale bioreactors is an important concern as it results in an heterogeneous environment, with the potential exposure of cells to cyclic variations in substrate concentration, dissolved carbon dioxide (dCO2) concentration, temperature, and pH. Such a fluctuating environment can affect microbial physiology, leading to decreased yields in biomass, formation of toxic by-products, and loss of productivity. In the present study, the effect of dCO2 fluctuations was determined to simulate conditions that can occur during large-scale operations. The metabolic and transcriptional response of E. coli expressing recombinant green fluorescent protein (GFP) was studied. The dCO2 fluctuations were simulated in a two-compartment scale-down system consisting of two interconnected stirred tank bioreactors, one of them maintained at low dCO2 concentration (about 33 mbar) and the other at high dCO2 concentration (from 53 to 210 mbar) through a proportional control algorithm that manipulated the gas composition to the bioreactors. Culture broth was continuously circulated between both vessels of the scale-down system to mimic an overall circulation time (tc) of 50, 170, and 375 s. At the largest tc evaluated the performance of E. coli was affected, specific growth rate decreased by 11%, maximum acetate accumulation was 23% higher than reference culture (without dCO2 control). A delay in maximum GFP concentration, associated with an increased lag phase, was observed. To further investigate the effects of dCO2 fluctuations, the relative transcription levels of genes involved in regulating the expression of the glutamate decarboxylase system (gadE, gadX, gadA, and gadC) were determined by RT-PCR. In addition, the transcription levels of genes involved in the production or uptake of acetate and regulation of the glyoxylate shunt (poxB, acs, fadR, and iclR) were determined. No changes in the expression levels of these genes were observed under either control or flucutating dCO2 cultures. The transcriptional analysis suggested that the main effect of dCO2 fluctuations was on the enzymatic activity rather than at the transcriptional level.

METABOLIC ENGINEERING OF VANILLIN PRODUCTION IN S. CEREVISIAE

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Vanillin is one of the most widely used aromatic flavour compound, thus it assumes a crucial role in food, cosmetic and pharmaceutical industry. Great majority of the vanillin being marked at present is chemically synthesised, thus contributing to non-green industry. Because it is important to find an environmental friendly alternative for synthesising vanillin, we use *de novo* synthesis of vanillin from glucose in *S. cerevisiae* as an industrial relevant case study. A vanillin producing pathway was expressed in *S. cerevisiae*. Here we use an evolutionary algorithm applied to a genome-scale stoichiometric model (OptGene) to design a rational strategy to improve vanillin production in *S. cerevisiae*.

Recently available genomic data of several microorganisms, including *S. cerevisiae*, has enabled the reconstruction of their metabolic networks (Förster J et al., 2003). The availability of such models enables a rational approach to strain improvement for production of a desired metabolite. Different algorithms for identification of metabolic engineering strategies have been developed, e. g. OptKnock (Burgard AP et al., 2003) OptGene (Patil KR et al., 2005) and OptStrain (Pharkya P et al., 2004) among others.

Despite the potential of the developed algorithms, the accuracy/success of the suggested strategies is highly dependent on the reconstructed metabolic network. Microbial metabolism is often subjected to tight regulation which is very difficult to reproduce *in silico*, especially due to the lack of knowledge of the cellular regulatory systems (Patil K et al., 2005). Despite several efforts, the need to implement regulation on the genome-scale models is far from being accomplished. One possible approach requires first reconstructing the transcriptional regulatory network structure in the form of Boolean rules based on various available data sources and then integrating this network with the metabolic network model (Herrgård MJ et al. 2006). Most of the approaches to introduce regulation into these models imply an accurate knowledge, as much as possible, of a transcriptional regulatory network structure, which is very often incomplete. We propose a new heuristic method to introduce certain regulatory rules in the stoichiometric genome-scale models. Within this new approach, we collect information from different sources, namely literature, physiological data and metabolic control analysis to build a simple regulatory network for some of the pathways involved in the central carbon metabolism. The new model will be evaluated by comparing the corresponding new gene manipulation strategies for improvement of vanillin production in yeast with those obtained from the model without added regulatory information.

AN INTEGRATED FLUX ANALYSIS AND METABOLIC PROFILING STUDY TO IDENTIFY PATHWAYS CAUSING HEPATIC LIPOAPOPTOSIS

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Cell dysfunction and death induced by elevated serum free fatty acid (FFA) concentrations is a key contributor to the pathogenesis of obesity and type 2 diabetes. In particular, fat accumulation in the liver can lead to a condition known as non-alcoholic steatohepatitis (NASH), which involves tissue inflammation and damage that may culminate in cirrhosis. Hepatic apoptosis is a prominent feature of NASH and correlates with disease severity. Previous *in vitro* studies have demonstrated that saturated fatty acids (SFAs; e.g., palmitate) induce reactive oxygen species (ROS) generation and apoptosis, while mono-unsaturated fatty acids (e.g., oleate) lead to lipid accumulation but do not trigger apoptosis. Ceramide accumulation has been considered a primary factor responsible for these effects, because ceramide is synthesized de novo from palmitate and serine and also has been shown to activate apoptotic signaling. Recent studies, however, have reported that SFAs can induce apoptosis through ROS formation and endoplasmic reticulum stress without altering intracellular ceramide levels. Thus, a consensus mechanism linking SFA-induced metabolic alterations to apoptosis has been difficult to establish.

To identify metabolic pathways causing hepatic lipoapoptosis, we applied metabolic flux analysis (MFA) using [U-$^{13}C_5$]-glutamine as an isotopic tracer to quantify phenotypic changes in H4IIEC3 hepatoma cells treated with either palmitate alone (PA-cells) or both palmitate and oleate in combination (PA/OA-cells). Our results indicate that palmitate inhibited glycolysis and lactate dehydrogenase fluxes while activating TCA cycle flux and glutamine uptake. This decoupling of glycolysis and TCA cycle fluxes occured during the period following palmitate exposure but preceding the onset of apoptosis. Oleate co-treatment restored most fluxes to their control levels, resulting in increased lipid accumulation while preventing apoptosis. In addition, palmitate strongly increased the cytosolic NAD^+/NADH ratio, while oleate co-treatment had the opposite effect on cellular redox.

We next examined the influence of amino acids on these FFA-induced phenotypic changes. Increased medium amino acids enhanced ROS generation and apoptosis in PA-cells, but these effects were not observed in PA/OA-cells. Overloading the medium with non-essential amino acids induced apoptosis, but essential amino acid overloading partially ameliorated apoptosis. Glutamate was the most effective single amino acid in promoting ROS. Amino acid overloading also increased cellular palmitoyl-ceramide; however, ceramide synthesis inhibitors had no effect on measurable indicators of apoptosis. Our results therefore suggest that FFA-induced ROS generation and apoptosis are accompanied by the decoupling of glycolysis and TCA cycle fluxes leading to abnormal cytosolic redox states. Amino acids play a modulatory role in these processes, not through ceramide, but possibly through the control of TCA cycle flux.

BIOTECHNOLOGICAL METHIONINE PRODUCTION – POTENTIAL, PITFALLS, PROSPECTS

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Methionine is a sulphur containing essential amino acid and is also one of the last feed amino acids still produced in petroleum based chemical synthesis. This is mainly due to a very efficient process and the presence of racemases in higher animals, enabling the feed of racemic mixtures. Nevertheless, some studies suggest a preferred metabolism of the L-isomer in poultry, the most important market for feed methionine. In recent years, efforts have been made to replace the chemical synthesis with a water based fermentation process using renewable feedstocks that would yield the pure L-isomer. To date, however, the success of these projects is limited and biotechnological methionine yields are far from competitive.

In the present work, in silico modelling was used to discover promising strategies for high yield fermentation and to compare the potential of Escherichia coli and Corynebacterium glutamicum, the most important organisms used for industrial amino acid production [1]. Especially the reduction state of the used sulphur source will be critical.

Moreover, a systems approach combining proteomics, metabolomics and fluxomics was applied to C. glutamicum. A strain comparison between the wild type and a knockout mutant with a lack in transcriptional control over the methionine and cysteine biosynthetic genes revealed that engineering of the methionine pathway and sulphur reduction pathway has a broad impact on the cell, ranging from the activation of new overflow pathways [2] to oxidative stress. The studies revealed an important, if not the most important, metabolic bottle neck in the methionine pathway of C. glutamicum, the actual methylation reaction. The data suggests, that the supply with precursors is sufficient, but the activity of methionine synthases is limiting.

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METABOLIC FLUX ANALYSIS OF MAIZE (ZEA MAYS, GS3 X GASPE) CELL SUSPENSION CULTURE BY USING 13C LABELING EXPERIMENT AND 2-DIMENSIONAL NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

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Quantification of fluxes in plants is an inevitable tool for the development of strategies for genetic modification, the recognition of metabolic regulation and control, and the identification of correct operating metabolic network topology. For the application of metabolic flux analysis, it is crucial to construct metabolic networks for different plant systems and quantify the fluxes by using labeled substrates. We fed a mixture of uniformly ^{13}C-labeled sucrose (10%) and naturally labeled sucrose on maize (*Zea mays*, GS3 X Gaspe) cell suspension used as a model plant. We then quantified central metabolic fluxes based on the three compartmented (cytosol, plastid, and mitochondrion) model from 2-dimensional [^{13}C, ^{1}H] NMR spectra of protein and starch hydrolysates of the suspension cell at late exponential growth stage. We obtained 133 ^{13}C isotopomer abundances from the NMR analysis and a generic computer program, NMR2Flux, previously developed in our research group was used for the flux evaluation. When the evaluated flux values of maize cell suspension were compared to corresponding fluxes previously reported for other storage plant systems, such as soybean and canola embryos, considerably higher flux to the tricarboxylic acid (TCA) cycle indicated that high respiration is a main metabolic activity in the maize cell suspension culture. In addition, larger fluxes in the mitochondrial malic enzyme reaction and the plastidic oxidative pentose phosphate (OPP) pathway were observed suggesting high demand of reductants like NAD(P)H. Substantial flux in cytosolic non-OPP pathway was seen, but still less compared to its counterpart in the plastid. Negligible flux in the glyoxylate shunt pathway was also found. Similar flux values were acquired in the replicate experiment that was conducted about 4 months after the first ^{13}C experiment indicating the values are biologically reproducible. Metabolic flux analysis of maize cell suspension can be extended to studies on metabolic changes in central carbon metabolism of the plant under genetic and environmental perturbations.

RATIONAL ENGINEERING OF NADPH METABOLISM IN CORYNEBACTERIUM GLUTAMICUM FOR IMPROVED LYSINE PRODUCTION

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Lysine production by *Corynebacterium glutamicum* with a current world market of 850.000 tons per annum is one of the major industrial processes in biotechnology (Wittmann and Becker, 2007). Metabolic engineering displays a powerful strategy to improve *C. glutamicum* for biotechnological lysine production. Identification of beneficial genetic modifications is, however, not trivial and often requires a detailed understanding of the underlying metabolic pathways and their regulation. In this regard metabolic flux analysis, providing quantitative insights into *in vivo* activities of intracellular enzymes and pathways has proven especially useful. In the case of lysine, the effective supply of NADPH has been identified as important property of superior production strains. Metabolic flux analysis hereby revealed that the pentose phosphate pathway (PPP) is the major NADPH providing pathway in *C. glutamicum*, and thus one of the key fluxes in high-efficient lysine-producers (Wittmann and de Graaf, 2005).

In this work, we applied different strategies to increase the flux through the PPP, thereby providing more NADPH for lysine production. Over expression of fructose 1,6-bisphosphase (FBPase) in a feedback-deregulated strain resulted in a 40 % increase of lysine production yield. As shown by metabolic flux analysis, this modification was linked to an increased PPP flux by redirection of the carbon flux from glycolysis (Becker, et al., 2005). Moreover, over expression and A243T nucleotide exchange of glucose 6-phosphate dehydrogenase (G6PDH), the flux controlling enzyme of the PPP (Becker, et al., 2006), resulted in increased lysine production as well as increased PPP flux. Implementation of all modifications further improved the production characteristics of the strain. The obtained mutant showed a significant NADPH excess, suggesting a high remaining potential, which could be fully utilized by additional metabolic engineering of the lysine pathway and the precursor metabolism.

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IMPROVING SESQUITERPENE PRODUCTION IN SACCHAROMYCES CEREVISIAE THROUGH INTEGRATION OF THE MEP PATHWAY

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Sesquiterpenes are a structurally diverse group of isoprenoids with a broad range of properties and commercial applications. Sesquiterpenes are derived from farnesyl diphosphate (FPP), the biological precursor for all sesquiterpenes. In Saccharomyces cerevisiae, the mevalonate (MVA) pathway that leads to the formation of ergosterol as the major end product of the pathway is the functional pathway for the biosynthesis of FPP. In plants and bacteria a different pathway contributes to the production of isoprenoids: the Methyl Erythritol Phosphate (MEP) pathway (Fig.1). Even though its substrates and intermediates for the first enzymatic steps are different, the MEP pathway merges with the last part of the MVA pathway at the level of isopentenyl diphosphate (IPP) In order to evaluate the potential of this pathway in yeast as the only source of IPP, the MEP pathway from E.coli was stabilized by integration into the S. cerevisiae genome using gene targeting methods. To further improve the properties of the strain, a strain improvement approach was used. The clones gathered from these experiments were characterized genetically and physiologically

TARGET SELECTION BY TOP-DOWN SYSTEMS BIOLOGY

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Increasingly, systems biology is used as a tool to identify targets in metabolic engineering. At its extreme, two different systems biology approaches can be discriminated: bottum-up and top-down systems biology. In bottom-up systems biology, biological knowledge is used as the starting point and a mathematical model of the biological system under study is built. In contrast, in top-down systems biology (functional genomics) data are used as the starting point and statistical data mining approaches are applied to identify the biomolecules important for the biological question under study. Currently, over 95% of the scientists working in the field of systems biology use a bottum systems biology approach. This does not do justice to the potential of top-down systems biology.

A basic problem with top-down systems biology is that large data sets are used as the starting point which bears the risk of "drowning" in the data. The successful application of top-down systems biology therefore depends on balancing three key factors (i) definition of the biological question, (ii) experimental design and (iii) the choice of the data analysis tool. Once these factors are properly balanced, top-down systems biology can result in new insights and above-average improvements in strain improvement.

To demonstrate the merits of the top-down systems biology approach, we have studied vitamin B12 production by *Propionibacterium freudenreichii*. An experimental design was defined that resulted in large differences in the vitamin B12 productivity when *P. freudenreichii* was cultivated on different media. Samples were taken from these fermentations, quenched to halt cellular metabolism and subsequently analyzed by comprehensive metabolomics [van der Werf *et al*., 2007]. The metabolomics data was analyzed with the multivariate data analysis tool Partial Least Squares, in order to rank the metabolites important for vitamin B12 production. The data were biologically interpreted, genetic targets were identified and mutant strains with altered expression of these target-genes were constructed by metabolic engineering. These recombinant strains indeed demonstrated an above average increase in vitamin B12 titer. In a second example, the use of top-down systems biology to identify the interactions of the phenylalanine biosynthesis pathway of *Escherichia coli* with the remainder of the metabolism will be discussed. To this end the biostatistical tool Canonical Correlation Analysis was employed.

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ANAEROBIC GROWTH AND POTENTIAL FOR AMINO ACID PRODUCTION BY NITRATE RESPIRATION IN *CORYNEBACTERIUM GLUTAMICUM*

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Various L-amino acids including L-lysine, L-arginine, L-glutamine, and L-glutamate are widely manufactured by fermentation using regulatory mutants of *Corynebacterium glutamicum*. Under oxygen limitation, production strains usually accumulate undesirable organic acids, which results in damaged fermentation with decreased production yields. For this reason, it is necessary to keep dissolved oxygen above a certain level during fermentation. To this subject, considerable effort has been dedicated to investigation of aeration efficiency of fermentation tanks from the viewpoint of mechanical engineering. Nevertheless, capital investment is hampered by the recent situations where the world market is highly competitive and process economics are of primary importance. If we can develop strains that produce amino acids efficiently even under oxygen limitation, a significant benefit would likely arise industrially. However, there is no such technology at present. The presenters paid attention to which nitrate (NO_3^-) can serve as an alternative electron acceptor to oxygen in *C. glutamicum*, namely, utilization of an another respiration system independent of oxygen. Early taxonomic studies had been shown that most wild-type strains belonging to the organism grow by the properties, so-called nitrate respiration, but there was no genetic and physiological study on the character. The presenter will report that the nitrate respiration can support the anaerobic production of amino acids even at low level, and genes responsible for the nitrate reduction had been identified.

Our study was initiated by analysis of the oxygen-requiring properties of *C. glutamicum*, generally regarded as a strict aerobe. This organism formed colonies on agar plates up to relatively low oxygen concentrations (0.5% O_2), while no visible colonies were formed in the absence of O_2. However, in the presence of nitrate, the organism exhibited limited growth anaerobically with production of nitrite (NO_2^-), indicating that *C. glutamicum* can used nitrate as a final electron acceptor. Assay of cell extracts from aerobic and hypoxic cultures yielded comparable nitrate reductase activities, irrespective of nitrate levels. Genome analysis revealed a *narK2GHJI* cluster potentially relevant to nitrate reductase and transport. Disruptions of *narG* and *narJ* abolished the nitrate-dependent anaerobic growth with the loss of nitrate reductase activity. Disruption of the putative nitrate/nitrite antiporter gene *narK2* did not affect the enzyme activity but impaired the anaerobic growth. These indicate that this locus is responsible for nitrate respiration. Agar piece assays using L-lysine- and L-arginine-producing strains showed that production of both amino acids occurred anaerobically by nitrate respiration, indicating the potential of *C. glutamicum* for anaerobic amino acid production.

METABOLIC FLUX ANALYSIS OF DIFFERENTIATING MOUSE EMBRIONIC STEM CELLS (MES CELLS)

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Basic metabolic analysis was performed on mouse embryonic stem cells considering 3 differentiation conditions: a) classical embryoid body formation (EB), and two differentiation conditions on a surface coated with b) gelatine (GEL) and c) matrigel (MAT). This approach has never been applied to differentiating embryonic stem cells, and the information that can be obtained is valuable in terms of understanding the metabolic changes that these cells undergo. The chosen analysis was MFA (Metabolic Flux Analysis) measuring only 4 metabolites (glucose, lactate, glutamine and glutamate). 20 stoichiometric reactions and 3 transport steps were considered for the MFA model. Concurrently, a genetic profile was performed using microarray technology, which can give a broad idea of what is happening with the inner machinery of differentiating cells.

Carbon balance was calculated and almost all point conditions closed with error values lower than 10% (except 5 points out of 18) and only 3 points above 20%. Among the interesting results is that the flux towards biomass production is maintained nearly constant, whereas the flux towards energy production changes 100% to 200% (increase) depending on the differentiation condition, on day 5 of the differentiation protocols. In particular, the fluxes corresponding to the reactions: MAL->OAA, PYR->AcCoA and OAA->aKG increase by 100% in the case of EB and GEL. In MAT these fluxes also increase, but only to a minor degree. The other big change noticeable at day 5 is the flux of the reaction of oxidative phosphorylation. In the EB condition this flux increased by 166%, while with GEL it increased 100% and with MAT 200%. Comparing the behavior of the biomass flux, the increase is only 25% in the case of EB, while in the other two conditions, the flux is maintained in a range no higher than 5%. These large changes correlate with changes in the genetic profile, which was obtained using microarrays.

When analyzing the particular genes involved in oxidative phosphorylation, the behavior of one gene is noticeable. This gene, Aralar (Scl25a12), a mitochondrial gene which functions as an aspartate-glutamate carrier, shows a high expression ratio at day 0 (the undifferentiated state of embryonic stem cells) which abruptly decreases at day 3 (the first day which was analyzed with microarrays) and then increases constantly until the level reaches almost the same level of expression at day 7 of differentiation. This behavior is observed across all different differentiation protocols. All these observations point to an increase in the energy produced, while the flux of biomass is maintained constant.

TRANSCRIPTIONAL RESPONSE OF THE TERPENOID INDOLE ALKALOID PATHWAY TO THE OVEREXPRESSION OF ORCA3 ALONG WITH JASMONIC ACID ELICITATION OF *CATHARANTHUS ROSEUS* HAIRY ROOTS OVER TIME

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Two important TIAs produced in small quantities by *C. roseus* are vinblastine and vincristine which are used clinically as anticancer drugs. Our lab is interested in engineering *C. roseus* hairy roots to increase the production of the TIAs. Jasmonic acid has been shown to activate the transcriptional regulator ORCA3 which regulates numerous terpenoid indole alkaloid (TIA) genes and causes an increase of TIA pools within *Catharanthus roseus*. In this work, we have explored the transient effects of overexpressing ORCA3 under the control of the glucocorticoid inducible promoter in *C. roseus* hairy roots along with the simultaneous feeding of jasmonic acid (JA). Time profiles of both mRNA and alkaloid levels were analyzed by sampling 0, 6, 12, 24, 48, and 72 hours after feeding of JA and/or induction of ORCA3.

Analysis of the alkaloid levels showed that JA treatment alone caused the greatest increase in TIA pools with significant increases occurring around 24 hours with the concentration of most metabolites leveling off afterwards. Overexpression of ORCA3 caused the pools of lochnericine, hörhammericine, and tabersonine to decrease significantly by 12 hours. Overexpression of ORCA3 along with JA feeding caused a significant decrease in hörhammericine and tabersonine by 24 hours and lochnericine by 72 hours. Using Q RT PCR we analyzed the mRNA levels of TIA genes and regulators at each time point. Upon induction and elicitation with JA, ORCA3 transcripts increased 170-fold whereas induction alone caused an 89-fold increase and elicitation alone caused a 5-fold increase in transcripts. In general, JA treatment alone caused the largest increase in the mRNA levels with the maximum increase occurring at 12 hours (for genes early in the indole pathway) or 24 hours. A subsequent decrease in mRNA levels of the JA treated samples was observed with mRNA levels returning to levels similar to the un-treated samples by 72 hrs. In the induced samples overexpressing ORCA3, the largest increase in the transcripts of ZCT1 and ZCT2, TIA transcriptional repressors, coincided with the largest increase in ORCA3 transcripts.

These results show that a coordinate overexpression of many enzymes may be necessary to increase the production of the terpenoid indole alkaloids and point us towards a multigene strategy to engineer *C. roseus* hairy roots for increased production of TIAs. These results also point to a complex regulatory network whose components are beginning to be discovered and their roles elucidated.

APPLICATION OF METABOLIC FLUX ANALYSIS TO IDENTIFY THE MECHANISMS OF PALMITATE TOXICITY IN HUMAN HEPATOMA CELL LINE

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Chronic exposure to elevated levels of free fatty acids (FFAs) has been shown to cause cell death (lipotoxicity), but the underlying mechanisms of lipotoxicity in hepatocytes remain unclear. We have previously shown that the saturated FFA, palmitate, causes much greater toxicity to human hepatoma cells (HepG2) than the unsaturated ones. In this study, metabolic flux analysis (MFA) was applied to identify the metabolic changes associated with the cytotoxicity of saturated FFA. Application of MFA revealed that cells exposed to palmitate had a consistently reduced flux of glutathione (GSH) synthesis but greater de novo ceramide synthesis. These predictions were experimentally confirmed. In silico sensitivity analyses identified that the GSH synthesis was limited by the uptake of cysteine. Western blot analyses revealed that the levels of the cystine transporter xCT, but not that of the GSH-synthesis enzyme glutamyl-cysteine synthase (GCS), were reduced in the palmitate cultures, suggesting the limitation of cysteine import as the cause of the reduced GSH synthesis. Supplementing with N-Acetyl L-cysteine (NAC), a cysteine-provider whose uptake does not depend on xCT levels, reduced the FFA-toxicity significantly. Further studies suggest that general protein synthesis is unaffected, which was confirmed by the unchanged protein level of xCT in palmitate cultures with cycloheximide (inhibitor of general protein synthesis) and actinomycin-D (inhibitor of general transcription). Thus, these results leaves open the possibility that, in addition to metabolic factors, biophysical effects may contribute to the reduced expression/activity of transmembrane proteins, such as system xc-, observed with palmitate.

USING THE "OMICS" TECHNOLOGIES AS COMPLEMENTARY TOOLS TO STUDY THE MOLECULAR MECHANISMS INVOLVED WITH THE ADAPTATION OF MYELOMA CELL LINE TO PROTEIN-FREE MEDIUM

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Production of recombinant therapeutic proteins, especially monoclonal antibodies, in myeloma cell lines represents a significant segment of the pharmaceutical market, and therefore striving for increased productivity of these lines represents a major investment of resources. The elucidation of biologically important markers for the adaptation of NS0 myeloma cell line to protein-free medium and the recombinant protein production are a major emphasis of our research. These markers could potentially be used in a variety of ways to improve culture conditions, including active approaches to agonize/antagonize important pathways within a medium formulation or diagnostic approaches indicative of improved conditions during the culture. In this work, we used two-dimensional electrophoresis/ mass spectrometry and the iTRAQ technology to analyze different protein levels in adapted and non-adapted NS0 myeloma cell line. Several proteins with differential expression profile were characterized and quantified. Changes in lactate production rate with respect to glucose consumption rate were observed according to the changes observed by proteomic. Also a comparison of the myeloma cell line in different medium conditions was performed using flux balance analysis (FBA). Carbohydrate metabolism, protein synthesis and membrane transport were the principal pathways that change after the adaptation by proteomic. The same results were obtained using FBA in a murine metabolic network with selected medium conditions.

RAPID GENERATION OF FUT8 KNOCKOUT CHO CELL LINES USING ENGINEERED ZINC FINGER NUCLEASES

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Improvements to the in vivo efficacy and potency of therapeutic antibodies via enhancement of antibody dependant cell-mediated cytotoxicity (ADCC) show considerable promise in the treatment of human disease. One of the approaches to improve the ADCC activities of IgG1 class monoclonal antibodies is via alteration of N-linked glycan composition. Reducing the relative content of alpha-1,6-fucose within the core oligosaccharide present in the Fc region of IgG1 class MAbs can increase ADCC activity inversely proportionate to fucose content, with complete removal giving maximal in vitro and in vivo activity.

One of the validated approaches for reducing alpha-1,6-fucose levels is via targeting expression of the fucosytransferase 8 (FUT8) gene, which is responsible for the addition of fucose to the core N-linked oligosaccharide. In order to maximize process and parameter fit within Genentech's cell line and bioprocess development timeframes, we undertook to generate a CHO cell host in which both alleles of the FUT8 gene locus were functionally deleted at the genomic level, via targeted disruption within the coding sequence. In order to enable this approach, we utilized engineered zinc finger nucleases (ZFNs) supplied by Sangamo BioSciences Inc to target a specific sequence within a coding exon of the FUT8 gene for disruption.

After transient transfection of an expression plasmid encoding the ZFN reagents into the Genentech CHO host line, single-cell cloning was performed and the resulting clones analyzed at a cellular and molecular level to determine Fut8 status and functionality. A series of clones were identified that exhibited specific mutations (deletions and additions) within the targeted region of both alleles of the FUT8 gene at the genomic level. These clones exhibited a complete absence of Lens culinaris agglutinin (LCA) lectin mediated cellular toxicity and a marked reduction in LCA binding to modified cells, as assayed via FACS. Modified clones also exhibit similar growth profiles and behaviour as unmodified CHO cells and produce recombinant proteins that contain no detectable alpha-1,6-fucose, as assayed via MALDI-TOF. Using this approach, we have rapidly generated a modified CHO host line where the endogenous FUT8 gene has been functionally deleted and which produces afucosylated recombinant proteins.

DEVELOPMENT OF LARGE SCALE KINETIC MODELS FOR METABOLIC NETWORKS: CHALLENGES, PITFALLS, PRACTICAL SOLUTIONS

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Construction of dynamic models of large scale metabolic networks is one of the central issues of engineering of living cells. This endeavor aims not only to get deeper understanding of the complexity beyond intuitive comprehension, but also to e. g. perform predictions for gene targets to improve the performance of the cell. From a system biology perspective, these models should not only describe the dynamic behavior of metabolic reaction networks, but, they should also ultimately allow combining several pathways (horizontal modeling) and/or "omic" levels (vertical modeling) in the cell. Nevertheless, construction of such large models are often hampered with a number of challenges e.g. data availability, compartmentalization and parameter identification coupled to design of *in vivo* perturbations. Hence, a modeling approach that would handle construction of large scale kinetic models while providing solutions to the mentioned challenges is of great interest.

We will present in our contribution, the pitfalls in constructing kinetic models and possible solutions are discussed. We exemplify our approach by developing a large scale kinetic metabolic model of our model organism, *Penicillium chrysogenum* which aims to encompass all the major pathways. In constructing the kinetic model, we departed from the stoichiometric model which consists of 188 metabolites and 167 reactions located in 3 compartments (cytosol, mitochondria and peroxisome) (van Gulik *et al.*, 2000). We used the short-term (<300 sec.) dynamic perturbation data presented in Nasution *et al.*, (2006) for parametrization.

Considering the challenges in available experimental information, we reduced the network by taking into account time-scale considerations and compartmentalization. For the rate expressions, we used approximative linlog kinetics, allowing to represent the enzyme-metabolite kinetic interactions by an elasticity matrix. Information on the presence and absence of mass action and allosteric enzyme kinetic information was obtained from literature survey and database search. The final values of the elasticities are estimated by fitting the model to the available short term kinetic response data (Nikerel *et al.*, 2006). The final model consists of 70 metabolites and 75 reactions located in one compartment. With approximately 200 parameters, the model describes the glycolysis, TCA cycle, Penicillin biosythesis, amino acid production and pentose phosphate pathways. Having obtained a large scale kinetic model for *Penicillium chrysogenum*, it is concluded that the linlog modeling framework was effective and facilitated model reduction and parameter identification of this complex system.

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TANDEM MASS SPECTROMETRY METHOD FOR METABOLIC FLUX ANALYSIS

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Recent progress in methods for metabolic flux analysis using stable-isotope labeling experiments and gas chromatography/mass spectrometry (GC/MS) and nuclear magnetic resonance (NMR) has allowed us to make accurate flux estimates. Here, we introduce a novel method for quantifying isotopic labeling and estimating metabolic fluxes based on new technique, tandem mass spectrometry, which can dramatically increase the resolution with which fluxes can be determined in vivo. Tandem mass spectrometry is a relatively new tool that can provide an order of magnitude more labeling information than can be obtained from GC-MS. However, experimental and computational methods for measuring and analyzing data from tandem mass spectrometry have not been fully developed. Here, we present an extension to the elementary metabolite unit (EMU) framework for measuring metabolic fluxes from mass spectrometry and tandem mass spectrometry data. In this novel modeling framework the isotopic labeling of EMUs are represented by mass isotopomer distribution matrices (MIDX) instead of mass isotopomer distribution vector (MIDV). The MIDX is composed of daughter mass spectra collected for a given parent fragment. We demonstrate that MIDX of any metabolite can be computed by a convolution of MIDVs of the daughter and its complement fragment. We measured MIDXs for two molecules, glucose and phenylalanine, and compared them to theoretically predicted values. The agreement between theoretical and measured values was better than 0.2%. This demonstrates that tandem mass spectrometry can provide highly accurate isotopomer labeling information for flux studies. Furthermore, we illustrate that our modeling method allows us to trace and simulate isotopic labeling in complex metabolic networks and ultimately estimate fluxes. Since our method is based on the highly efficient EMU modeling framework it can be applied to any compounds and metabolic networks of any complexity.

RELATIONSHIP BETWEEN ENERGETIC METABOLISM AND SIALIC ACID LEVELS OF R-TPA: FLUX BALANCE ANALYSIS UNDER DIFFERENT HEXOSE CONCENTRATION IN CONTINUOUS CULTURE OF CHO CELLS

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Animal cell cultures have been widely used in the last decades for protein production with therapeutic applications, due to their ability to perform post-translational modifications, including glycosylation. Protein glycosylation has shown to be an essential step to achieve the desired biological activity. However, because of the high complexity of these systems, the knowledge about these metabolic processes and mechanism affecting protein production and glycosylation is still limited. It has been seen that a decrease in glucose concentration has a detrimental effect on both productivity of recombinant protein and sialic acid levels in the glycosylations. Furthermore, the hexose use as carbon source has an important effect. Mannose, though similar to glucose as carbon source, in batch cultures prevents the negative effects of ammonium, demonstrating that cells have different responses to these sugars. For these reasons, we have studied the effect of different concentrations of glucose and mannose in continuous cultures on recombinant tissue plasminogen activator (r-tPA) produced by CHO cells using flux balance analysis (FBA). The stoichiometric model considered hexose and amino acids consumption, lactate and ammonium production, as well as biomass, recombinant protein and glycosylation synthesis. Besides, ATP flux form cellular maintaining processes were estimated from central metabolism reactions and oxidative phosphorylation. Three feeding concentrations (2.5, 10 and 15 mM) were tested. The cultures were performed in spinner flask using 150 mL of medium and dilution rate 0.015 h^{-1}. Differences between mannose and glucose cultures were observed in hexose and amino acids specific consumption rates, lactate, ammonium and protein production rates. r-tPA specific and volumetric production rate were the highest when 10 mM of hexose was used, though sialic acid:tPA ratio was 3.1-3.5 for mannose cultures and 2.5-3.3 form glucose. FBA shows that intracellular flux distribution changes as a function of both concentration and type of hexose. The flux of ATP for maintaining calculated increased when hexose concentration changed from 2.5 to 10 mM, but decreased when concentration of hexose was 15 mM, showing an inhibitory effect by the carbon source. Moreover, we have found out that there is a linear dependence between fluxes of ATP for maintaining and sialic acid:tPA suggesting that the effects of different types and concentrations of hexose observed on the glycosylation process is directly related to energetic state of cells, rather than a limitation by nucleotide sugars concentration in the cell.

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A SYSTEMATIC METHOD FOR MODELING THE DYNAMICS AND HETEROGENEITY OF CELLULAR METABOLISM IN BATCH CULTURE

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High production costs and increasing demand are driving the need to increase the productivity of industrial cell culture processes. A metabolic model that can simulate the dynamics of these processes would significantly reduce the experimental time and resources necessary for process optimization.

A systematic modeling approach is presented that can simulate the changes in cell density and metabolite concentrations throughout a cell culture process. The methodology involves three steps. First, from a genome-scale metabolic reaction network, a graph-theoretical strategy is applied to systematically reduce the network to a manageable set of functional modules. Second, kinetic rate expressions are defined for each module to characterize the initial state of the system during balanced growth. Third, the transition periods following a system perturbation, e.g. a temperature shift, are explained by the generation of multiple, metabolically distinct sub-populations. The dynamics of such transitions are then simulated through a method of predicting the sub-population distributions.

The methodology was applied to an industry-relevant cell culture process of Chinese Hamster Ovary (CHO) cells producing a recombinant protein. Using the above strategy, a network of more than 200 reactions was reduced approximately by 90% while preserving stoichiometric relationships. Balanced growth was modeled with a steady state flux approximation. Dynamic simulation results suggested that the subsequent departure from this initial steady state resulted from the depletion of essential internal metabolites (TCA cycle intermediates). The associated alteration in metabolite profiles and reduction in growth rate was attributed to the reversal of specific reactions to compensate for the depletion.

OVERCOMING LACTATE ACCUMULATION IN MAMMALIAN CELL CULTURE

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Minimizing lactate accumulation is one of the major objectives when developing biotherapeutics manufacturing processes in mammalian cell culture. Our small scale process development data showed that both low dissolved oxygen (dO2) and high pH bioreactor conditions resulted in elevated glucose consumption rate and lactate accumulation. However, when comparing the two conditions, the low dO2 condition displayed a decrease of 50-60% in both cell growth and volumetric cell productivity. This result indicated that lactate in itself might not be the damaging factor. Instead, significant cell growth and productivity decrease in low dO2 culture are likely due to inefficient energy supply rather than excessive lactate. Lactate accumulation caused by high pH and low dO2 conditions were differentiated by comparing cell performance and total glucose consumption. The low dO2 condition culture exhibited a significant decrease in viable cell density which impacted volumetric productivity and total glucose consumption. Controlling glucose at a low level to increase glucose utilization efficiency and decrease metabolic by-product lactate is a common practice in the industry. However, in a shake flask study, glucose controlled at 10 and 20g/L concentrations did not increase lactate accumulation. Cell performance was negatively affected when both glucose and lactate were depleted in the chemically-defined culture. Another study investigating the addition of lactate to the culture was observed to increase glucose utilization efficiency and decrease lactate production. Although, the detrimental effect of low dO2 on cell performance was more profound than high pH, data from our pilot scale bioreactor run showed that dO2 fluctuation didn't induce high lactate or negatively impact cell performance. Interestingly, a short term pH excursion initiated higher lactate accumulation. It is well known that lactate production and secretion are coupled with proton flux. Therefore, pH was identified as a key optimization parameter to control lactate accumulation at minimum levels. A pH control strategy utilizing a larger dead band decreased base addition by 500%. An improved cell culture process consistently demonstrated a 30% lactate decrease for multiple runs at different bioreactor scales. These preliminary results provided targets for future molecular modifications to improve cell metabolism.

METABOLOMICS AS MOLECULAR ANALYSIS TOOL IN CELL CULTURE ENGINEERING

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In the post-genomic era, quantitative molecular fingerprinting enables the analysis of gene expression and protein activity in the context of networks and systems of interacting genes and gene products, enabling thus the reconstruction of more accurate models of cellular function. To succeed, however, in the challenge of quantitative systems biology, major issues concerning the quantification capabilities of the high-throughput molecular analysis techniques for each level of cellular function need to be resolved. They range from limitations in the available experimental protocols for many biological systems and problems, to lack of data analysis techniques for upgrading the information content of the acquired measurements. So far, cell culture engineering has used the high-throughput transcriptional and more recently protein profiling techniques attempting to further understand and resolve significant issues regarding the cell culture performance and its optimization. At the metabolic level, flux analysis has been used to monitor changes in the metabolic state that could affect the successful outcome of cell cultures.

Metabolomic profiling, referring to the simultaneous quantification of the (relative) concentration of the free small metabolites, is the most recently introduced high-throughput method for the measurement of the metabolic fingerprint of a biological system. Considering the role of metabolism in the context of the overall cellular function, it is easily understandable why quantifying a complete and accurate metabolomic profile is foreseen to have a major positive impact in cell culture engineering research. In the case of the metabolomic analytical platform, however, its broad deployment to biotechnology requires its standardization for accurate, reproducible and validated performance. Failure to achieve this technological status may end up limiting the application of metabolomic analysis, despite its great potential.

Taking all these issues into consideration, we will present and discuss application of Gas Chromatorgaphy-Mass Spectrometry metabolomics [1] to monitor and analyze mammalian cell culture systems. Specifically, baby hamster kidney cells were cultivated in high-cell density perfusion reactors at both laboratory and manufacturing scales and samples were analyzed over the course of the cultivation. Metabolomic profiling enabled the differentiation between cell cultures based on bioreactor type, and stage of cell growth. In addition, use of multivariate statistical analysis techniques enabled the identification of the metabolites whose change in concentration was characteristic of the modification in any of these macroscopic parameters, providing thus important information about the *in vivo* state of the cultures. The acquired results support the usefulness of metabolomics as a molecular analysis tool in cell culture engineering. It is expected that its integration with the already established flux analysis methodologies and other “omics” will prove significant for bioprocess development and optimization.

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METABOLOMIC ANALYSIS OF SEX-SPECIFIC PATHWAYS IN ADULT ZEBRAFISH

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Environmental factors play a key role in sex determination in teleost fish [1]. Therefore, furthering our understanding of the sex-specific pathways in these fish could have a positive impact in environmental research and the development of sensoring systems for aquatic ecology . The modern high-throughput molecular analysis techniques can assist towards this goal[2].

In this study, gas chromatography–mass spectrometry (GC-MS) metabolomics [3-4] was used for the identification of sex-specific metabolic pathways in adult zebrafish, a model organism of gonochoristic teleost fish. Presently, there is no other published report about metabolomics of zebrafish, nor GC-MS metabolomics of any type of fish. Thus, this work contributes novel holistic data of zebrafish metabolic physiology that could be used in the relevant environmental research [5]. Moreover, the acquired information provided additional to the currently existing evidence that GC-MS metabolomics is an effective fingerprinting method for the monitoring of aquatic ecology. The major finding was that the energy metabolism is largely stimulated in the female compared to the male zebrafish. This metabolic difference could accommodate the energy-consuming process of oogenesis in females. Indications were also obtained about higher osmotolerance of female zebrafish. These results provide the basis for further “omic” analyses to elucidate the relationship between the alteration of these gender-differentiating pathways’ activity due to environmental factors and sex determination in teleost fish.

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METABOLIC PROFILING OF A RECOMBINANT E. COLI IN FERMENTATION PROCESS

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Unbiased global metabolic profiling is an approach to obtain a “snapshot” of the metabolism of a population of cells in a bioreactor. As such, it has potential utility in the area of upstream bioprocess optimization. Here, metabolic profiling was used to monitor a single E. coli clone within two similar medium formulations. Despite the similarities, the clone produced disparate cell densities and titers in the two medium formulations. Using a metabolic profiling approach from Metabolon, 258 distinct biochemicals were profiled and several distinct differences in the basal and feed medium formulations were identified. Further many biochemical differences in the cells were observed including increased levels of glycolytic and TCA cycle intermediates, amino acids, and other metabolites. Such elevations possibly from divergent metabolic fluxes of the aforementioned metabolites exhibit a clear correlation to different levels of cell growth and recombinant protein productivity. Hence, using the above described approach, one can gain insights into the array of complexities that exist within medium formulations and cellular metabolism.

GETTING THE RIGHT NUMBERS: HOW TO AVOID SOME (COMMON) MISTAKES IN METABOLOMICS-BASED RESEARCH IN *S. CEREVISIAE*

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Accurate measurement of intracellular metabolite levels is of prime interest in the study of metabolic reaction networks and their regulation *in vivo*. Despite recent advances in analytical tools, which led to the advent of the field of metabolomics, obtaining accurate, reliable data on intracellular metabolite levels is still not straightforward. We shall analyze some of the limitations in yeast metabolomics and describe the approaches we have used to tackle them.

Currently, one of the major constraints in metabolomics of eukaryotes is compartmentation. Direct measurements provide whole-cell averages. On the other hand, existing cell fractionation techniques allow changes in the very metabolite pools to be measured. A further constraint is that, in addition to the free species (kinetically and thermodynamically relevant), certain metabolites are partly present in protein-bound form. We have addressed compartmentation and protein-binding of NAD(H), a key redox carrier and signalling molecule, by determining the cytosolic free NAD/NADH ratio from the product/substrate ratio of a suitable near-equilibrium redox reaction, which we introduced in yeast specifically for this purpose[1]. Using this method we found that the cytosolic free NAD/NADH ratio was more than 10-fold higher than the whole-cell total NAD/NADH ratio. We also demonstrate the use of this method under highly dynamic conditions and discuss the implications of the newly available data for quantitative physiology, particularly in the study of redox metabolism, and thermodynamic reaction network analysis.

Another aspect that can be problematic in microbial metabolomics is sampling and sample treatment. Quenching in 60% methanol at -40ºC is the standard method for sub-second arrest of metabolic activity but there have been contradictory reports in the literature on whether metabolites leak from yeast cells. By determining the levels of a large range of metabolites in different sample fractions and establishing mass balances we could trace their fate during the quenching procedure and confirm that leakage does occur, to such an extent that the levels of most metabolites have been previously underestimated by at least 2-fold[2]. Using this quantitative approach we evaluated the effect of different quenching conditions and developed a method where leakage is entirely prevented. Making use of improved data on intracellular metabolite levels we re-evaluate the need of sub-second quenching and of removing the extracellular medium. We also discuss the implications of these findings for *in vivo* kinetic modelling and non-stationary ^{13}C flux analysis.

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MICROFLUIDIC DROPLETS AS NANOBIOREACTORS FOR SCREENING METABOLIC ENGINEERING LIBRARIES

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Metabolic engineering has contributed significantly to the rational improvement of strains for industrial applications. In addition to enzymatic steps closely associated with the product-forming pathway, other, so-called distal genes may also impact production in a profound way due to (often unknown) kinetic and regulatory effects. Such genes are identified by combinatorial methods whereby libraries of the host strain are constructed harboring random variants of the basic strain genes, as well as random combinations of gene knock outs and over-expressions. Cells with superior properties are selected from these libraries and the specific genetic alteration identified in a process known as Inverse Metabolic Engineering. However, these approaches necessitate the use of high throughput screening methods to select the most desirable clones from these libraries of engineered strains. For many metabolic engineering libraries, the selection criterion is the production of a secreted metabolite or the consumption of a medium component. Thus, a strategy for compartmentalizing clones is necessary so that each clone grows in a separate environment allowing for the measurement of clone-specific metabolite concentrations. Traditional methods such as microwell plates for culturing and assaying can be utilized but are not sufficiently high throughput. Here, we will present our development of a high throughput screening platform which utilizes microfluidics to encapsulate yeast cells in nanoliter aqueous droplets surrounded by an immiscible fluorinated oil phase. This system contains modules for cell culturing, measurement of the metabolite of interest with a fluorescent enzymatic assay, and sorting. To demonstrate the capabilities of this platform, we screened an incoming population which contained equal numbers of two *Saccharomyces cerevisiae* strains and enriched for the higher xylose consuming strain by over 20 times. Furthermore, we screened a *S. cerevisiae* xylose transporter library for high xylose consumption and selected a mutant which consumes 5% more xylose than the library as a whole.

UNDERSTANDING OF E. COLI IN VIVO EVOLUTION UNDER NADPH ACCUMULATION STRESS

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Bacteria are robust organisms as they can overcome natural or induced perturbations on their environment, such as changing in carbon source or gene deletions, through adaptative evolution. In this study, we have tried to understand if bacteria are able to overcome a major stress, such as an imbalance intracellular redox power. With a view to answer this problematics, a metabolic engineering strategy was used to modify E. coli MG1655 genotype so as to increase NADPH ratio. Indeed, the carbon flux was redirected, with glucose as carbon source, through Pentose phosphate pathway, thanks to pgi and edd deletions, and depleted NADPH reoxydation pathway, with qor and udhA deletions. This strain has undergone two parallel independent evolution process by prolonged exponential growth thanks to successive passage of cultures into fresh minimal medium complemented with glucose. After eleven successive cultures, approximatively 60 generations, two final evolved populations were obtained with the same endpoints of evolution. From these populations, several clones that showed the same genotype were isolated. A final evolved clone was characterized with a growth rate 12-fold higher than parental strain, and this genome has been entirely sequenced by Nimblegen CGS (Comparison Genome Sequencing) technology. Two mutations were identified and were confirmed to be the sole mutational events occurred during adaptative evolution because these only two mutations are responsible for growth rate increase. One is a single mutation on nuoF (nuoF*), which encodes for a subunit of the respiratory complex I. This evolved enzyme exhibits a novel enzymatic activity: NuoF can use both of NADPH and NADH as electron donors. Thus, this evolved complex I establishes a new NADPH reoxydation pathway in the cell. The second mutation is a two nucleotide deletion on rpoA, which encodes for the alpha subunit of the RNA polymerase. Transcriptional experiments on rpoA and rpoA* strains for understanding this role in adaptative evolution reveal pleiotropic effects on E. coli metabolism. Principally, they suggest energy save by under expressed useless genes, such as genes involved in flagellar function and chemotaxis, and a probably establishment of glyoxylate shunt. Moreover, these analyses show over expressed genes involved in amino acids biosynthesis, known to be NADPH consuming reactions. This study enlightens the capacity of E. coli to find effective solution for adaptative evolution by selection of two original mutations, that leads modified strain to a quasi wild type phenotype.

MODELING, RATIONAL DESIGN AND *IN VIVO* EVOLUTION OF A 1, 2 PROPANEDIOL PRODUCER

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1, 2 propanediol is a bulk chemical, with a huge market, which is currently exclusively produced from chemical process using propylene as a raw material. As an alternative to this chemical process dependent on fossil carbon, we have modeled and developed a biological process for the continuous conversion of glucose to 1, 2 propanediol and an oxidized co-product at very high carbon yield and very low production of carbon dioxide. Based on our MetaVista™ metabolic model, we engineered *Escherichia coli* by a rational metabolic approach and combined it to our MetEvol™ technology for *in vivo* evolution.

We will present both the genetic targets of the evolution in the evolved strains and the performances of the further optimized bioprocess to produce propylene glycol at high yield, titer and productivity.

ENGINEERING COMPLEX PHENOTYPES TO ENABLE BIOFUELS AND BIOCHEMICAL PRODUCTION

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The production of biofuel and biorefining chemical products requires the engineering of complex phenotypes, which are defined by their sensitivity to multiple genetic and environmental causes. Examples of complex phenotypes include tolerance to feedstock contaminants (acetate), process conditions (pH, osmolarity), and fermentation products (alkanes, alcohols, acids). Prior efforts to engineer complex phenotypes have been limited by an inability to effectively map the genetic and environmental basis of such phenotypes. As a result, conventional approaches rely upon recursive adaptation of target strains, which are slow, often result in dead-end or "sick" strains, and do not generate the basic understanding required for further engineering or intellectual property protection. The effective engineering of such phenotypes represents a fundamental challenge for the future of metabolic engineering. We have previously reported that development of the SCalar Analysis of Library Enrichments (SCALEs) method to not only monitor enrichment patterns across comprehensive genomic libraries but also to better understand the mechanisms directing selection for complex phenotypes. We have applied this approach to gain insights into the genetic and biochemical mechanisms underlying tolerance to a variety of chemicals, including 3-hydroxypropionic acid, acetate, succinate, ethanol, napthol, and various antimicrobials. While this approach has provided unique insights for biofuels and biochemical production, it is limited in it's ability to simultaneously detect genes acting in-trans to alter a phenotype and does not detect disruptional mutational events. Thus, we have developed a multiplex-recombineering approach that allows for simultaneous engineering multiple "up" or "down" mutations into a single strain as well as the highly parallel tracking of such mutations via a molecular barcoding strategy. We have demonstrated the complete construction and application of required genetic vectors and are currently applying this design at the genome scale. We will report upon the insights we have uncovered through the application of such tools for engineering complex phenotypes.

AN INTEGRATED "LOW VOLUME HIGH THROUGHPUT CULTIVATION PLATFORM" FOR INDUSTRIAL SYSTEMS BIOLOGY: *STREPTOMYCES COELICOLOR* A CASE STUDY

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Streptomycetes are known for production of numerous secondary metabolites. At present more than half of the antibiotics in clinical use are produced by *Streptomyces* species. Traditionally *Streptomyces* strain improvement programmes have been dominated by random mutagenesis, followed by screening and selection in controlled environments for a desired phenotype. Today, with the advent of Industrial systems biology[1], it is possible to identify high-probability targeted genetic strategies to increase yield, titer, productivity, and/or robustness [2, 3]. The fate of these high throughput approaches in capturing the high producing mutant lie in the successful scaled down version of the production process i.e. to keep the physiological traits constant when the scale is changed. Moreover, such low volume high throughput cultivation platform (HTCP) can be used in other process optimization projects also. Scaling down of existing fermentation processes is a challenge due to volume restrictions on process control and monitoring tools. Furthermore, particular challenges faced when growing *Streptomycetes* in small scale are high demand for oxygen and filamentous growth. Here, we implement an **omics** and physiology based validation approach for evaluation of HTCP. We introduced frozen mycelia based inoculum protocol instead of spores to obtain better reproducibility and to reduce the process turn around time. To achieve dispersed morphology, glass beads were used during cultivation. For process monitoring, we used in-house developed automated image analysis software to capture the onset of antibiotic production. Further, to evaluate the scale down process, we used Macro and Micro analysis based validation. In macro analysis we compared fermentation kinetics (mu, Ysx, Yps, qp) between batch reactor (scale: 1liter) and HTCP (scale: 3ml). For micro analysis, we compared ^{13}C flux distribution and transcriptome profiles. We observed that the process scale down is not only reproducible on macro scale but also the physiological traits are reproducible on fluxome and transcriptome level. The major factor which attributes to successful scale down is underlying control of morphology of *S. coelicolor*. We show that the beads are responsible for reproducible morphology. Moreover, we used particle size distribution as a tool to check variation among HTCP wells. Finally, some key results related to i) process scale down ii) large scale analysis of *Streptomyces coelicolor* mutant library and iii) non invasive process monitoring will be discussed as a proof of concept.

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METABOLIC FLUX ANALYSIS OF SHEWANELLA SPP. REVEALS EVOLUTIONARY ROBUSTNESS IN CENTRAL CARBON METABOLISM

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Shewanella spp. are a group of facultative anaerobic bacteria widely distributed in marine and freshwater environments. In this study, we profiled the central metabolic fluxes of eight recently sequenced Shewanella spp.â€™s grown under the same condition in minimal medium with [3-13C] lactate. Although the tested Shewanella species had slightly different growth rates (0.23 - 0.29 hr-3) and produced different amounts of acetate and pyruvate during early exponential growth (pseudo-steady state), the relative intracellular metabolic flux distributions were remarkably similar. This result indicates that Shewanella species share similar regulation in regard to central carbon metabolic fluxes under steady growth conditions: the maintenance of metabolic robustness is not only evident in a single species under genetic perturbations (Fischer and Sauer 2005), but also observed through evolutionary related microbial species. This remarkable conservation of relative flux profiles through phylogenetic differences prompts us to introduce the concept of metabotype as an alternative scheme to classify microbial fluxomics. On the other hand, Shewanella spp displays flexibility in the relative flux profiles when switching their metabolism from consuming lactate to consuming pyruvate and acetate.

IMPROVED THERMOSTABILITY AND ACETIC ACID TOLERANCE OF ESCHERICHIA COLI BY DIRECTED EVOLUTION OF HOMOSERINE O-SUCCINYLTRANSFERASE

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In Escherichia coli, growth is limited at elevated temperatures mainly because of the instability of a single enzyme, homoserine o-succinyltransferase (MetA), the first enzyme in the methionine biosynthesis pathway. The metA gene from the thermophile Geobacillus kaustophilus cloned into the E. coli chromosome was found to enhance growth of the host strain at elevated temperature (44C), thus confirming the limited growth of E.coli due to MetA instability. In order to improve E. coli growth at higher temperatures, we used random mutagenesis to obtain a thermostable MetA. Sequencing of the thermotolerant mutant showed five amino acid substitutions. An E. coli strain with the mutated metA chromosomally inserted showed accelerated growth in the temperature range 34-44C. Among the site-directed metA mutants, we identified two amino acid residues responsible for the heat and acid sensitivity of MetA. Replacement of asparagine-267 with aspartic acid and isoleucine-229 with threonine stabilized the protein. The thermostable MetA showed less aggregation in vivo at higher temperature and after acetic acid treatment. The mutated MetA showed increased catalytic activity at elevated temperatures and in the presence of acetic acid. The data presented here are to show improved E. coli growth under stress conditions solely because the MetA protein has been stabilized.

URACIL-EXCISION BASED CLONING: A FAST AND EFFICIENT METHOD FOR THE CREATION OF DNA-CONSTRUCTS

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Metabolic engineering requires the creation of DNA constructs which often is the bottleneck in the construction of strains of interest. For example, when assembling a DNA construct composed of several independent units, overlapping PCR and successive restriction/ligation reactions are the preferred techniques. While overlapping PCR carries a high risk of PCR-introduced errors, restriction sites present in the fragments to be joined pose a limitation to the use of restriction/ligation reactions. One promising technique to overcome this bottleneck is uracil-excision based cloning which was first described in the early 1990s. The uracil-excision cloning event relies on the ability of app. 7-15 nt long complementary overhangs generated at the ends of, respectively, a PCR amplified DNA fragment and a linearized destination vector, that make a stable hybridization product, which can be used to transform E.coli without prior ligation. These overhangs are generated on PCR fragments by placing a single uracil residue in each primer used to amplify the target DNA and subsequently treating the resulting PCR product briefly with uracil DNA glycosylase and DNA glycosylase-lyase Endo VIII. This technology has been available as a commercial kit for several years (USER kit) however, the technology has remained largely unused. We have made several essential modifications to this technology which now allows simultaneous fusion and cloning of multiple PCR products independently of restriction sites. We are able to simultaneously fuse and clone up to six PCR fragments with more that 90% efficiency. Apart from the single PCR amplification and the bacterial transformation, the procedure takes approximately 1 hour, therefore, our cloning output has increased by more than ten-fold which has led to that creating DNA constructs is no longer a bottleneck. We expect this method to replace overlapping PCR and also the use of restriction/ligation reactions in many of their applications for metabolic engineering in bacteria, plants and fungi.

DYNAMIC METABOLIC FLUX ANALYSIS WITH LINEAR FLUX FUNCTIONALITY

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Metabolic flux analysis (MFA) using stable isotopes has been established as a practical tool for developing flux mappings of complex biological reaction networks. At the present time, work involving MFA has been limited to systems at metabolic stationary state. In order to develop a framework capable of analyzing fully dynamic systems, a reaction rate functionality must be assumed. In the current work the validity of approximating reaction rates as a linear functionality of time is examined using principles of stoichiometric metabolic flux analysis. A model was developed using linear variations in fluxes to analytically simulate concentration profiles from initial concentration and fluxes at discrete time points. Weighted least squared principles were applied to estimate optimum values for these flux parameters from concentration and flux measurements in order to elucidate flux profiles. An error minimization routine was applied to select optimum discrete time points for fluctuations in reaction rate in order to minimize error while preventing overfitting. The model was applied to a previously collected data set, concentration profiles for production of 1,3-propanediol obtained from a fed-batch fermentation of a high yielding strain of E. coli. The new model was demonstrated successfully and flux profiles generated were in agreement with flux estimates by traditional means. In addition, a statistical analysis of the model was conducted allowing determination of flux confidence intervals and parameter sensitivities for the first time. Establishment of parameter sensitivity to measurements will allow the model to serve as a guide for sample time point selection. Having demonstrated the validity of the linear flux mappings approximation, future work will involve application of linear flux mapping for analysis of dynamic isotope labeling experiments. Additional work will also involve extension of the methods within the current work to analyze the validity of more complex flux mapping functionalities.

EVOLUTIONARY MULTIOBJECTIVE ALGORITHMS FOR *IN SILICO* METABOLIC ENGINEERING

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In Metabolic Engineering, the identification of genetic manipulations that lead to mutant strains able to produce a given compound of interest is a promising, while still complex process. Several different algorithms have been proposed to address this problem, namely mixed integer linear programming (MILP) [1] and more recently stochastic meta-heuristics, such as Evolutionary Algorithms (EAs) [2] and Simulated Annealing (SA) [3].

The most common approach consists in solving a bi-level optimization problem, where the strain that maximizes the production of some compound is sought, while keeping the biological objective of maximizing biomass. Although these approaches have provided good results, they give only one single optimal (or near optimal) solution to the problem. In many situations, a set of solutions with different trade-offs between the production of the desired compound and the biomass production would be desirable.

In this work, an approach based on Multi-Objective Evolutionary Algorithms (MOEAs) is proposed to this problem. In fact, since the mid-1980's, MOEAs are being used to solve all kinds of multiple-criterion problems in distinct scenarios and the multiobjective nature of the *in silico* Metabolic Engineering problem suggests that this is a good candidate for MOEAs. The MOEAs chosen for this task are two of the most popular algorithms, namely the SPEA2 and the NSGA-II, widely accepted as two of the algorithms with best overall performance.

The MOEAs are validated using a case study that considers the production of succinic acid with *E. coli*, using the available genome scale metabolic model [4]. The results are compared to previous work [2][3] regarding the same case study, where single objective EAs and SA approaches have been proposed. The results obtained are quite promising, since the MOEAs were able to find in a single run, a set of trade-offs between the two optimization aims that could only be reached by single-objective algorithms with several runs varying a threshold parameter.

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13C-METABOLIC FLUX ANALYSIS FOR THE TRANSIENT IN THE BATCH CULTURE USING CE-TOF/MS

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Metabolic flux analysis (MFA) based on 13C-labeling experiments has been recognized to be a powerful tool for quantitative analysis of metabolic networks, in particular for metabolic engineering and metabolic regulation analysis etc. The application of this approach, based on the labeling patterns of the proteinogenic amino acids measured using GC-MS and/or NMR, is essentially limited to the continuous culture due to the stationary assumption. Since most of the industrial fermentations are conducted in batch mode, and the metabolism changes with respect to time, it is strongly desired to evaluate the metabolic fluxes with respect to time. Although the above method may be applied to batch and fed-batch cultures with slow growth condition, the changes of the metabolic fluxes cannot be evaluated with respect to time due to the nature of proteinogenic amino acids [1]. To overcome this problem, we developed a procedure for MFA from the labeling patterns of the intracellular metabolites measured by CE-TOF/MS [2].

In the present research, we measured the labeling patterns of intracellular metabolites such as F1,6P, DHAP, 2/3PG, PEP, PYR, Ru5P, R5P, S7P, and MAL as well as those concentrations using CE-TOF/MS with respect to time in the batch culture of *Escherichia coli* BW25113. The metabolic fluxes were evaluated at 4, 5, 6, 7 and 9 hours from the start of batch culture, where M9 synthetic medium with 4 g/l of glucose was used. The result indicates how the metabolic fluxes changed with respect to time, and in particular, the TCA cycle flux tended to increase with respect to time. After depletion of glucose, the metabolism drastically changed and the fluxes at 9 h indicates the activation of gluconeogenic and anaplerotic pathway such as glyoxylate pathway. The statistical analysis was also made to assure the reliability of the result. Recently some flux analysis studies using LC-MS(/MS) for measurements of mass distribution of intracellular metabolites applied to non steady state conditions such as fed-batch culture or short time isotopic dynamics were reported [3, 4]. However, the batch culture involves more drastic changes in the metabolism than theses conditions, and CE-TOF/MS is high throughput as compared with LC-MS(/MS). The present approach may give new paradigm with widespread application to the efficient metabolite production by batch cultures in industry.

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SIMPLE LOCAL FLUX QUANTIFICATION USING ^{13}C-TRACER SUBSTRATE IN ISOTOPIC NON-STATIONARY EXPERIMENTS

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^{13}C flux analysis has become an important tool in the omics technolgies for strain improvement due to its advantage in resolving parallel and reversible fluxes. Classical ^{13}C labeling experiments for metabolic flux analysis (MFA) are mostly limited by either the requirement of isotopic steady state or, for the isotopic non-stationary state, the extremely high computational effort due to the size and complexity of large metabolic networks. The presented novel approach is based on a ^{13}C tracer flux and circumvents these limitations by applying the isotopic non-stationary approach to a local metabolic node of interest. By introducing a small (5%) flux of labeled tracer, to the node of interest, metabolic fluxes can be estimated accurately, using mass isotopomer analysis of only a few intracellular metabolites, without simulation of the complete network. Moreover, the metabolite pool size and flux data are integrated by the wash-in dynamics of the mass isotopomer fraction, which allows accurate quantification of the flux. The much larger time constant of the tracer wash-in dynamics compared with the intracellular metabolites allows, using time scale separation, simplification of the ordinary differential equation model into isotopomer pseudo steady state mass balances. Experimental design showed that short isotopic non-stationary experiment can be achieved by using high cell density cultivation. The new method was applied to the oxidative branch of pentose phosphate pathway of *Penicillium chrysogenum* as a case study, using [U-$^{13}C_6$]-gluconate as tracer. The obtained flux is highly comparable with the result from the isotopic stationary tracer method (Kleijn *et al*. 2006) as well as the conventional metabolic flux balancing analysis with assumptions on NADPH balances. This result is a lead for further metabolic engineering of the strain for productivity improvement.

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RECOMBINEERING WITH RED®/ET® - MODIFICATION OF THE BACTERIAL CHROMOSOME

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Metabolic engineering to design and construct microorganisms suitable for the production of industrial products like ethanol or aromatic amino acids requires the disruption of specific genes on the bacterial chromosome. Regulatory circuits, the uptake of carbon and amino acids, the glycolytic and pentose phosphate pathway, as well as the common aromatic amino acid pathway have to be manipulated. The complexity of the necessary modifications requires a tool allowing the precise knock-out or alteration of multiple genes without leaving antibiotic selection markers. Red/ET recombination (1) also known as recombineering is an easy to use modification system for prokaryotic functional genomics. There is proven evidence that Red/ET works not only in E.coli, but also in Salmonella, Shigella, Yersinia, Serratia and Citrobacter. Here we demonstrate the easy and precise knock-out/knock-in of genes as well as the possibility to use Red/ET as a powerful subcloning-tool. References: (1) Zhang Y. et al 1998: “A new logic for DNA engineering using recombination in Escherichia coli“ Nature Genetics 20, 123-138.

ENZYME STATES ALLOW IDENTIFICATION OF RATE-LIMITING STEPS

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A precise quantification of the effect of perturbations in a metabolic network depends on explicit knowledge of the kinetic properties of the enzymes of the individual reactions. However, a comprehensive knowledge of enzymes kinetics in a metabolic network is very difficult, if not impossible, to obtain. Furthermore, experimental data obtained under different conditions introduce uncertainty in enzyme kinetic parameters. In this work, we model the uncertainty in the kinetic data and we predict quantitatively the responses of metabolic networks in the presence of genetic, biochemical, and environmental variations. The proposed methodology accounts explicitly for mechanistic properties of enzymes and physicochemical and thermodynamic constraints and is based on formalism from process control and metabolic control. The method employs a novel, efficient Monte Carlo sampling procedure that allows us to simulate all possible meaningful states of a metabolic network and to compute the corresponding values of the kinetic constants of the individual reaction steps. We demonstrate the properties of the proposed framework through a number of case studies

IDENTIFICATION OF THE DESIGN PRINCIPLES OF SIGNALING PATHWAYS FOR METABOLIC ENGINEERING

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Signal transduction pathways relay extracellular stimuli from the plasma membrane to targets in the cytoplasm and nucleus, initiating diverse responses involving cell growth, mitogenesis, differentiation and stress responses in mammalian cells. The mitogen-activated protein kinase (MAPK) cascades are ubiquitous in eukaryotic signal transduction, and these pathways are conserved in cells from yeast to mammals. Metabolic engineering of mammalian cells requires the redesign of the steady-sate and the dynamic responses of signal transduction pathways. Therefore, understanding the design principles of these pathways is a key to success of metabolic engineering for cell culture development and drug target discovery.

In recent years, much effort has been devoted in the development of detailed kinetic models of MAPK networks as tools for the rational design of metabolic engineering strategies. These models are mostly large systems of differential-algebraic equations (DAEs) and they link molecular (protein-protein, protein-DNA, and protein-RNA) interactions, gene expression and chemical reactions to cellular behavior. In this DAE description of signaling patwhays, the ordinary differential equations express the mass-action kinetics, whereas the algebraic equations enforce conservation relations among the constituents. Moreover, these models typically involve a relatively large number of parameters, such as the rate constants and strength of protein-protein interactions, the values of which are not directly accessible in vivo and are subject to large uncertainty.

In this work, we investigate the application of dynamic optimization techniques to study the relationships between the biophysical and biochemical parameters and the functions of the MAPK cascades. Large-scale, nonlinear DAE models can be handled within this framework, as well as a large variety of objective functions and constraints. We first consider a single kinase-phosphatase cycle and we employ dynamic optimization methods to identify ranges of the parameters that confer optimal dynamic response properties. We find that values of the kinetic parameters that confer optimal dynamic properties, such as short response times and large amplification capabilities, also allow optimal threshold response profile to a graded input (ultrasensitivity). In the second series of studies, we extend the analysis to the general case of multiple cycles in the MAPK cascade, with emphasis on a linear three-kinase model. The same response properties as in the single cycle case are considered, and dynamic optimization is employed to identify parameter values that optimize the dynamic and steady-state response properties.

SYSTEMATIC REDUCTION OF MODELS OF TEMPLATE POLYMERIZATION PROCESSES

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The engineering of gene regulatory circuits forcefully requires the use of systematic mathematical modeling to provide guide lines and make quantitative predictions. The components of such circuits posses exceedingly diverse and rich forms of interactions, it is therefore indispensable that mathematical models incorporate the essential details of the processes involved for making accurate predictions. It has been shown that incorporating time delays in reduced models of gene networks is often essential to capture the whole range of behavior. From a mechanistic model for protein translation in the form of a large system of ODE's, we systematically derive a reduced time-delay model by approximating the ODE system by a linear PDE with a nonlinear, integral boundary condition. We find quantitative agreement in protein synthesis rates between models. The region in parameter space where self-sustained oscillations exist is found, via bifurcation analysis, for both the reduced model obtained and a heuristic model proposed elsewhere. Qualitative differences are found between the models and explained in terms of the undergoing biological process.

AN ELEMENTARY METABOLITE UNITS (EMU) METHOD FOR RATIONAL DESIGN OF LABELING EXPERIMENTS FOR METABOLIC FLUX ANALYSIS

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Quantitative assessment of metabolic pathway fluxes is critical for metabolic engineering of microbial cells and in health sciences. The development of Elementary Metabolite Units (EMU) framework is seen as one of the most important developments in this area in the past decade. The EMU framework opened the door for highly advanced metabolic flux analysis (MFA) methods that include the use of multiple isotopic tracers and analysis of nonstationary systems in vivo. In this presentation, I will describe further novel developments of the EMU framework for optimal and rational design of labeling experiments for MFA. The amount of information that is obtained from tracer experiments is highly dependent on experiment design, in particular (i) choice of isotopic tracer, (ii) positional labeling of substrates, (iii) choice of sampled metabolite pools, and (iv) time of sample collection. Until now it has been difficult to assess the optimal combination of measurements and tracers in tracer experiments. Here, I will demonstrate how the EMU framework provides an elegant solution to this problem. Based on EMU decomposition of metabolic networks one can derive expressions of measurements in terms of basis vectors, which solely depends on the network structure and substrate labeling. As such, for a given network model the number of independent basis vectors, and hence the number of independent fluxes that can be estimated, depends solely on the choice of tracer input and measurements. The problem of finding the best combination of measurements and tracers is therefore reduced to the problem of maximizing the rank of a matrix consisting of basis vectors. This method allows intuitive and rational design of optimal tracer and measurement combinations. This method was successfully applied in small scale example models, where the results were compared to an exhaustive search of all possible labeling combinations using a grid search algorithm, and in realistic large scale models. The advantage of this extended EMU approach lies in the fact that it is (i) rational, (ii) general, i.e. can be applied to networks of any size and complexity, and (iii) computationally fast. Further extensions of this method provide novel methodologies for optimal design of isotopic tracer experiments in dynamic systems.

IDENTIFICATION AND EVALUATION OF APPROXIMATIVE KINETIC MODEL STRUCTURES

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Background: Nowadays, the focus in metabolic engineering is shifting from the massive overexpression and inactivation of genes towards the model-based fine tuning of gene expression. Hence, target identification is more than ever at the core of metabolic engineering. Especially, the potential of dynamic metabolic models to identify genetic targets appears huge, since these models incorporate enzyme kinetics and regulatory mechanisms.

Still, the targets identified by these dynamic metabolic models are subject to uncertainty, which can be due to uncertainty on the model structure and/or inaccurately known model parameters. Hence, being able to assess the uncertainty on the identified targets is essential for the use of the model's outcome to steer the process of metabolic engineering.

Results: For an illustrative pathway the uncertainty on the calculated flux control coefficients has been assessed.

To assess the uncertainty on the calculated flux control coefficients due to inaccurately known model parameters, these parameters have been identified using transient *in silico* data.

To assess the uncertainty on the calculated flux control coefficients due to uncertainty on the model structure, the performance of several rival model structures has been evaluated. The state of the art approximative kinetics, the linlog kinetics, the GMA type power law kinetics, and the linear in metabolite levels kinetics, have been used to do so. All kinetics adequately described the data.

Conclusions: A Bayesian method is proposed to properly assess the uncertainty on the calculated flux control coefficients in view of increasing the trustworthiness of the identified metabolic engineering targets. All of the state of the art approximative kinetic formats: the linlog kinetics, the GMA type power law kinetics, and the linear in metabolite levels kinetics adequately described the data, even though the original kinetic equations used in this study are highly nonlinear and the metabolite range observed during the transient is not small.

It is shown that to a large extent the uncertainty on the calculated flux control coefficients is due to an uncertain model structure and consequently it is worth the effort to increase the trustworthiness of the identified metabolic engineering targets by means of optimal experimental design for model discrimination.

APPROXIMATE FLUX FUNCTIONS

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A method for the construction of approximate steady-state flux functions that describe and predict steady-state flux changes in response to any number of simultaneous, large enzyme activity changes is presented. The methodology for constructing these approximate flux functions and an assessment of their predictive power is illustrated using a detailed kinetic model of *L. lactis*'s glycolytic and fermentative pathways as a test case. The predictions of the approximate flux functions proved to be in excellent agreement with the fluxes calculated with the detailed kinetic model. The method is further challenged to describe and predict the fluxes of *L. lactis* mutants with altered expression of glycolytic enzymes.
Metabolic systems comprise many components interacting in non-linear fashion. Metabolic system properties, such as steady-state fluxes, are thus complex functions for which an explicit analytical solution cannot be found. Analytical solutions for systems of components interacting linearly, on the other hand, are readily attainable. Within the framework of Metabolic Control Analysis (MCA) the dependence of steady-state fluxes and metabolite concentrations upon small (infinitesimal) changes in the system parameters have been solved explicitly and several generalizations have been proposed as MCA theorems. The important results and theorems of MCA are, however, limited to the abstraction of infinitesimal changes in the system's parameters.
A general property of enzyme catalyzed reactions is that the rate of catalysis is directly proportional to the amount of enzyme present. In MCA, changes in the rate of enzyme catalyzed reactions as functions of enzyme amounts and metabolite concentrations are approximated by linear combinations of small changes in these variables. This procedure enables the analytical solution of steady-state fluxes as functions of enzyme activity changes, but also results in rate changes that are not directly proportional to the amount of enzyme present. Here we've used approximate kinetic functions that preserve the direct proportionality between enzyme rates and amounts and used these in combination with the theorems of MCA to construct expressions that describe flux changes as functions of enzyme activities. In contrast to traditional approaches within MCA, our approximate flux functions predict fluxes changes in response to any number of simultaneous, large enzyme activity changes. In agreement with the available experimental observations, our method predicts that fluxes are hyperbolic functions of enzyme activities.

DYNAMIC LINLOG MODELLING OF THE CENTRAL METABOLISM OF E. COLI: ESTIMATION OF ELASTICITIES FROM PULSE-RESPONSE DATA USING GPROMS

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Future developments in metabolic engineering shall greatly benefit from the availability of dynamic metabolic models. However, efforts on dynamic metabolic model identification are still challenging.

As an alternative to highly nonlinear mechanistic equation, linlog kinetics containing elasticities as linearly appearing parameters have been proposed. Focus of this contribution is the estimation of elasticities in a dynamic model for *E. coli* on the basis of pulse-response data. As a model environment, gPROMS (www.psenterprise.com) is exploited. gPROMS is commonly used in the chemical process industry for large scale systems and process optimization. The software evaluates the complete set of sensitivity equations and can readily solve stiff and high-index DAE (differential algebraic equation) systems. This makes it also attractive for biochemical modeling.

A chemostat culture of *Escherichia coli* K12 MG1655 (D = 0.1 1/h) was subjected to a moderate glucose pulse. Intra- and extracellular metabolites were monitored using appropriate sample handling and analytical techniques [1]. Available measurements were: glucose, glucose-6P, fructose-6P, fructose-biphosphate, pool of 2- and 3-phosphoglycerate, phoshoenolpyruvate, pyruvate, and 6-phosphogluconate. The dynamic linlog model was derived from the network stoichiometry in [2] containing glycolysis, the pentose phosphate pathway and biosynthetic fluxes. Adjustments were made to embed by-product formation and correct dynamics in biosynthetic fluxes. Cofactors were approximated by analytical expression based on data and considered as inputs.

Due to the lack of information in the data, not all 59 elasticities could be estimated. Intermediate results revealed that a number of elasticities are negligible (zero). Calibration of the remaining model to the data yields an excellent data description. Most parameter estimates are, however, highly correlated and uncertain. Only seven out of 30 elasticities are statistically significant. Extension of the identified model with mass balances for cofactors can not predict the cofactor data, indicating the importance of incorporating oxidative phosphorylation, TCA and (probably) amino acid synthesis to fully capture cell dynamics.

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13C-EMU FLUX: A SIMPLE APPLICATION FOR ^{13}C-BASED STEADY-STATE METABOLIC FLUX ANALYSIS

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^{13}C fluxomics is an important component of systems biotechnological approaches for metabolic engineering. However, ^{13}C-MFA requires significant upfront investment to construct the underlying isotopomer balance model and numerical optimization. We have developed 13C-EMU FLUX, in order to meet the need for a simple yet robust application to perform large-scale steady-state ^{13}C-MFA. This is a universal application that facilitates a streamlined workflow from metabolic model setup to parameter estimation and confidence interval determination. The application was developed on JAVA and MATLAB.

13C-EMU FLUX is significantly faster than an existing cumomer-based software, 13C-FLUX. Using a ^{13}C experiment derived from literature as a sample problem, we showed that the computation time for each optimization was reduced from hours to seconds (by at least 600 fold). This is attributed to the use of the EMU (Elementary Metabolic Unit) framework for constructing the isotopomer balance model. We also introduced a straightforward coordinate system to describe bi-directional fluxes, which allowed us to implement an automated system for free flux assignment. As a result, the model setup becomes simple and intuitive. In addition, the application supports large-scale MFA, whereby metabolites bearing no relevance to the isotopomer balance, such as cofactors, can be incorporated as additional constraints. Lastly, the definition of the simulated data vector is flexible, thus various experimental data type can be applied.

13C-EMU FLUX was validated against published ^{13}C-MFA studies for *C. glutamicum* and yeast. We were able to reproduce the simulated GC-MS data, as well as the estimated flux parameters and corresponding confidence intervals, with a few exceptions. The discrepancies detected in two of the estimated fluxes could be explained by the existence of alternative optima. Conventional linear or non-linear confidence interval algorithms do not account for the presence of multiple optima and provides a false sense of accuracy of estimates. The presence of multiple optima in relatively simple labelling experiments (glucose-minimal medium) highlights the need for fast algorithms to explore the solution space using Monte-Carlo simulations both in the experimental design phase to identify suitable labelled substrates and metabolite for measurement and in the analysis phases to identify such optima.

DEVELOPMENT OF AN ACCURATE METHOD FOR INTRACELLULAR METABOLOME ANALYSIS IN *ESCHERICHIA COLI* FOR *IN VIVO* KINETIC ANALYSIS

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Biotechnology needs metabolic engineering strategies which aim at increasing production of certain desired substances in a cell, especially of agricultural, food and pharmaceutical products. The targets for engineering of the cells are generally selected using metabolic network models. The modeling of these metabolic networks is important for understanding metabolic functionality of the microorganisms. These models require *in vivo* kinetic information of the metabolism. One of the ways to get knowledge on *in vivo* kinetics is observing the response of the extra/intracellular metabolite concentration to a sudden perturbation of the organism which was initially at steady state. Accurate measurement of these intracellular metabolite concentrations requires efficient removal of the extracellular medium in which these metabolites often are present in significant amounts. Glycolytic, pentose phosphate (PP) pathway and TCA cycle intermediates, free amino acids and nucleotides were measured in steady state chemostat ($D=0.1h^{-1}$) cultivated aerobic glucose-limited *Escherichia coli* K12 cells. LC-ESI-ID-MS/MS measurements showed that significant amounts (ìM-concentration range) of almost all metabolites measured were present outside the cells in the fermentor. The most commonly applied method for the required removal of extracellular medium, quenching with cold methanol and subsequent washing of the cell pellet, resulted in severe (> 80%) leakage of metabolites into the cold methanol. The leakage mechanism was proved with a rigorous balancing approach, by showing in a quantitative manner that the metabolites lost from the cell pellet are found back in the quenching solution. Different quenching solutions did not decrease leakage. Therefore a differential method was developed whereby metabolite levels in broth and culture filtrate samples (obtained by direct filtration) were quantified and subtracted from each other. This method was successfully applied to obtain a thermodynamically consistent intracellular metabolite dataset for the steady state.

The differential method was then applied for metabolite measurements during a rapid pulse (< 600 seconds) experiment. Chemostat cultivated aerobic glucose-limited *Escherichia coli* cells were disturbed with sudden increase of glucose in the culture (from 20 to 500 mg/L). Considerable dynamics, suggesting oscillations, was observed for glycolytic, PP pathway and TCA cycle metabolites, nucleotides and free amino acids.

RANKING OF MOST INFLUENTIAL KINETIC PARAMETERS IN METABOLIC NETWORKS THROUGH GLOBAL SENSITIVITY ANALYSIS

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Dynamic models for metabolic networks comprise a nonlinear differential algebraic system of equations, which arise from mass balances for metabolites and have a large number of kinetic parameters that require tuning for a specific growth condition. However, uncertainty in input parameters has different effect on model outputs. In this work, we have performed a global sensitivity analysis through variance-based techniques to identify most influential parameters on model output and which of them account for most of the uncertainty in that output. Sensitivity indices have been calculated for each parameter, based on Sobol's approach (2001), which makes use of Monte Carlo methods for the calculation of times profiles for main effect variances in input parameters for main state variables. The global sensitivity analysis has been carried out on a large-scale differential algebraic system representing a dynamic model for the Embden-Meyerhof-Parnas pathway, the phosphotransferase system and the pentose phosphate pathway of Escherichia coli (Chassagnole et al., 2002). The model comprises eighteen dynamic mass balance equations for extracellular glucose and intracellular metabolites, thirty kinetic rate expressions and seven additional algebraic equations to represent the concentration of co-metabolites. The model involves around one hundred parameters (Di Maggio et al., 2008). We have implemented the large-scale metabolic network model in g-PROMS (PSE Enterprise, 2007). In this environment, two different sets of random parameters have been generated for k=20 parameters, which were selected with a preliminary screening. Sample size of N=2500 scenarios have been considered. We have performed the N(2k+1) Monte Carlo simulations in g-Proms and output temporal profiles for state and algebraic variables have been exported for subsequent variance and sensitivity indices calculation within a Fortran 90 environment. Calculated sensitivity indices show, for example, that all parameters affect the concentration of ribu5p, but the most influential one is Nptsg6p, which is involved in the kinetic expression for phophotransferase system. Pgp concentration is sensitive to only four parameters, Kpglumueq, Kgapdhgap, Kgapdhpgp y Rgapdhmax which are involved in the kinetic expressions for glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate mutase. Global sensitivity analysis has provided a ranking of most influential input parameters for the formulation of a dynamic parameter estimation problem.

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PAIRWISE SELECTION ASSEMBLY FOR METABOLIC PATHWAY CONSTRUCTION AND ENGINEERING

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The engineering of biological components has been facilitated by de novo synthesis of gene-length DNA. Biological engineering at the level of pathways and genomes, however, requires the scalable and cost-effective assembly of DNA molecules that are longer than approximately 10 kb, and this remains a challenge. Here we present the development of Pairwise Selection Assembly (PSA), a method that involves hierarchical construction of long-length DNA through the use of a standard set of components and operations. In PSA, recyclable activation tags at the termini of assembly sub-fragments activate vector-encoded selectable markers, enabling stringent selection for correctly assembled product in vivo, often obviating the need for clonal isolation. Importantly, construction via PSA is sequence-independent, and does not require primary sequence modification (e.g., the addition or removal of restriction sites). Restriction sites within the activation tags are made to be unique through a RecA-assisted cleavage technique, ensuring that the fundamental operations performed on two sub-fragments to be paired are not dependent on the sequence being constructed, and do not change throughout the assembly process. PSA is useful for the combinatorial assembly of pathways and pathway variants from native or engineered 'modules,' consisting of promoters, genes, and other regulatory elements.

The utility of the PSA method for assembly of long-length DNA is demonstrated in the construction of various pathways, including a completely synthetic 91 kb fragment of DNA derived from *Saccharomyces cerevisiae*. Importantly, the sequence-independent PSA method enabled assembly of the entire 91 kb DNA fragment without introducing sequence modifications to accommodate the construction method. This advantage over conventional ligation-based assembly methods becomes substantial when designing and constructing pathways or chromosome-sized DNA molecules where the tolerance for sequence modification is unknown. The method was effective for pairing sub-fragments ranging from ~1.5 to ~45 kb and may be limited only by the ability to propagate assembled product in the chosen host. Other key PSA features, including hierarchical assembly, reaction standardization, and stringent selection for correct product make this process amenable to large-scale, automated assembly of pathways and pathway variants.

PRODUCTION OF SHIMIKIC ACID IN AN *ESCHERICHIA COLI* STRAIN LACKING THE PHOSPHOENOLPYRUVATE:CARBOHYDRATE PHOSPHOTRANSFERASE SYSTEM.

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Shikimic acid (SHK) is an intermediate of the chorismate biosynthesis pathway. This metabolite is used as precursor for the chemical synthesis of the antiviral drug oseltamivir phosphate (OSF) that inhibits the neuraminidase enzyme of the avian influenza virus H5N1. SHK used as substrate for OSF synthesis was obtained from plants like *Illicium anisatum* or *I. verum*, however potential risk of an avian influence pandemic, request the availability of large amounts of this precursor. In this work we report the SHK production in an engineered *Escherichia coli* strain lacking the phosphoenolpyruvate:glucose phosphotransferase system (PTS^-) that is able to grow in glucose by using the GalP system and phosphorylates the incoming glucose by the housekeeping Glk (PTS^- Glc^+). This genetic background and introduction to this strain of an additional copy of the *tktA* and *aroG*^{fbr} genes in a plasmid (pJLB*tktA aroG*^{fbr}), resulted in and increased carbon flow from the glycolytic and pentose-phosphate pathways to the aromatic biosynthesis metabolism. SHK production was achieved by inactivation of the *aroK* and *aroL* genes codindg the SHK kinase K and L respectively (PB12 &Delta*aroL* &Delta*aroK::cm*), introduction of an additional copy of the *aroB* and *aroE* genes coding 3-Dehydroquinate synthase and SHK dehydrogenase, respectively (TOPO *aroB aroE*) and inactivation of the *pykA* gene coding the Pyruvate synthase K (&Delta *pykF::gm*). Fermentor cultures with engineered strains were performed in order to evaluate the effect of each genetic modification performed on SHK production in 1 L reactors using a mineral broth complemented with 25 g/L glucose and 15 g/L yeast extract during 50h at 37°C, pH constant at 7.0 and speed of 500-700 rpm to achieve 20% dissolved oxygen. Fermentations were performed by duplicate with/without addition of 10mM IPTG. Growth was determined by optical density (OD_{600nm}) and the concentration of glucose, DAHP, quinic acid, galic acid, DAHQ, SHK and acetic acid were determined during the process. SHK was produced both during exponential growth and stationary phase, but maximum production was achieved during late stationary phase. Engineered strain designated as PB12.SHK (PTS^- Glc^+ &Delta *aroL* &Delta *aroK::cm* &Delta *pykF::gm* pJLB*tktA aroG*^{fbr} TOPO *aroB aroE*) produced 7 g / L SHK wit ha yield of 0.28 g SHK / g glucose and a theoretical aromatic yield of 0.33 g aromatic compounds / g glucose.

METABOLIC MODELLING OF THE N - ALKANE BIOCONVERSION: FUNCTIONAL MODELLING PACKAGE DEVELOPMENT

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The conversion of linear paraffins (n - alkanes) can take place through a number of chemical processing techniques. Due to the low activity of the carbon backbone, low reactivity demands fairly extreme operating conditions while product reactivity typically exceeding reactant reactivity poses challenges with respect to product selectivity. As an alternative, microbial systems can be used to convert the linear paraffins to higher value fine chemicals. Biological systems are well-recognised for their operation at ambient conditions and their selectivity. An increase in the production in linear paraffins as by-products, especially from the rapidly growing synthetic fuel and chemical industries has been predicted.

While many microbial systems are recognised to metabolise linear alkanes, very little quantitative research has been conducted into process optimisation and more specifically into microbial cell design. Such research is ideally conducted through juxtaposition of rigorous modelling of the system, system prediction and experimental validation. This paper centres on the development of a modelling package that provides robust numerical analysis as well as a user friendly interface to facilitate this research approach. By developing a suitable metabolic modelling tool providing linear flux analysis and fundamental dynamic kinetic analysis of a specific biochemical reaction network, research time and cost can be significantly reduced. This is of greater significance when working with non - conventional substrates due to the limited knowledge of the genetic and physicochemical system. By providing the two different metabolic modelling techniques in a single package, limitations around using steady state flux analysis can be mitigated while facilitating early stage simulations in the absence of sufficient kinetic data.

The modelling package has been developed to provide general functionality and can therefore be focussed on the system defined by the user. A user friendly GUI has been developed in Delphi ® along with numerical class wrappers that are used to access numerical routines compiled in FORTRAN or C. Robust numerical procedures provide the model with sparse linear, ODE (including stiff) and Parameter Sensitivity solving capabilities. It also has robust built-in parameter estimation and constrained optimisation capabilities.

Validation of the linear flux analysis component of the model is done using the data of Huang (2003). Model population with appropriate kinetic data will occur from experimental and literature sources, the finished model is expected to contribute to the optimisation of the kinetic parameters in the n - alkane reaction network through identifying targets for both genetic and physiochemical modification.

HIGH CELL DENSITY ACCUMULATION OF ENGINEERED ESCHERICHIA COLI WITHOUT EXTERNAL GLUCOSE FEEDING FOR THE PRODUCTION OF BIOPHARMACEUTICALS: OVERCOMING OVERFLOW METABOLISM IN BATCH CULTURES

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Culture productivities are usually increased by reaching high cell concentrations in the bioreactor. A major bottleneck to attain high cell-densities Escherichia coli cultures is the overflow metabolism, which is characterized by the production of acetate under aerobic conditions when the specific glucose uptake rate (qS) surpasses a threshold value. Typically, overflow metabolism is avoided by restricting qS to a value below the threshold through external feeding. However, a reduction of qS also decreases the specific growth rate, which results in longer culture times. Additionally, fed-batch processes require sophisticated control systems that might not be convenient during early stage microbial screening and drug development. In this contribution, we present an alternative molecular approach to avoid overflow metabolism. By replacing the glucose phosphotranspherase transport system by galactose permease, the qS value was decreased in about 66 %, whereas the specific growth rate was only slightly affected as compared with the parental strain. This allowed the use of high amounts of glucose in batch mode without requiring external glucose feeding. This strategy was tested for the production of recombinant green fluorescent protein (GFP) using 30 and 100 g/L of initial glucose in batch mode. When 30 g/L of glucose were used, 20, 3.3 and 0.15 g/L biomass, GFP and acetate, respectively, were produced by the modified strain. In contrast, 18, 1.7 and 4 g/L of biomass, GFP and acetate were accumulated by the parental strain. Biomass and GFP concentrations reached levels of 52 and 8 g/L, respectively, in the engineered strain cultures using 100 g/L of initial glucose concentration, and only 34 and 4 g/L in the parental strain culture. Under these conditions, acetate accumulated only to a maximum of 2 g/L in the engineered strain cultures, while the maximum value for the parental strain was 14 g/L. The engineered strain was also tested for the production of a plasmid DNA vaccine candidate using initial glucose concentrations of 10 and 100 g/L. In cultures at 10 g/L of initial glucose, 8.4 g/L of biomass were attained for the engineered strain, whereas only 5.5 g/L were produced by the parental strain. Acetate accumulated up to 3 g/L in the wild-type strain, whereas it was not detected in the engineered strain cultures. In cultures at 100 g/L of initial glucose concentration, biomass and acetate produced by the engineered strain were 47 and 2 g/L. In contrast, the parental strain produced 27 and 14 g/L of biomass and acetate, respectively. The potential advantages of the approach presented here for the production of plasmid DNA by each strain is currently being evaluated. The results described here show the potential of applying metabolic engineering tools to overcome process limitations and to accelerate the development of biopharmaceuticals.

GENOMIC AND PROTEOMIC ANALYSIS OF LYCOPENE-OVERPRODUCING *ESCHERICHIA COLI* STRAINS

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Systems biology represents a powerful method to describe and manipulate phenotypes of interest by incorporating biological information from various levels of cellular organization. Such an approach is illustrated from a library of both rationally-directed and combinatorial gene knockout strains of *E. coli* containing the pAC-LYC plasmid which have been shown to produce elevated levels of the representative isoprenoid lycopene. Global genomic and proteomic expression changes associated with increased lycopene production of mutant *E. coli* constructs were discovered using whole-genome *E. coli* DNA microarrays and a novel LC-MS technique, respectively.

Following validation of the DNA microarray method by ANOVA analysis, transcriptional profiling was applied to five high lycopene-producing knockout mutants. One observation was greater than 4 times more differential expression for strains with a deletion in *hnr*, an important regulator of $sigma_s$ factor, as compared to mutants with metabolic targets. Additionally, the conservation of expression trends across a majority of distantly-related mutants for specific genes was seen. Within a specific mutant family of *DgdhA, DgdhA DaceE,* and *DgdhA DaceE* D_P*yjid* strains, *hisH* of the histidine biosynthetic pathway was down-regulated more than 10-fold while a gene encoding for a critical component of ATP synthase, *atpE*, was up-regulated more than 3-fold.

To supplement the genomic data, proteomic expression changes between the strains were analyzed using a novel LC-MS technique for simultaneous identification and label-free quantitation of proteins. The combined database search results from the LC-MS analysis of eight samples provided greater than 500 protein identifications. While a majority of proteins showed little expression change in the mutants relative to the pre-engineered strain, some key proteins were up- or down-regulated by nearly 10-fold. In particular, WrbA, a NADPH quinone oxidoreductase which reduces the quinone pool, was observed up-regulated over 5-fold while MdoG, a periplasmic protein which may affect the membrane storage capacity for lycopene, was down-regulated over 2-fold.

A simultaneous examination of both genomic and proteomic data revealed that the TCA cycle appears generally down-regulated while the PEP-glyoxylate cycle appears generally up-regulated across the various mutants. This is interesting due to the effect of the PEP-glyoxylate cycle upon cellular NADPH/NADH balance since lycopene biosynthesis requires large amounts of NADPH. Accordingly, a repressor of the glyoxylate pathway, IclR, and a membrane-bound pyridine nucleotide transhydrogenase, PntB, were targeted as potentially important to lycopene production.

Current work is progressing to overexpress and delete the *hisH, wrbA, iclR,* and *pntB* genes and determine resulting effects upon lycopene production.

ENHANCEMENT OF ANTI-HIV PEPTIDE T-20 PRODUCTION IN RECOMBINANT ESCHERICHIA COLI BY ANALYSIS OF METABOLIC LOAD

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Analysis of metabolic load has revealed the enhanced production of the anti-HIV peptide T-20 in recombinant *Escherichia coli*. Marked activity of the protein expression system can increase production of a target protein, but imposes a considerable metabolic load to the host cell. A consequence is the stringent response, a complicated prokaryotic response system that transcriptionally regulates gene expression to enable cell survival under nutrient depleted conditions. The response includes cessation of the production of foreign proteins and switched gene expression to genes critical for cell survival. The protein producing machinery of the host cell is not fully utilized over time. Guanosine tetraphosphate (ppGpp) is the signal and effector molecule of the stringent response. By monitoring the intracellular ppGpp level, the occurrence of the stringent response and the resultant metabolic load can be determined.

Presently, fed batch fermentation was performed for T-20 mass production with *E.coli* BL21/pET23a-G3T20, which has an isopropyl-beta-D-thiogalactopyranoside inducible expression system. During fermentation, intracellular ppGpp was measured; ppGpp increased from 1.55umol/g dry cell weight to 4.5umol/g dry cell weight 1h after induction. Cell growth retardation was also observed. It might be that induction of target protein expression caused the metabolic load and resulting stringent response.

To clarify metabolic load-related physiological changes, a proteome analysis was done after induction. Prominant spots were selected and malonyl CoA-acyl carrier protein transacylase (FabD) was finally chosen. *E.coli* BL21/pET23a-G3T20/pACYC-fabD, of which fabD expression is enhanced, was constructed and a fed-batch fermentation experiment was performed. Introducing fabD increased the percentage of T-20/total protein and T-20 production, but decreased cell growth. Expression of fabD also maintained the intracellular ppGpp at half that of the control. These results indicate that enhancing FabD caused the decrease of intracellular ppGpp, and that a low level of ppGpp could not trigger the stringent response under metabolic load. As a consequence, in *E.coli* BL21/pET23a-G3T20/pACYC-fabD, 6.14% of T-20/total protein and 392.49 of T-20 production (cell growth*T-20%) was obtained. These values were about 3.5 times higher than *E.coli* BL21/pET23a-G3T20, which produced 1.77% of T-20/total protein and 143.72 of T-20 production (cell growth*T-20%).

COMBINATORIAL ENGINEERING OF *ESCHERICHIA COLI* FOR OPTIMIZING L-TYROSINE PRODUCTION

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Although the rational design of strains has been the hallmark of traditional metabolic engineering, direct genetic manipulations often fail to expose the maximum potential of a cell. This shortcoming stems from the sheer complexity and interconnectivity of biological systems which makes it difficult, if not impossible, to predict a) the (oftentimes simultaneous) modifications needed to alter cellular phenotype and b) the unexpected ancillary effects introduced by even simple genetic changes. Thus, the most successful strain improvement strategies often couple rational design with combinatorial engineering. With the latter approach, chemical and/or genetic techniques are used to create large libraries of strains which are then subjected to screening or selection for phenotypes of interest.

Here, we describe the application of novel combinatorial engineering tools for enhancing L-tyrosine production in *Escherichia coli*. Starting from a previously engineered strain exhibiting a modest level of L-tyrosine production (~ 400 mg/L), we were able to utilize global transcription machinery engineering (gTME) to reprogram cellular transcription and hence, cellular phenotype. Specifically, we began by constructing libraries of strains through the mutagenesis of *rpoD* and *rpoA*, genes that encode two subunits of the bacterial RNA polymerase. Screening these libraries with a melanin-based assay for L-tyrosine production led to the discovery and isolation of three strains exhibiting significant increases (up to two-fold) in L-tyrosine production. We are currently conducting an in-depth analysis of the transcriptional changes introduced in these mutants in order to formulate new, directed strategies for optimizing L-tyrosine production in *E. coli*.

MODELING OF BATCH FERMENTATION KINETICS FOR SUCCINIC ACID PRODUCTION BY *MANNHEIMIA SUCCINICIPRODUCENS*

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M. succiniciproducens has been studied more extensively based on its complete genome sequence, and has been shown to be an efficient succinic acid producer. In order to develop a cost-competitive fermentative succinic acid production process, strain improvement maximizing the production of succinic acid but minimizing the formation of by-products through rational metabolic engineering is of vital importance. Kinetic modeling is regarded as an indispensable step in developing a fermentation process since the models can be used to determine an optimal operation condition for the production of a target metabolite. However, little kinetic study on the fermentative production of succinic acid has been performed to date.

We present empirical kinetic models to describe the batch production of succinic acid by *M. succiniciproducens* MBEL55E. Experimental data collected from a series of batch fermentations with different initial glucose concentrations were used to estimate parameters and also to validate the models proposed. The optimal values of the parameters were approximated by minimizing the discrepancy between the model predictions and corresponding experimental data. The growth of *M. succiniciproducens* could be expressed by a modified Monod model incorporating inhibitions of glucose and organic acids accumulated in the culture broth. The Luedeking-Piret model was able to describe the formation of organic acids as the fermentation proceeded, in which succinic, acetic, and formic acids followed a mixed-growth-associated pattern. However, unexpectedly, lactic acid fermentation by *M. succiniciproducens* was nearly nongrowth-associated.The models developed were able to successfully explain cell growth, succinic acid production, by-products (acetic, formic, and lactic acids) formation, and glucose utilization.

In conclusion, the model simulation matched well with the experimental observations, which made it possible to elucidate the fermentation characteristics of *M. succiniciproducens* during efficient succinic acid production from glucose. These models thus can be employed for the development and optimization of biobased succinic acid production processes.

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TOWARDS NOVEL BIOPOLYAMIDES - METABOLIC ENGINEERING OF *CORYNEBACTERIUM GLUTAMICUM* FOR PRODUCTION OF 1,5-DIAMINOPENTANE

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The shortage of fossil fuels with a continuously rising oil price, escalating CO_2 production and global warming have intensively stimulated the development of biotechnology processes producing chemicals from renewable resources. In the field of polymers, comprising a significant fraction of todayâ€™s chemical products, polylactic acid and polyhydroxyalcanoates are examples of biologically derived materials successfully produced in industrial scale. Their material properties, however, resemble those of low cost standard polymers like polyethylene and do not allow an application in the field of polymers with increased properties used e. g. in the automotive industry or in high value consumer products. Polyamides with an annual production volume of 3.500.000 tons are an important fraction in the field of polymers with increased material properties. They are still produced chemically from fossil fuels, so that biotechnological processes towards polyamides possess an enormous ecological and economical future potential.

In this regard we describe the biotechnological production of 1,5-diaminopentane as building block of future biopolyamides through metabolic engineering of the soil bacterium *Corynebacterium* glutamicum. *C. glutamicum* is one of the most important microorganisms in industrial biotechnology, currently used to produce more than 2.000.000 tons of glutamate and lysine per year. 1,5-Diaminopentane is hereby derived from glucose via the lysine biosynthetic pathway, including expression of a feedback deregulated aspartokinase. The key modification is the heterologous expression of lysine decarboxylase from *E. coli*, catalyzing the formation of 1,5-Diaminopentane from lysine. Utilizing systems biotechnological approaches such as metabolome and fluxome profiling for analysis and rational engineering of the underlying metabolic pathways, key questions concerning the supply of building blocks, cofactors and energy as well as the formation of undesired by-products, are addressed to optimize diaminopentane production. This includes novel strategies for engineering of central catabolic pathways such as pentose phosphate pathway or TCA cycle.

The development of a superior strain for efficient production is accompanied by developments in down-stream-processing, polymerisation and further processing to finally derive a fully integrated industrial process for biologically derived polyamides.

IN-SILICO DESIGN OF MULTIPLE MUTATIONS FOR AMINO ACID PRODUCTION IN C. GLUTAMICUM

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An optimal design of production mutants usually requires the introduction of multiple mutations. Up to now mutations are usually introduced one by one. Useful targets are then identified in a stepwise procedure following a detailed analysis of the physiology of single mutations, particularly using metabolic flux analysis [1]. However, it is generally very difficult to predict the effect of combinations of multiple mutations. We have earlier shown that in-silico analysis allows the identification of maximum yield pathways and optimal sulphur sources for the production of methionine [2].
The first level to identify useful combinations of mutations is on the level of the stoichiometry of metabolic networks, often called flux balance analysis. We applied the method of elementary mode analysis to identify combinations of gene deletions promising to force cells to increased overproduction of amino acids. Most of the mutations were found in the central metabolic pathways and combinations of mutations were very unlikely to be identified by direct reasoning or consecutive introduction of single mutations followed by physiological investigations.
Selected combinations of mutations were implemented into a regulatory mutant of the aspartokinase [3] in *Corynebacterium glutamicum* and resulted in interesting phenotypes with partly substantial increase in the overproduction of amino acids, e.g. lysine. We studied the metabolite secretion patterns of the resulting metabolites and used 13C metabolic flux analysis to get a detailed picture of resulting metabolic redirection of fluxes.
We expect that the method developed will be of general use particularly with the advent of high sequencing technologies and the possibility to get genomic pathway information from any interesting strain very easily.

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PROTEOME ANALYSIS OF HYALURONIC ACID PRODUCING BACTERIA FOR STRAIN OPTIMISATION

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Hyaluronic acid (HA) is a biopolymer with valuable applications in the pharmaceutical and cosmetic industries. Many medical products require specific rheological properties (viscoelastic, pseudoplastic), which are dictated by the molecular weight (MW) of HA. While if cross-linked HA has delivered good performance in viscosupplementation, it has failed to provide satisfactory results in ophthalmic viscoelastic devices, where a linear polymer is required. Currently high molecular weight HA is produced commercially mainly from animal tissues (i.e. rooster comb). Increased concerns over the contamination of animal derived products with infectious agents have made bacterial fermentation a more desirable production system to meet future demands.

Little is known about the mechanism of molecular weight control of beta-polysaccharides such as HA, cellulose or chitin. In the commercial production strain, *S. zooepidemicus*, molecular weight is greatly affected by medium and culture parameters suggesting that – in addition to intrinsic features of the HA synthase – metabolite levels affect the control of Mw. We have recently demonstrated that overexpression of enzymes involved in UDP-N-acetylglucosamine production (Pgi and GlmU) increases MW up to 79%. However, over-expression of enzymes involved in UDP-glucuronic acid production do not suggesting that UDP-glucuronic acid levels are important for MW control. Surprisingly, the empty plasmid control also displayed higher Mw despite limited impact on growth.

Proteomics was employed to compare wild type, empty plasmid control and Pgi overexpressing strains. The strains were characterised using differential gel electrophoresis (2D-DIGE) to evaluate changing levels of protein expression and to identify target proteins using mass spectrometry (LC/MS/MS and MALDI TOF/TOF). Seventy proteins were identified by MS and a reference map of the proteome of *S. zooepidemicus* was completed. One enzyme, UDP-N-Acetylglucosamide 1-carboxivinyltransferase, was identified as a possible target for further improvement of the strain. Our 2D-DIGE experiment showed that this enzyme was over-expressed in the wild type strain compared to both the empty plasmid and Pgi strains. The enzyme consumes UDP-N-acetylglucosamine and is part of the peptidoglycan biosynthesis pathway. Our genome annotation (zoocyc) shows two entries for this particular enzyme, both of which where identified in the 2D gel, indicating that it might be possible to knock out one of the genes for further improvement of the strain.

Thus, proteomics confirms the results from targeted metabolome analysis that UDP-N-acetylglucosamine is critical for MW control and have identified a possible candidate for further strain improvement.

EFFECT OF THE PYRUVATE KINASE ACTIVITY ON THE PHENYLALANINE YIELD OF E COLI STRAINS THAT LACKS PHOSPHOTRANSFERASE SYSTEM

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The aromatic compounds are widely used in the food and pharmaceutical industries as precursors of a wide variety of compounds like L-DOPA or TAMIFLU. The first step in aromatic aminoacids biosynthesis in the bacterium Escherichia coli is the condensation of the eritrose4phosphate (E4P) with phosphoenolpyruvate (PEP) by DAHP synthase. In E.coli glucose transport is performed by the phosphotransferase (PTS) system, which uses the phosphoryl group of PEP through a phosphorylation cascade. This process consumes the 50% of PEP generated by glycolisis. Stoichiometric analyses indicate that the yield for aromatic compounds from glucose could significantly increase in a strain that transports glucose without coupling this process to PEP utilization. For this reason, E. coli strains that can uptake glucose without coupling this process to PEP-dependent phosphorylation have been developed. This approach has proven partially successful, as yield from glucose was significantly increased, but the maximum theoretical value was not reached. In this work we generated and characterized strains with increased PEP availability by inactivating pyruvate kinases pykA, pykF in a PTS- background. These isozymes catalyze the transformation of PEP into pyruvate (PYR) using one ATP. Starting from wild type E. coli strain W3110, the PTS- derivative VH33 was constructed. Using VH33 as progenitor, strains VH34 (PTS- pykA- genotype) and VH35 (PTS- pykF- genotype), were constructed. These strains were then transformed with plasmids carrying genes encoding enzymes that direct carbon flow to the L-phenylalanine (PHE) biosynthetic pathway. The capacity of these strains to produce PHE under growth conditions in shake flask in minimal medium (M9W) with glucose (10g/l) as carbon source and minimal medium with yeast extract (M9WYE) and glucose (22.5g/l) was determined. In M9W the PHE yield was 0.04, 0.37, 0.13 and 0.04 (molCPHE/molCglc) for W3110, VH33, VH34 and VH35 respectively. The same behavior was observed in the fermentation in M9WYE with PHE yields of 0, 0.11, 0.08 and 0.06 respectively. However when the shikimate pathway intermediate dehydroshikimate was measured the following yields were calculated; 0.01, 0.19, 0.08 and 0.35 for M9W fermentations and 0, 1.13, 0.85 and 1.58 for M9WYE. This shows that for early stages of the aromatics aminoacids biosynthesis pathway the deletion of pykF gene in a PTS- background has positive effects on the carbon flux committed, but for the terminal stages this strategy is not working, this is likely because of the lack of precursors like NADPH, ATP, PEP and glutamate that are used in the PHE biosynthesis.

METABOLIC NETWORK STRUCTURES DURING GROWTH AND XANTHAN GUM PRODUCTION IN XANTHOMONAS CAMPESTRIS GROWING ON SUCROSE.

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Industrial production of xanthan gum using *Xanthomonas campestris* has improved over several decades due to both genetic selection and empirical process improvement. To date, pragmatic engineering of this organism's metabolic network has not been widely employed other than as a means to modify organic acid composition of the polysaccharide. This is due largely to a restricted understanding of the metabolic constraints governing carbon and energy flux within the central pathways. The absence of phosphofructokinase and the resulting use of the Entner-Doudoroff pathway, coupled to the presence of a direct oxidative pathway situated in the periplasm lead to a complex pathway structure when the biosynthetic pathways for polysaccharides synthesis from glucose-6P and fructose-6P are included. In glucose excess condition, the periplasmic oxidation of glucose to gluconate can by-pass the sugar phosphates necessary for xanthan synthesis with a significant modification of the yield of xanthan. Under such conditions, the fuelling of the biosynthesis pathways becomes carbon limited (Letisse *et al.*, 2002). Improved rates of production were achieved using sucrose as carbon substrate though yields are slightly diminished due to an increased maintenance requirement correlated to a modified carbon flux pattern at high metabolic rates. Quantitative analysis of intracellular metabolites coupled to thermodynamic estimations and in some cases kinetic analysis of enzyme activity, enables flux through key reactions to be estimated. This approach enables global network flux to be estimated without the inherent uncertainty of such analyses in complex networks. High flux is observed through the pentose-phosphate pathway during growth on sucrose recycling hexose-6Ps via the pentose-phosphate-pathway and thereby providing precursors and redox power (NADPH, H+) necessary for anabolic needs and xanthan gum production. Late in the fermentation, the growth is blocked because of the depletion in organic phosphate (Letisse *et al.*, 2001) diminishing the metabolic fluxes. The carbon flux is then redirected towards the Entner-Doudoroff pathway. This shift in carbon flux leads to more efficient generation of metabolic energy and an increase in xanthan yields. Energetic analysis and cofactor pool concentrations indicate that this shift in carbon flux distribution is linked to the internal redox status of the cell.

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TECHNOLOGY SWITCH TOWARDS A FERMENTATION BASED PRODUCTION PLATFORM IN FINE CHEMICAL INDUSTRY

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In contrast to conventional chemical synthesis, industrial biotechnology uses enzymes or micro-organisms to produce chemicals – in the majority of cases based on renewable raw materials. We develop a technology platform for fermentation processes that are both an environment friendly and more economic substitution for chemical processes. An evaluation of the raw material situation showed that not only raw oil prices are volatile, but also prices for renewable resources like sugar were increasing remarkably during the last years [1]. Since the carbon source is one of the key contributors to fermentation costs, we aim at optimizing the yield of the process by integrating choice of carbon source, strain development, fermentation and down stream processing from the very beginning of project design. An approach resulting in diversified white biotechnology processes. As an example we present the development of 2 -ketoglutaric acid which is a fermentation process to produce the fine chemical applied as physiological ingredient for infusion solutions, in cosmetics and in sports and wellness nutrition. The micro-organism platform used consists of yeast Yarrowia lipolytica which is transformed into efficient production strains using state of the art metabolic engineering. In a fermentation using the renewable raw material rapeseed oil as carbon source a concentration of 104 g/L 2-ketoglutaric acid was produced.

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COMBINING QUANTITATIVE METABOLOMICS AND THERMODYNAMIC MODELLING TO REVEAL REGULATORY SITES IN CENTRAL CARBON METABOLISM

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Understanding metabolic regulation is almost a prerequisite for rational strain development. A particularly important target of regulation are metabolic fluxes through a network since they are the functional endpoint of the nonlinear interactions of transcripts, proteins, and metabolites [1].

To identify the enzymes that are key to establishing different modes of metabolic network operation we integrated metabolomics and flux data on a global level. For this purpose, we grew *Bacillus subtilis* on minimal medium with the glycolytic and the gluconeogenic substrates glucose and malate and a mixture of both. During exponential growth we quantified 77 intracellular metabolites with LC-MS/MS and GC-TOF methods, thereby covering most of central carbon metabolism. Through interpretation of these metabolite concentrations with a thermodynamic model, we predicted several flux directions in central carbon metabolism [2], which were subsequently used as constraints for 13C based metabolic flux analysis. On malate we found a surprisingly low TCA cycle flux. During co-metabolization of glucose and malate the flux distribution above the PEP-pyruvate node was similar to that on glucose, whereas the flux distribution below this node was similar to that on malate. By combining thermodynamic and flux data, pyruvate dehydrogenase and fructose bisphosphate phosphatase were found to be key targets of regulation.

Beyond the above steady state conditions, we wanted to identify dynamic changes in regulatory sites after the addition of glucose or malate to the respectively other substrate at a time resolution of up to 40 s. Thermodynamic modelling in combination with time-resolved, quantitative metabolite data identified regulatory sites in the metabolic network. Currently we are using proteomics data from the same experiment to validate these predictions.

In conclusion, we present a novel approach to establish a solid basis for rational strain development by identifying regulatory sites in the metabolic network using metabolomics data, flux analysis and thermodynamic modelling.

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IDENTIFICATION OF ESSENTIAL MUTATIONS FOR THE OPTIMIZATION OF SUCCINATE PRODUCTION WITH E. COLI

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The environmental concerns and the increasing scarcity of oil are factors prompting the renewed interest in so called green production processes, based on renewable feedstocks. One of the products that has become the focus of the bio-based industry, next to bio-ethanol and bio-diesel, is succinic acid. This compound has been designated in 2004 as one of the top twelve added value chemicals (1) and has ever since enjoyed increasing attention of both the scientific community and industry. In this work, we present a novel way to evaluate candidate targets for gene disruption or enhanced expression for the production of chemicals from renewable feedstocks. By analysing elementary flux modes data (2) with partial least squares regression (3) we were able to identify several gene targets. This strategy yielded easily a 30% glucose carbon conversion to succinate and a maximal volumetric succinate production rate of 3 g/l/h in aerobic conditions. A key to production revealed by the model is enhanced succinate efflux. This was achieved by overexpressing the anaerobic C4-dicarboxylic acid exporter protein DcuC and knocking out the aerobic C4-dicarboxylic acid importer protein DctA. By merely adding this system to a succinate dehydrogenase negative E. coli strain the succinate yield was increased about 45% (c-mole/c-mole glucose) and the specific production rate about 40% (c-mole/c-mole biomass/h). Such a “pull” methodology has, to our knowledge, never been reported before and seems to be a very promising tool to enhance yields and production rates. The model also pointed to the fact that an upregulated glycolysis is essential for succinate production. A first step towards a higher flux through the glycolysis was knocking out the first reaction of the Entner Douderoff route. This yielded the peculiar result of a doubling in growth rate, which evidently led to an increase in specific production rate. It can be concluded that this novel model based approach for identifying gene targets has been implemented successfully for succinate production. In the future, this methodology can easily be applied to other industrial interesting compounds. References 1. Werpy, T., Petersen, G., Aden, A., Bozell, J., Holladay, J., White, J., and Manheim, A. (2004) Top Value Added Chemicals from Biomass. Volume I: Results of Screening for Potential Candidates from Sugar and Synthesis Gas. In., US Department of Energy, Oak Ridge, USA 2. von Kamp, A., and Schuster, S. (2006) Bioinformatics 22(15), 1930-1931 3. Wold, S., Sjostrom, M., and Eriksson, L. (2001) Chemometrics and Intelligent Laboratory Systems 58(2), 109-130

THE ATP LIMITATION IN A PYRUVATE FORMATE LYASE MUTANT OF ESCHERICHIA COLI INCREASES GLYCOLYTIC FLUX TO D-LACTATE

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Lactate demand has been increasing mainly for bio-polymer manufacture. Biodegradable polylactates can substitute oil derived plastics. Renewable lignocellulosic biomass is an important feedstock for lactate production. Few lactic acid bacteria can metabolize pentoses and complex nitrogen sources are needed to obtain elevated productivities. Escherichia coli is the most studied bacteria which can metabolize pentoses and hexoses. When it is grown in anaerobic conditions E. coli produces D-lactate as part of a mixture of fermentation products. In this work a derivative of E. coli MG1655 (named CL3) was constructed for D-Lactate production by deleting the pyruvate formate lyase (pflB), alcohol dehydrogenase (adhE) and fumarate reductase (frdA) genes. Due to the inactivation of the main acetyl-CoA production pathway under non-aerated conditions, this pfl mutant grew one third slower than its parental strain in mineral media-glucose, but lactate yield was 95% of the theoretical. Experimental data shows that the ATP/ADP ratio remains the same between CL3 and parental strain. Due to the lack of acetate formation, in CL3 the ATP yield from glucose to lactate is low (2 ATP/Glucose), hence CL3 improves its glucose uptake rate in order to fulfill the ATP pool and support a high volumetric productivity of D-lactate (1.6 g/L.h). W3110 derivatives have a poor growth in mineral media with glucose as the sole carbon source thus lactate productivities are limited. E. coli B derivatives engineered for D-lactate production rapidly ferments 10% W/V glucose but complex media were required, a metabolic evolution strategy has been used to improve those strains achieving high lactate yields and productivities in mineral media. Also, it is suggested that the better performance, in terms of biomass formation and lactate production rate, of the MG1655 pfl mutant is due to a residual flux of pyruvate to acetyl-coA (probable as a result of the activation of anaplerotic pathways), and this flux is able to fulfill the acetyl-CoA pool required for fermentative growth.

CHARACTERIZATION OF AN *ESCHERICHIA COLI* MUTANT THAT GROWS ON ACETATE THREE FOLD FASTER THAN THE WILD TYPE STRAIN

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An *Escherichia coli* (*E. coli*) mutant (PB122) that grows with a unusual high specific growth rate (μ) on acetate was spontaneously obtained from a strain that lacks PEP:carbohydrate phosphotransferase system (PTS) but that is capable of utilize glucose (PB12, PTS^- Glc^+). On minimal medium with acetate as the sole carbon source (2 g/l), PB122 has a μ of 1.0 h^{-1} while parental PB12 and wild type JM101 strains have a μ of 0.13 and 0.28 h^{-1}, respectively. As far as we know, no *E. coli* derivative has been reported to have that μ on acetate. Interestingly, growth on glucose was not substantially altered in PB122 since its μ on this carbon source is similar to that of parental PB12 strain (approximately 0.4 h^{-1}). Simultaneous utilization of acetate and glucose is possible in PTS^- strains because of the lack of glucose catabolite repression [1]. If the inocula is adapted on glucose, PB122 and PB12 co-utilize glucose and acetate, with the former strain finalizing first acetate while the latter strain glucose. However, when PB122 inocula is adapted on acetate before the glucose and acetate co-utilization experiment, the glucose is consumed until acetate is totally exhausted, as if acetate was exerting catabolic repression over glucose. Also, strain PB122 grows faster in high acetate concentrations (5 and 10 g/L) as compared to PB12 and JM101. Finally, PB122 does not grow in other gluconeogenic substrates as citrate, succinate or malate, indicating that the properties acquired by this mutant are specifically for acetate. We are working in the transcriptome analysis of this strain to better understand its particular metabolism. This information may be useful as an example of pathway optimization and could be used for the defining novel metabolic engineering strategies that involves acetate and its derived metabolites.

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COUTILIZATION OF GLUCOSE AND GLYCEROL ENHANCES THE PRODUCTION OF PHOSPHOENOLPYRUVATE: CARBOHYDRATE PHOSPHOTRANSFERASE SYSTEM

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Escherichia coli strains lacking the phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) are capable of coutilizing glucose and other carbon sources due to the absence of catabolite repression by glucose. In these strains, the lack of this important regulatory and transport system allows the coexistence of glycolytic and gluconeogenic pathways. Strains lacking PTS have been constructed with the goal of canalizing part of the phosphoenolpyruvate (PEP) not consumed in glucose transport to the aromatic pathway. The deletion of the *ptsHIcrr* operon inactivates PTS causing poor growth on this sugar; nonetheless, fast growing mutants on glucose have been isolated (PB12 strain). However, there are no reported studies concerning the growth potential of a PTS $^{-}$ strain in mixtures of different carbon sources to enhance the production of aromatics compounds.

PB12 strain is capable of coutilizing mixtures of glucose-arabinose, glucose-gluconate and glucose-glycerol. This capacity increases its specific growth rate (μ) given that this strain metabolizes more moles of carbon source per unit time. The presence of plasmids pRW300*aroG*^{fbr} and pCL*tktA* reduces the μ of strain PB12 in all mixtures of carbon sources, but enhances the productivity and yield of aromatic compounds, especially in the glucose-glycerol mixture, as compared to glucose or glycerol cultures. No acetate was detected in the glycerol and the glucose-glycerol batch fermentations.

Due to the lack of catabolite repression, PB12 strain carrying multicopy plasmids containing *tktA* and *aroG*^{fbr} genes is capable of coutilizing glucose and other carbon sources; this capacity, reduces its μ but increases the production of aromatic compounds.

RATIONAL DESIGN OF MICROBIAL CHEMICAL FACTORIES

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The growing interest in a "biomass-based" economy has led to new efforts to construct and improve microorganisms capable of producing chemicals. The current focus is largely on liquid biofuels; however, a successful "biorefinery" is likely to be a mixed-product facility, with many compounds produced from one or more biomass-derived feeds. Identifying methods for the production of both novel biofuels and "value-added" compounds is both a challenge and an opportunity. The potential for biological conversion of feedstocks to bulk chemicals is enhanced by the availability of tools and techniques from the established discipline of Metabolic Engineering, which has enjoyed tremendous successes in the development of highly productive microorganisms for a variety of products of interest. We can also gain insights from Biocatalysis, where the choice of enzymes to mediate biotransformation of chemical substrates is based largely on consideration of the required functional group conversion without being limited by prior evidence of transformation of the full structure.

Our group is interested in applying principles from each of these intellectual arenas towards the design and construction of novel biosynthetic pathways for specified target compounds. This "retro-biosynthetic design" approach relies on the tremendous advances in directed enzyme evolution and rational protein design to propose enzymatic conversions by considerations of only the functional groups involved. Novel pathway designs for two value-added compounds will be presented. In the first case, we are exploring alternative biosynthetic routes towards a natural product (glucaric acid) found in fruits, vegetables, and mammals. In the second, we have proposed a novel pathway towards a compound with no identified natural source (3-hydroxybutyrolactone). The latter has provided additional motivation for the production of chiral hydroxyacids. Considerations for the design of these pathways as well as progress-to-date in their construction will be presented.

GENOME-SCALE METABOLIC NETWORK MODEL OF ARABIDOPSIS

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Genome-scale metabolic maps are useful tools to represent and analyze the metabolism of an organism. Flux balance analysis has been useful for qualitative and quantitative analysis of the metabolic reconstructions. A metabolic network model for a plant cell has been reconstructed based on Arabidopsis genome, Aracyc, KEGG database and biochemical information found in the literature. It represents the first attempt to collect and characterize a plant cell to perform fluxomics analysis at genome-scale. More than 1300 unique reactions and 1436 metabolites represent the carbon flux through the metabolic network, compartmentalized between cytosol, mitochondria, vacuole, plastid, and peroxisomes, including more than 130 transporters. The model has been validated using a number of classical plant physiological scenarios such as the diurnal starch cycle in leaves and ammonia assimilation in leaves and roots. The plant genome-scale model is being used to perform fluxomics analyses for a better understanding of plant metabolic capabilities under different conditions and in the presence of additional drains such as PHA production. This is a potential platform to test hypotheses in silico and derive biological insights from the metabolism of plants. Fluxome profiles integrated to other omics dataset represent an important step toward genome-scale plant systems biology.

AUTOMATED CONSTRUCTION AND CURATION OF GENOME-SCALE METABOLIC MODELS

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Currently, over 700 genomes (including eleven plant species) have been fully sequenced, however, only about 20 organism-specific genome-scale metabolic models have been constructed. It appears that metabolic model generation can only keep pace with about 1% of the fully sequenced genomes. In response to this flood of present and future genomic information, automated tools such as Pathway Tools and SimPheny™ have been developed that, using homology comparisons, allow for the automated generation of draft organism-specific metabolic models. This has shifted the burden towards curating the accuracy and completeness of the automatically generated, though draft, reconstructions. All of these reconstructions remain to some extent incomplete as manifested by the presence of unreachable metabolites and some growth inconsistencies between model predictions and observed *in vivo* behavior.

In this work, we will highlight the construction of *in silico* metabolic models for a minimal organism *Mycoplama genitalium*, *i*PS189 and a human pathogen *Salmonella enterica* ser. Typhimurium. Key challenges for *M. genitalium* included estimation of biomass composition, handling of enzymes with broad specificities and the lack of a defined medium. Our computational tools were subsequently employed to identify and resolve connectivity gaps in the model as well as growth prediction inconsistencies with gene essentiality experimental data. The resulting curated model, *M. genitalium i*PS189 (264 reactions, 276 metabolites) was 87% accurate in recapitulating *in vivo* gene essentiality results for *M. genitalium*. The *Salmonella* model includes a number of pathways such as cobalamin (vitamin B12) biosynthesis that differentiate it from other enteric bacteria. The *Salmonella* model is compared with *in vivo* data for growth on various carbon, nitrogen, phosphorus, and sulfur substrates using BioLog Phenotype MicroArrays™. Furthermore, we explore the phenotypic differences of *S.* Typhimurium with *Escherichia coli* (both *in vivo* and *in silico*) under these same conditions.

Despite their careful and laborious construction, even widely used metabolic reconstructions (e.g., *E. coli*) are known to have gaps, omitted functionalities and inconsistencies with growth experiments or gene essentialities. In response to this challenge, we discuss integrated computational frameworks that will automatically identify and suggest hypotheses of correcting model deficiencies. Specifically we describe research in our group on filling gaps in metabolic reconstruction (i.e., GapFill) and on restoring model growth prediction inconsistencies with experimental data (i.e., GrowMatch). We will comment on how these curation tools helped us generate the above-mentioned metabolic reconstructions.

THE ENVIROSTAT - A NEW BIOREACTOR CONCEPT FOR STUDYING SINGLE CELL PHYSIOLOGY

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The analysis of phenotypic changes of living cells in response to environmental perturbations is arguably the most prominent technique in such diverse fields as drug efficacy research, fermentation optimization, and systems biology. Here scientists face two questions: Firstly, is the cellular response due to the chosen perturbations or is the outcome biased by the population under study, which itself constantly changes the culture conditions? Secondly, how much can one rely on the averaged read out of a cell population without knowing the single cell response? We present a novel bioreactor concept, the Envirostat, answering these two questions.

The Envirostat allows cell cultivation experiments under defined growth conditions at single cell resolution. Similar to a standard bioreactor, culture conditions can be controlled and altered as required. Adjustment of media composition, pH, gas saturation, and temperature is achieved within seconds. The Envirostat allows the selection of a single cell by a non-disturbing negative dielectrophoretic (nDEP) field cage, followed by long term contactless capture in a micro flow that enables constant supply of nutrients. The identical growth rates of *Saccharomyces cerevisiae* during unlimited growth in the Envirostat or traditional shake flasks, i.e. starting from a single cell or a cell population, respectively, suggest that no or minimal cellular stress was caused by the experimental conditions. We combined the Envirostat with a semi-automated cell recovery and culturing system that allows isolation of the single or few cells under study and closes the gap between single cell and population analysis.

We used the Envirostat to investigate the impact of nutrient availability on the morphology of *S. cerevisiae*. Baker´s yeast can undergo growth differentiation for example under nitrogen starvation, which can initiate formation of pseudohyphae and thus filamentous growth. In an industrial setting, this filamentous morphology can cause foam formation that in turn can cause overflow of the fermenter and thus reduces bioprocess availability. Therefore, active control of filamentous growth is advantageous, but not readily achieved as the understanding of regulation and its interplay with metabolism of filamentous *S. cerevisiae* is limited and does not directly allow preventing filamentous growth. We investigated, on a single cell level, the response of *S. cerevisiae* to nitrogen limitation in order to determine the heterogeneity of differentiation that is an outcome of the involved regulatory network. The interplay of this regulation with the metabolic network was quantified by ^{13}C tracer based flux analysis.

We present the construction, characterization, and use of the Envirostat. In addition, we discuss applications of the Envirostat concept, focusing on the analysis of cellular regulation.

SHIKIMATE PRODUCTION IN BACILLUS SUBTILIS STRAINS WITH CENTRAL METABOLISM AND AROMATIC AMINO ACIDS BIOSYNTHESIS PATHWAY MODIFICATIONS.

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Better known as the anionic form, shikimate is an aromatic intermediate used as starting compound in the synthesis of the neuramidase inhibitor oseltamivir (Tamiflu®). Industrial production of this antiviral has been of great importance because it is the best alternative to treat the avian flu caused by H5N1 virus. Most of the shikimate (90%) is obtained by extraction from the pod of a plant commonly named star anise (Illicium anisatum). However, the global concern is that in case of pandemic there is not enough source of extraction to supply the hypothetical demand. Microbial production of shikimate is an attractive alternative to its isolation from plant tissue. Several Metabolic Engineering efforts have been achieved to modify microbial strains of Escherichia coli to increase shikimate yield from glucose. Differences in central metabolism between E. coli and Bacillus subtilis propose the latter as an attractive model to overproduce aromatic compounds. It is reported that B. subtilis pyruvate kinase mutant strains accumulate intracellular phosphoenolpyruvate (PEP) 30-fold higher than in E. coli isogenic strains. Taking this into account, this work suggests that shikimate yield from glucose could be increased in Bacillus subtilis strains with a central metabolism modification (pyruvate kinase inactivation). B. subtilis strains with the capacity to accumulate shikimate were generated by insertional inactivation of the gene coding for shikimate kinase in wild type and a pyruvate kinase deficient strain, to generate CLC58-168 and CLC6-PYKA, respectively. The effect of pyruvate kinase inactivation on shikimate production capacity was determined in resting cells experiments, measuring shikimic acid, dehidroshikimic acid (DHS) and fermentation sub products (lactic acid, acetic acid, acetoine) by an HPLC system. The measured shikimate specific production rates (qSHIK) were 0.013 and 0.018 g/g/h and the shikimate yields from glucose (YSHIK/glc) were 0.043 and 0.068 g/g for strains CLC58-168 and CLC6-PYKA, respectively. With the purpose of reaching higher quantities of shikimate, 1 l batch and fed-batch cultures (using complex medium) were carried out finding a higher shikimate titer in the former. Strain CLC6-PYKA produced 4.7 g/l of shikimate in 42 hours with a (YSHIK/glc) of 0.0578 g/g. As a conclusion we postulate that strain CLC6-PYKA has the better characteristics to be applied in a biotechnological process for shikimate production.

METABOLIC ENGINEERING OF *ESCHERICHIA COLI* FOR L-TYROSINE PRODUCTION BY THE EXPRESSION OF THE GENES CODING FOR THE CHORISMATE MUTASE DOMAIN FROM NATIVE P-PROTEIN AND A CYCLOHEXADIENYL DEHYDROGENASE FROM *ZYMOMONAS MOBILIS*

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L-tyrosine (L-TYR) is an aromatic amino acid with several applications, including its use as a precursor in the synthesis of some drugs, polymers and phenylpropanoids. In nature, there are two routes for L-TYR biosynthesis. First, chorismate is converted to prephenate, in a reaction catalyzed by chorismate mutase. The next reaction is catalyzed by a group of dehydrogenases that belong to TyrA protein family. *Escherichia coli* synthesizes L-TYR using the bifunctional enzyme chorismate mutase-prephenate dehydrogenase ($TyrA_{pEc}$), which is inhibited by L-TYR.

Cyclohexadienyl dehydrogenase from *Zymomonas mobilis* ($TyrA_{cZm}$) uses as substrates prephenate, L-arogenate and NAD^+ in L-TYR biosynthesis and is not inhibited by the three aromatic amino acids (1). On the other hand, chorismate mutase-prephenate dehydratase (P-protein) from *E. coli* is involved in L-phenylalanine biosynthesis. When the chorismate mutase domain (residues 1-109) of the P-protein is expressed alone (CM_{PEc}), it retains catalytic activity and becomes insensitive to L-phenylalanine inhibition (2).

Modifications of the aromatic amino acid pathway for the construction of an *E. coli* L-TYR overproducer strain involves alleviation of feedback control of key enzymes in the common aromatic and in L-TYR biosynthetic pathways. In this work, the expression of the feedback insensitive $TyrA_{cZm}$ and CM_{PEc} was explored as an approach for engineering L-TYR production strains.

An *E. coli* L-TYR auxotroph was capable of growth in minimal media when it was transformed with the genes that code for $TyrA_{cZm}$ and CM_{PEc}. On the other hand, in enzymatic assays, it was found that the CM_{PEc} domain of P-protein was weakly inhibited by L-TYR, while $TyrA_{cZm}$ was not inhibited by L-TYR (1). Shake flasks experiments using a *E. coli* strain modified to increase carbon flow to chorismate, showed that the activity of $TyrA_{cZm}$ increased by 6.8-fold the yield of L-TYR from glucose ($Y_{L\text{-}TYR/glc}$), when compared to $TyrA_{pEc}$, no further enhancement was observed when both $TyrA_{cZm}$ and CM_{PEc} were expressed. In bioreactor experiments, a strain expressing both $TyrA_{cZm}$ and CM_{PEc} produced 3 g/L of L-tyrosine with a $Y_{L\text{-}TYR/glc}$ of 66 mg/g. These values are 46% and 48% higher when compared to a strain expressing only $TyrA_{cZm}$. The results show that these feedback insensitive enzymes can be employed for strain development as part of a metabolic engineering strategy for L-TYR production (3).

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WASH-IN OF U-^{13}C GLUCOSE INTO *E. COLI* CELLS CULTIVATED IN A CARBON LIMITED CHEMOSTAT

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Metabolic and isotopic stationary ^{13}C flux analysis based on the measurement of isotopomers of amino acids in biomass protein is commonly applied. One alternative, thereby avoiding that assumptions have to be made on amino acid biosynthesis pathways, would be to switch from biomass compounds to the direct measurement of intracellular intermediate metabolites. Direct measurement of the ^{13}C-labeling distribution of the intermediates of the central carbon metabolism has been restrained by their high turnover rates and low concentrations. However, by the development of reliable and accurate methods for rapid sampling and quenching of biomass followed by extraction [1] and very sensitive detection of intermediates of the primary metabolism using LC–MS [2], this approach becomes also feasible for *E. coli*. Due to leakage, quenching of cells leads to drastic lost of metabolites [1,3]. Hence, a differential approach correcting a whole broth sample for medium-contained metabolites is the most appropriate method. A problem hereby is that in *E. coli* cultures a large fraction of the intracellular metabolites is present outside the cells. Therefore, in order to correctly interpret the results obtained from a labeling experiment, first the ratios of intracellular and extracellular metabolites have to be determined.

To determine these ratios a wash-in experiment was performed, whereby the feed is switched from non-labeled glucose to U-^{13}C-labeled glucose and maintained for 1 h after reaching steady-state. From the results, it was found that this short period of labeling allowed the glycolytic intermediates to reach a stationary level and that only the intracellular fraction was labeled. Furthermore, the labeling of TCA-cycle intermediates appeared relatively slow, probably due to transaminase and protein turnover reactions.

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^{13}C FLUX ANALYSIS IN NON-GROWING AND FED-BATCH CULTURES OF *BACILLUS SUBTILIS*

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Most ^{13}C flux analyses reported so far are based on detection of ^{13}C labeling patterns in abundant and information rich protein-bound amino acids with inexpensive GC-MS. Unfortunately, protein-bound amino acids are not suited to determine fluxes in non-growing cells or metabolic transients, due to a lack of *de novo* protein biosynthesis and the long time span necessary to attain isotopic steady-state. These limitations hamper the application of ^{13}C flux analysis to industrially relevant fed-batch cultivations, where both metabolic transients and very slow growth rates are typically observed.
Here, we present a novel method for analyzing metabolic fluxes in slowly or non-growing cells using the mathematical frameworks pertinent to isotopically stationary systems. Using a method based on GC-MS derived ^{13}C labeling patterns in free intracellular amino acids, we determined flux ratios in a riboflavin-producing fed-batch process. *B. subtilis* was cultivated under increasing glucose-limitation, resulting in a decreasing growth rate over time. Based on physiological measurements, three distinct metabolic quasi steady states were identified in the transient process. We showed with ^{13}C label experiments that isotopic stationarity in free intracellular amino acids is obtained during these states, which enables us to utilize stationary flux analysis methods. Key differences between the state of highest (0.45 h^{-1}) and lowest growth rate (0.03 h^{-1}) were a decreasing anaplerotic contribution to tricarboxylic acid cycle and an increasing contribution to pentose phosphate pathway reflecting the actual network operation.
Further, we demonstrate stationary flux analysis in non-growing cells by investigation of a nitrogen-starved, resting *B. subtilis* culture. As amino acid synthesis is limited, we developed a novel LC-MS/MS approach to determine ^{13}C labeling patterns in entire and fragmented intracellular intermediates of central carbon metabolism. At least 10 intermediates can be measured depending on the investigated biological condition with a precision comparable to GC-MS derived ^{13}C labeling patterns (error < 1 mol %). Intracellular net fluxes were quantified by isotopomer modeling. This approach paves the road to determine dynamic flux responses during transient biological phenomena under a variety of conditions.

FED-BATCH FERMENTATION OF A TOLERANT 3-HYRDOXYPROPIONIC ACID PRODUCING *E. COLI*

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3-Hydroxypropionic acid (3-HP) has been identified as a top value-added chemical which may be produced from biomass. 3-HP is a 3-carbon, building block chemical which can be chemically converted to both commodity and specialty chemicals. Some high-value derivatives of 3HP include acrylic acid, 1,3-propanediol, and acrylamide. These three compounds alone have a combined current market value of nearly $10 billion. Utilizing our patented, platform technology, we have engineered microbial strains capable of producing commercially relevant titers of 3-hydroxypropionic acid at commercial productivities in inexpensive growth conditions. In this work, we present the results of fermentation scale-up from 10 mL batch culture to 10 L fed-batch controlled fermentation, all in minimal medium.

ENHANCED PRODUCTION OF 1,2-PROPANEDIOL BY TPI1 DELETION IN SACCHAROMYCES CEREVISIAE

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1,2-propanediol, also called as propylene glycol, is a major commodity chemical for food, drug and cosmetic industries. It is used as less-toxic antifreeze, solvent in mixing chemicals in the photographic industries, solvent for coloring, flavors and humectants food additives in the food industries, and moisturizer, lubricants, a carrier of fragrance oils in the cosmetic and pharmaceutical fields. There have been a few reports to produce 1,2-propanediol from several microorganisms using biochemical process. Two pathways have been used to produce 1,2-propanediol. First, deoxy sugars can be used as a carbon source to form lactaldehyde, which is subsequently converted to 1,2-propanediol. Second pathway is the conversion of DHAP (dihydroxyacetone phosphate) to 1,2-propanediol via methylglyoxal. The studies about this pathway identified mgs (methylglyoxal synthase) and gldA (glycerol dehydrogenase) genes for 1,2-propanediol production. Saccharomyces cerevisiae was metabolically engineered to improve 1,2-propanediol production. We chose S. cerevisiae because it is well studied, easy to manipulate and known as a good host to produce glycerol that is also produced from DHAP. Deletion of tpi1 (triosephosphate isomerase) gene in S. cerevisiae increased carbon flux to DHAP (dihydroxylacetone phosphate) in glycolysis, resulting in increased glycerol production. Then, mgs and gldA genes of which the products convert DHAP to 1,2-propanediol were introduced to the tpi1 deficient strain using a multicopy plasmid. As expected, the intracellular level of methylglyoxal was increased by introduction of mgs gene in S. cerevisiae and that of 1,2-propanediol by introduction of both mgs and gldA genes. As a result, 1.11 g/§¤ of 1,2-propanediol was achieved in flask culture. The deletion of tpi1 resulted in 120% improvement from the wild type strain.

PRODUCTION OF OPTICALLY PURE KETOALCOHOLS: COMPARISON OF E.COLI AND S.CEREVISIAE AS BIOCATALYSTS.

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Optically pure ketoalcohols can be produced through asymmetric carbonyl reduction of diketones. This reaction can be catalysed by microorganisms expressing carbonyl reductases. Utilization of whole cells as biocatalysts has the advantage over pure enzymes since they are easy and cheaper to prepare. Moreover intracellular enzymes have increased stability and in some hosts NAD(P)H is readily regenerated. E. coli is often used as biocatalyst for production of ketoalcohols since high expression of recombinant reductases can be achieved. It also has a relatively low background activity of competing reductases, which potentially would lead to a higher selectivity of the reaction. However carbonyl reductions in this host are limited by cofactor regeneration, so there is a need for over expression of an additional dehydrogenase. The use of S. cerevisiae as biocatalyst has the advantage over E. coli that it has a more efficient NADPH regeneration system. However the drawback of using S. cerevisiae is the presence more reductases responsible for a higher background activity resulting in a lower optical purity of the ketoalcohol products. In this study carbonyl reduction of the bicyclic diketone bicyclo[2.2.2]octane-2,6-dione (BCO2,6D) to its corresponding ketoalcohols (1R, 4S, 6S)-6-hydroxy-bicyclo[2.2.2]octane-2-one (endo-alcohol) and (1S, 4R, 6S)-6-hydroxy-bicyclo[2.2.2]octane-2-one (exo-alcohol) was used as a model reaction for the comparison of E. coli and S. cerevisiae as biocatalysts.

DETERMINATION OF 3-DEOXY-D-ARABINO-HEPTULOSONATE 7-PHOSPHATE YIELD FROM GLUCOSE IN *BACILLUS SUBTILIS* DEVOID OF THE GLUCOSE PHOSPHOTRANSFERASE TRANSPORT SYSTEM AND PYRUVATE KINASE.

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Bacillus subtilis has been the organism of choice for the production of several important industrial products, including antibiotics, enzymes, nucleosides, and vitamins. Several aspects of the biochemistry, genetics, and physiology of *B. subtilis* have been studied extensively making it the best characterized gram-positive bacterium. Comparatively little data are available on the aromatic compounds synthesis capacity in *B. subtilis*. The aromatic compounds are widely used in the industry.

The aim of this work was the construction of central metabolism mutants of *B. subtilis* to determine the effect of inactivation of the activities of enzymes related to the phosphoenolpyruvate (PEP) node in *B. subtilis*; Pyruvate kinase and the phosphoenolpyruvate: Carbohydrate Phosphotransferase System on 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) specific production rate and molar yield from glucose, by using strains with a block in the *aroB* gene (dehydroquinate synthase) from the common aromatic pathway, the *aroB*$^-$ derivatives 168B, CVPYKB and CVPTSGHIB excrete DAH/DAHP to the culture medium. With this approach, it was possible to accurately measure total carbon flow directed to aromatic synthesis.

The DAHP production experiments were performed by using cell resuspension medium supplemented with 8 g/L of D- glucose (resting cells). The measured specific rates of DAHP synthesis were 0.19 ± 0.02; 0.53 ± 0.04, and 0.17 ± 0.01 mmol/g-dcw.h and the DAHP molar yields from glucose were 0.07 ± 0.01; 0.37 ± 0.04, and 0.11 ± 0.01 mmol/mmol for the 168B, CVPYKB and CVPTSGHIB, respectively. These results show that the inactivation of PykA causes a significant increase in carbon flow to the common aromatics pathway. However, the inactivation of PTSGHI did not increase the carbon flow to aromatics. The $Y_{DAHP/GLC}$ determined for the PykA$^-$AroB$^-$ strain corresponds to 43% of this maximun theoretical yield (0.86 mol/mol).

IMPROVING THE YIELD ON REDUCING EQUIVALENTS FROM GLUCOSE FOR WHOLE-CELL BIOCATALYSIS

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Whole-cell biocatalysis is often preferred over *in vitro* enzyme systems requiring cofactor regeneration. One classification of whole-cell transformations is those which are not growth-coupled, but instead in competition with growth-related pathways. Our representative transformation of this type is the NAD(P)H-dependent reduction of xylose to xylitol via xylose reductase (XR) in engineered *E. coli*, where reducing equivalents are derived from glucose oxidation via central metabolism. Simultaneous transport of glucose and xylose is achieved either by the constitutive expression of xylose transporters or a CRP mutant. An important parameter to assess the efficiency of this process is the yield, defined as moles of xylose reduced per mole of glucose consumed. In batch cultures, xylitol yield is lowest during growth (where NADPH is required for cell growth) and is increased during stationary phase. Yield on xylitol is significantly improved in resting cells (~4.2) compared to growing cells (~1.5). The impact of any given metabolic engineering strategy depended on the *E. coli* host strain chosen, and that will be discussed.

We are interested in understanding cofactor trafficking in the context of a heterologous, NAD(P)H-dependent pathway, and in improving the yield on reducing equivalents delivered to the heterologous reaction. Stoichiometric models of *E. coli* metabolism are used to understand the potential roles of enzymes and pathways in cofactor supply, and the effects of various genetic modifications on the theoretical maximum yield. Experimentally, we have quantified the effect of many single and double deletions in genes involved in central metabolism and respiration on the biocatalytic properties of xylitol-producing strains. We find that transhydrogenase plays a minor role in the cofactor yield, and that NADH availability is not readily translated to NADPH availability. NADPH-dependent transformations provide higher yields than NADH-dependent ones, and genetic modifications that increase glucose oxidation through NADP+-dependent reactions (e.g., *pgi* or *pfk* deletions, *zwf* overexpression) directly improve yields. Most notably, deleting phosphofructokinase (in combination with other genetic changes) increases carbon cycling through the pentose phosphate pathway and results in more than half of all available reducing equivalents from glucose oxidation delivered to xylose reductase. We will also present additional studies focused on NADH- and NADPH-dependent cytochrome P450 transformations in whole cells. Finally, notable results relating to co-utilization of glucose and xylose will also be described. For example, xylitol phosphorylation by *E. coli* xylulokinase (XylB) was identified as a bottleneck to the use of xylose as an energy source during xylitol production, and this was overcome by replacement of *xylB* with *XYL3* from *Pichia stipitis*.

UNDERSTANDING AND HARNESSING THE MICROBIAL FERMENTATION OF GLYCEROL: A NEW PATH FOR THE PRODUCTION OF BIOCHEMICALS

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Glycerol is an abundant, inexpensive, and highly reduced molecule generated as inevitable by-product of biofuels production. The use of glycerol as carbon source in fermentation processes offers the opportunity to produce reduced chemicals at yields higher than those obtained with the use of common sugars. Fully realizing this potential, however, would require the metabolism of glycerol in the absence of external electron acceptors. Unfortunately, fermentative utilization of glycerol is restricted to a small group of microorganisms, most of them not amenable to industrial applications. The synthesis of 1,3-propanediol (1,3-PDO) by these organisms has long been considered the metabolic property that determines their ability to ferment glycerol. For example, *E. coli* and *S. cerevisiae*, two microorganisms considered workhorses of modern biotechnology, are unable to synthesize 1,3-PDO and therefore thought to metabolize glycerol only via a respiratory process.

We have recently discovered that *E. coli* can indeed ferment glycerol in a 1,3-PDO-independent manner. We identified 1,2-propanediol (1,2-PDO) as a fermentation product and established the pathway that mediates its synthesis as well as its role in the metabolism of glycerol. Based on our findings, we proposed a new model for the fermentative utilization of glycerol in which: (1) the production of 1,2-PDO provides a means to consume reducing equivalents generated in the synthesis of cell mass, thus facilitating redox balance, and (2) the conversion of glycerol to ethanol, through a redox-balanced pathway, fulfills energy requirements by generating ATP via substrate-level phosphorylation. Other auxiliary or enabling pathways facilitating this metabolic process were identified along with the culture conditions triggering them, and therefore facilitating glycerol fermentation. We have also shown that this model for the 1,2-PDO-dependent fermentation of glycerol is valid for other microorganisms.

The knowledge base created by the aforementioned studies has been instrumental in the implementation of metabolic engineering strategies to convert glycerol to a variety of products, including lactate, succinate, 1,2-PDO, formate, and ethanol. We will present at the meeting our latest results in the understanding of this new metabolic competency and the metabolic engineering of these microorganisms to produce different chemicals from glycerol.

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GENE ESSENTIALITY ANALYSIS AND IMPLICATIONS FOR THE REDESIGN OF METABOLIC NETWORKS

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Essential genes are defined as genes whose deletion is lethal. By analogy, synthetic lethals refer to pairs of non-essential genes whose simultaneous deletion is lethal (i.e., no biomass formation). One can extend the concept of synthetic lethality by considering gene groups of increasing size where only the simultaneous elimination of all genes is lethal but individual deletion are not. We developed an optimization based framework using a bilevel representation to exhaustively identify multi-gene (and by extension reaction) lethals for genome-scale models. Specifically, this framework was applied to the *i*AF1260 model of *Escherichia coli* leading to the exhaustive identification of all double, triple and some higher-order synthetic lethals. Graphical representations of these synthetic lethals reveal a variety of motifs ranging from hub-like to highly connected sub-graphs. These results provide a birds-eye view of the redundant mechanisms available for redirecting metabolism and reveal complex patterns of gene utilization and interdependence. By analyzing the functional classifications of the genes involved (such as carbohydrate or nucleotide metabolism) we uncovered trends in connectivity within and between classifications. We find that there exists an invariant “essential core” of genes whose presence is required under all conditions. The size and composition of the minimal sets for the remaining genes are highly dependent on the uptake environment as well as the imposed growth requirement. We explore the diversity of alternative minimal gene sets of the same size that satisfy the same requirements. Finally, we compare our results against predictions of minimal gene sets based on homology studies and placed the work in context with recent *in vivo* advances in creating a reduced-genome *E. coli*.

We exploit the same mathematical concepts used to exhaustively explore multi-gene lethality, to classify all fluxes in the metabolic model depending upon whether or not they must increase, decrease, or become equal to zero to meet a pre-specified overproduction target. This classification is not limited to individual reactions but is extended for pairs, triplets, etc. while making use of all flux data available for the strain before engineering though metabolic flux analysis (MFA). The final output of this analysis is a logic tree-diagram that spans all possible sets of engineering interventions capable of meeting a pre-specified overproduction target. The developed methodology is tested by exhaustively identifying all engineering interventions for succinate production in a glucose-limited medium. The method recapitulates known engineering targets but also reveals new non-intuitive ones that boost succinate production by performing coordinated changes on pathways distant from the last steps of succinate synthesis.

ENERGETIC AND CELLULAR FEASIBILITY OF NOVEL PATHWAYS TO DEGRADE BIPHENYL

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Background

Increasing amounts of anthropogenic compounds, called xenobiotics, are released into the environment in many different forms, including industrial pollutants and pesticides. Accumulation of these recalcitrant compounds leads to contamination of air, water and soil [1]. Microorganisms provide a wealth of biodegradative potential in the reduction and elimination of xenobiotic compounds in the environment [2, 3]. Computational tools are used to predict biodegradation in order to address the information gap between the number of compounds thought to be biodegradable and information on the reactions through which microbial biodegradation occurs. The objective of this work was to generate novel biodegradation pathways that are feasible alternatives to the proposed pathway of biphenyl, a model substrate for polychlorinated biphenyls.

Methods

We have applied BNICE, a computational framework for the discovery of novel biochemical pathways, to generate novel biodegradation routes for biphenyl. Upon predicting the biodegradation of biphenyl, we estimate the feasibility of the pathway. One useful metric to evaluate potential biodegradation pathways is thermodynamic feasibility, where a group contribution method [4] is used to estimate the Gibbs free energy of the individual reactions and cumulative energy of the pathway. Secondly, we investigate the cellular feasibility of the novel pathway using metabolic flux analysis to study how implementation of the pathway influences the existing metabolism of *E. coli*, a reference organism. Lastly, the thermodynamic analysis is coupled to MFA, termed thermodynamic metabolic flux analysis (TMFA), to generate thermodynamically feasible flux profiles [5].

Results

BNICE generates more than 16,000 novel pathways to acetaldehyde and pyruvate, the endpoints of the known pathway. These pathways were distilled down to six distinct overall reactions and compared to the known pathway, revealing a trade-off between energetic favorability and cellular feasibility. A comparison of the flux distribution profiles for growth on biphenyl and growth on glucose indicated that the main differences in the essential reactions can be attributed to glycolytic, pentose phosphate cycle, and TCA cycle reactions. Oxygen uptake and the efflux of carbon dioxide and water contribute to the differences in the flux profiles. Additionally, we have identified the thermodynamic bottlenecks in the metabolic network.

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QUANTITATIVE PERSPECTIVE ON THE CRABTREE EFFECT IN DIFFERENT YEASTS

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Quantitative perspective on the Crabtree effect in different yeasts.

Aerobic metabolism in yeasts differs widely depending on whether the Crabtree effect occurs. Crabtree-positive yeasts, such as *Saccharomyces cerevisiae* exhibit a high glucose uptake and glycolytic rate. By glucose catabolite repression, they down-regulate their tricarboxylic acid (TCA) cycle and produce ethanol. In contrast, yeasts as *Yarrowia lipolytica* and *Candida rugosa* exhibit a low glycolytic rate and fully respiratory metabolism without fermentative by-products. Here, we characterize the Crabtree effect in the above three yeasts at the level of intracellular metabolites and carbon fluxes. To resolve the different carbon fluxes in central carbon metabolism, we performed ^{13}C-tracer experiments with subsequent GC-MS analysis of protein-bound amino acids. ^{13}C-labelling patterns were interpreted by flux ratio analysis. The different metabolic states of Crabtree-positive and –negative species were clearly reflected in the calculated flux maps: *S. cerevisiae* exhibited a high glycolytic flux (13 mmol g^{-1} h^{-1}), whereas the Crabtree-negative species had a 6-fold lower glycolysis. The TCA cycle flux was lowest in *S. cerevisiae* compared to *Y. lipolytica* and *C. rugosa* The pentose phosphate pathway did not seem to be influenced. Both *Y. lipolytica* and *S. cerevisiae* exhibited the same absolute flux (1.6 mmol g^{-1} h^{-1}). To elucidate potential correlations between flux and metabolite levels, we quantified the concentrations for over 30 intracellular metabolites in central carbon metabolism. The cells were quenched with -40 °C methanol (60%). After hot ethanol extraction the metabolite concentrations were measured by LC-MS/MS and GC-TOF. With the exception of fructose-6-phosphate and 3-phosphoglycerate, *S. cerevisiae* had elevated glycolytic intermediate concentrations, which were 2 to 9-fold higher than in *Y. lipolytica* and *C. rugosa*. As expected from fluxes, the TCA cycle intermediate concentrations were increased in *Y. lipolytica* and *C. rugosa*, although the differences were not as accentuated as in the glycolytic intermediates. The pentose phosphate pathway intermediates, adenine nucleotides and the amino acids did not show a dependency on the presence of the Crabtree effect. To summarize, we see at both the fluxome and metabolome, that the Crabtree effect is mainly manifested in the glycolysis and the TCA cycle. A ubiquitous correlation between fluxes and intermediate pools seems to exist. However, further measurements of more species are needed to shed light on the generality of these findings.

METABOLIC ENGINEERING FOR 3-HYDROXYPROPIONIC ACID PRODUCTION BY FERMENTATION: A ROUTE TO ACRYLIC ACID FROM RENEWABLE RAW MATERIALS

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Acrylic acid is a very high volume chemical (3.1 million tons produced in 2005) that is produced in the petrochemical industry by oxidation of propylene, a product from the refining of crude oil. Almost half of the crude acrylic acid produced annually is used to make glacial acrylic acid for superabsorbent polymers used in personal care items such as disposable diapers (more than 1 million tons annually). The remainder is used to produce acrylates that are components of acrylic fibers, coatings, paints and inks. The acrylic acid market grows approximately 4 percent annually. 3-Hydroxypropionic acid (3-HP) has been identified as a platform chemical (¡°building block¡±) that can be produced from renewable feedstocks by fermentation and subsequently can be chemically converted to several downstream products, including acrylic acid, that are today derived from petrochemical building blocks. Several years ago, Cargill initiated a program to develop recombinant microorganisms for the production of 3-HP by fermentation, and recently Cargill and Novozymes announced a joint development agreement to continue this development with the goal of achieving a commercially competitive route to acrylic acid starting from renewable feedstocks. This poster presentation will compare various biosynthetic routes to 3-HP, describe technical progress to date on the metabolic engineering of the various pathways, provide an overview of the options for recovery of 3-HP from fermentation broth and describe the chemical conversion of 3-HP to acrylic acid.

IDENTIFICATION OF THE *IN SILICO* TARGETS OF *ESCHERICHIA COLI* USING THE METABOLITE AVAILABILITY

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Complex biological systems are very robust to genetic and environmental changes at all levels of organization. Many biological functions of microorganisms can be sustained against single-gene or even multiple-gene mutations, by utilizing the redundant or alternative pathways. Thus, only limited number of genes were identified to be effective to the cell. While the studies on the topological and functional properties of metabolic networks have been increasingly populated, they still provide a limited understanding of the metabolic robustness. The conventional attempt to study such robustness relies on the identification of the genes or reactions indispensable to a cell. In this regard, the reaction-centric gene deletion study has a limitation in understanding the metabolic robustness.

Here, we report the use of flux-sum, which is the summation of all incoming or outgoing fluxes around a particular metabolite under pseudo-steady state condition, as a good conserved property from the metabolite availability point of view. The functional behavior as well as the structural properties of metabolites effective to the cell survival was investigated by means of a constraints-based flux analysis under perturbed conditions. The flux analysis was carried out under various genotypic and environmental conditions using the genome-scale *Escherichia coli* metabolic model.

The metabolites are capable of maintaining a steady flux-sum even against severe perturbation by actively redistributing the relevant fluxes. Disrupting the flux-sum maintenance was found to suppress the cell growth and increase the desired biochemical production. This approach of analyzing the metabolite-essentiality provides novel insight into the cellular robustness and concomitant fragility, which can be used for several applications including the development of improved strain for biochemical production and the identification of new drugs targets in pathogens. [This work was supported by the Korean Systems Biology Project of the Ministry of Science and Technology (M10309020000-03B5002-00000). Further supports by the LG Chem Chair Professorship and KOSEF through the CUPS are appreciated].

A GENOMICS APPROACH TO IMPROVE THE ANALYSIS AND DESIGN OF STRAIN SELECTIONS

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Strain engineering has been traditionally centered on the use of mutation, selection, and screening to develop improved strains. Although mutational and screening methods are well characterized, selection remains poorly understood. This is because overall biological fitness is a function of multiple phenotypes that contribute to different extents depending upon the selection strategy employed. We hypothesized that we could use a genome-wide method for assessing laboratory selections to design selections with enhanced sensitivity (true positives) and specificity (true negatives) towards a single desired phenotype. To test this hypothesis we first applied multi-SCale Analysis of Library Enrichments (SCALEs) to identify genes conferring increased fitness in continuous flow selections with increasing levels of 3-hydroxypropionic acid (3-HP). We found that this selection not only enriched for 3-HP tolerance phenotypes but also for wall adherence phenotypes (41% false positives). Using this genome-wide data we designed a serial-batch selection with a decreasing 3-HP gradient. Further examination by ROC analysis confirmed that the serial-batch approach resulted in significantly increased sensitivity (46%) and specificity (10%) for our desired phenotype (3-HP tolerance).

PRODUCTION OF NON-RIBOSOMAL PEPTIDES IN *SACCHAROMYCES CEREVISIAE*

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Non-ribosomal peptides (NRPs) represent a large group of secondary metabolites many of which have antibiotic, immunosuppressive or antitumoral activities and they show a remarkable structural diversity. This project aims at establishing yeast as a cell factory for production of existing as well as novel NRPs.

NRPs are synthesised by NRP synthetases (NRPSs), large enzymes that exhibit a modular structure, in which one module usually catalyses the activation and incorporation of one amino acid.
As a model NRPS, we have heterologously expressed ACV synthetase (ACVS) of *P. chrysogenum* in *S. cerevisiae*. Since NRPSs depend on a covalently bound 4´-phosphopatetheine cofactor for activity, three different 4´-phosphopantetheinyl transferase encoding genes were co-expressed with ACVS encoding *pcbAB*. In all three cases, ACV production was proven by LC-MS analysis.
In order to enhance ACVS expression and ACV production, the first 27 codons of *pcbAB* were optimised with regard to the yeast codon bias, which had no significant effect on ACV synthesis. However, when the yeast strains were grown at low temperature, the intracellular ACV concentration increased more than 30-fold.

The modular structure of NRPSs enables the combination of modules from different origin, which can then lead to the production of novel peptides. Recently, communication-mediating (COM) domains, which allow selective interaction between modules on separated polypeptide chains, have been identified in bacterial NRPSs.
We have tested the applicability of COM domains for NRPS expression in yeast by expressing a module of the tyrocidine gene cluster of *Bacillus brevis* together with its native COM domain and a module of the surfactin gene cluster of *Bacillus subtilis* containing a mutated COM domain from different plasmids. Yeast strains containing both plasmids are showing production of the expected dipeptide.

These results clearly indicate that *S. cerevisiae* is a suitable host for production of NRPs.

IMPROVING THE SYNTHESIS OF ANTHRANILATE FROM GLUCOSE IN *ESCHERICHIA COLI* BY METABOLIC ENGINEERING

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Anthranilate is an aromatic amine used as precursor for the synthesis of compounds with several industrial applications. Chemical synthesis of anthranilate is an unsustainable process since it implies the use of nonrenewable benzene and the generation of toxic by-products. In several microbial and plant species, anthranilate is an intermediate in the tryptophan biosynthetic pathway; but it is normally not accumulated. In *Escherichia coli*, anthranilate is synthesized from chorismate by anthranilate synthase (TrpE), and then converted to phosphoribosyl anthranilate by phosphoribosyl transferase (TrpD), to continue the biosynthetic pathway. With the purpose of generating a microbial anthranilate producer strain, *E. coli* W3110 *trpD9923*, a mutant in the *trpD* gene that displays a modest anthranilate production capacity, was characterized and modified using metabolic engineering strategies. In order to increase the carbon flux to the common aromatic pathway, the genes encoding a feedback inhibition resistant DAHP synthase (*aroG*^{fbr}) and transketolase (*tktA*) were expressed in strains W3110 *trpD9923* having either active or inactive phosphoenolpyruvate:sugar phosphotransferase system (PTS). Molecular characterization of the *trpD9923* mutant allele confirmed a nonsense mutation in the *trpD* gene, losing the phosphoribosyl transferase activity of TrpD. Fed-batch cultures of W3110 *trpD9923* strain produced 4.2 g/L of anthranilate with a glucose-yield ($Y_{Ant/Glc}$) corresponding to 22% of the $^{max}Y_{Ant/Glc}$. Inactivation of PTS in W3110 *trpD9923* strain caused a 2.2-fold increase in anthranilate production, with a $Y_{Ant/Glc}$ corresponding to 43% of the $^{max}Y_{Ant/Glc}$. However, a drastic decrease in q_{Glc} was observed, resulting in 65% growth reduction. Strain W3110 *trpD9923* expressing *aroG*^{fbr} and *tktA* genes displayed the best anthranilate production parameters. This strain produced 14 g/L of anthranilate with a $Y_{Ant/Glc}$ corresponding to 56% of the theoretical maximum. Also, it was observed a 19-fold reduction in the acetate accumulation in comparison to the W3110 *trpD9923* strain.

CORYNEBACTERIUM AS PLATFORM FOR PRODUCTION OF FINE CHEMICALS: CARBON CONTROL AND ACCESS TO NEW CARBON SUBSTRATES

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Amino acid production by *Corynebacterium glutamicum* amounts to about 2.5 million tons per year and, thus, is a proven large-scale biotechnological process. The traditional product spectrum has recently been widened by metabolic engineering approaches, e.g. for production of 3-aminopropionic acid or ethanol. The generally recognized as safe *C. glutamicum* has been shown to be robust against a variety of inhibitory compounds and to be able to efficiently co-utilize different carbon source mixtures. The characterization of genetic control mechanisms of carbon metabolism, which are distinct from those of the model bacteria *E. coli* and *B. substilis*, enabled strain development for improved carbon substrate utilization. In addition, pathways for access to new carbon sources have been engineered, e.g. for efficient use of glycerol, which arises in large quantities in the biodiesel process as major by-product of plant seed oil transesterification with methanol. Progress and future challenges to establish *C. glutamicum* as platform for the production of fine chemicals will be discussed.

STREPTOCOCCUS ZOOEPIDEMICUS ENGINEERED TO OVEREXPRESS PGI PRODUCES HIGH MOLECULAR WEIGHT HYALURONIC ACID

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Hyaluronic acid (HA) is a valuable biopolymer used in numerous pharmaceutical and cosmetic applications. High molecular weight HA is desirable in many of these applications. While identical in composition, HA produced from bacterial fermentation e.g *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) is typically of lower molecular weight (1-2 MDa) than HA extracted from rooster comb (up to 4-5 MDa with careful extraction). Cross-linking is an option for some applications (e.g., viscosupplementation in osteoarthritis), but other applications like eye surgery require native linear high molecular weight HA. Though the biosynthetic pathway of streptococcal HA production is well-established, little is known about what controls the molecular weight; this is generally true for beta-polysaccharide production despite their abundance (e.g., cellulose, chitin, beta-glucan).

HA molecular weight is greatly affected by medium and culture parameters. This suggests that in addition to intrinsic features of the HA synthase, metabolite levels affect the control of molecular weight. In this study, we manipulated metabolites in the HA pathway by overexpression of the 5 genes of the HA synthesis (*has*) operon in *S. zooepidemicus*. The greatest increase (79%) in molecular weight was observed in a recombinant strain overexpressing phosphoglucoisomerase (*pgi*). Under optimal aerobic conditions, this strain produced HA of 3.8 MDa with a yield of 10% on glucose.

Overexpression of the HA synthase resulted in a dramatic drop in productivity indicating that high levels of the synthase causes an acceptor substrate inhibition at the level of the HA chain initiation reaction. Overexpression of HasB and HasC led to a 19 and 13 fold increase in precursor pool of UDP-glucuronic acid, but did not affect molecular weight or productivity. Combined with the superior performance of the strain overexpressing Pgi (and to a lesser extent, the strain overexpressing GlmU), these data suggest that molecular weight is controlled by UDP-N-acetyl-glucosamine availability.

MECHANISTIC MODELING OF QUANTITATIVE MULTI-OMICS DATA: INTEGRATING POOLS, FLUXES, ENZYME ACTIVITIES, AND TRANSCRIPTS OF C. GLUTAMICUM

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Quantitative multi-omics data represent a huge source of information on the regulation of intracellular metabolic and genetic networks. However, consistent and complete data sets from well standardized series of experiments are hardly available. In the project “Systems biology of microbial amino acid producers” (funding: BMBF 0313704 managed by Evonik Degussa GmbH) several German research teams joined forces to supply such multi-omics data sets. A series of experiments was performed under quasi steady state conditions (continuous and fed batch cultivation) and cells were harvested and rapidly inactivated by methanol quenching. From these samples the following quantitative data was obtained in a coordinated and highly standardized way:
(1) metabolite pool sizes of 48 pools in the central metabolic pathways (glycolysis, pentose phosphate pathway, citric acid cycle (TCA), amino acid biosynthesis),
(2) metabolic fluxes in these pathways applying ^{13}C metabolic flux analysis,
(3) enzyme activities of 15 enzymes in the TCA obtained from enzyme assays, and
(4) genome wide transcriptome data from Affymetrix chips.

The complete data set contains 7 experiments; some more experiments will follow soon. Since the experiments were carried out with different knockout and over expression mutants of *C. glutamicum* the available data set can serve as a source for iteratively building up a mechanistic model of this organism which, finally, will be used to predict the outcome of new experiments. The following model features of the mechanistic model are important:
(1) Convenience kinetics approach is used to model enzymatic reaction steps.
(2) The model focuses on the description of TCA regulation and is less detailed in other parts of metabolism.
(3) Gene expression is modeled by phenomenological relations and state specific factors shared by all genes for integrating transcriptome data.

The complete model consists of 16 metabolite pools, 32 reaction steps, and 21 gene/enzyme pairs. The model is built up using the Modelica simulation language. As expected, reproduction of the underlying multi-omics data obtained by parameter fitting is not always satisfying. Particularly, from misfits in the proteome and metabolome data useful conclusion on the validity of data and possible errors in measurement procedures can be drawn.

Moreover sensitivities for model variables and parameters are calculated using an automatic differentiation method developed for Modelica source code (ADModelica). Using this sensitivity information parameter covariances are estimated. Moreover, as common in metabolic control analysis, control coefficients for pools and fluxes are determined leading to model based predictions for strain improvement.

ENHANCED CELL GROWTH AND RIBOFLAVIN PRODUCTION IN RECOMBINANT BACILLUS SUBTILIS CARRYING VITREOSCILLA HEMOGLOBIN GENE

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The Bacillus subtilis is an important microorganism used for industrial production. So far there were a few successful examples of VHb expression in B. subtilis: Expression of VHb in B. subtilis has been found that the total protein secretion and the production of alpha-amylase and neutral protease were enhanced (30%, 7-15% and 1.5-fold, respectively) (1). For hyaluronic acid (HA) production with the expression of VHb in B. subtilis, the cell concentration was enhanced 25%, HA production was increased by 100%(2).For the production of surfactin, a recombinant plasmid PEST-RDR-vhb was constructed and transferred into strain B. subtilis, the results showed that the expression of vhb gene could improve cell growth and enhance the production of surfactin with 31.8%(3).

Dissolved oxygen (DO) has great effect on cell growth and riboflavin production in B. subtilis, so a high oxygen supply rate is necessary. However, high oxygen supply depends on increasing the agitation speed, which will result in high cost for power consumption during the fermentation process. In this study, vgb gene was integrated into the chromosome of genetically engineered strain B. subtilis PY and under the control of sacB promoters, with an aim to increase the oxygen and energy supply and, consequently, to improve the cell growth and production of riboflavin. The influence of vgb on physiological characters has been investigated. The apparent phenotype with more rapid specific growth rate, higher oxygen uptake rate and higher biomass was achieved under oxygen-limited conditions in batch cultivation. In contrast, fermentation by-product, acetate accumulation was significantly reduced in VHb-expression strains. Metabolic flux distribution analysis discloses that VHb can significantly alter central carbon metabolism and direct a larger shift in carbon fraction from glycolysis toward the pentose phosphate pathway. VHb can also facilitate oxygen delivering, generate sufficient oxygen flux and direct interaction with the respiratory apparatus of the cell and tune the glucose consumption and TCA cycle to a proper ratio and thus relieve the overflow metabolism. Moreover, the metabolic analysis indicated that the intracellular ratio of ATP-ADP increased from 1.3-fold in host strains to 3.2-fold. The increased flux of pentose phosphate pathway, the high concentration of ATP, as well as decreased by-products could explain the increased biomass and riboflavin production in VHb-expression strains. The expression of VHb also improved the biomass and yield of riboflavin compared with the control strain under fed-batch fermentation, and the final concentration of biomass increased 28% and about 20 % improvement of the riboflavin titer were obtained.

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PRODUCTION OF POLYHYDROXYALKANOATES FROM OLIVE OIL IN METABOLICALLY ENGINEERED *ESCHERICHIA COLI*

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Polyhydroxyalkanoates (PHAs), good candidates for biodegradable plastic material, are polyesters of (R)-hydroxyalkanoic acids accumulated in numerous bacteria as an energy and carbon storage material under nutritional limitation condition in the presence of excess carbon source. Recently, it became possible to display proteins on the surface of microorganisms by fusing them to an surface anchoring motif, known as cell surface display. In this study, the direct fermentation system of olive oil to PHA by metabolically engineered *E. coli* displaying the lipase on the cell surface was investigated. We have developed a metabolically engineered *E. coli* strain producing polyhydroxyalkanoates (PHA) from olive oil by displaying the Bacillus sp. TG43 lipase using FadL as an anchoring motif. After confirmation of successful display of lipase on the cell surface through immunofluorescence microscopy, whole cell lipase activity was measured during the cultivation. Lipase activity was assayed by spectrophotometric method using p-nitrophenyl decanoate as a substrate. As the result, the fusion protein was continuously expressed on the surface of *E. coli* throughout the cultivation. To establish the PHA biosynthesis pathway in recombinant *E. coli* from olive oil, the Pseudomonas sp. 61-3 PHA synthase gene (phaC2) and the Pseudomonas aeruginosa enoyl-CoA hydratase gene (phaJ) were expressed in several different *E. coli* strains displaying lipase. Medium chain length-PHAs were produced by recombinant *E. coli* strains using olive oil as a carbon source. The PHA contents obtained in the recombinant strains were not beyond 10% of cell concentration. When the fadA mutant was used as a host strain, a higher PHA content of about 40% could be achieved. For PHA production, metabolically engineered *E. coli* strains were cultivated at 30 oC in 250 ml flask containing 100 ml of LB medium supplemented with 10 g/L of olive oil. PHA concentration and monomer composition were determined by gas chromatography equipped with a fused silica capillary column using benzoic acid as an internal standard. Finally, *E. coli* cell surface display system allowed direct conversion of oil to biodegradable polyesters, PHAs, resulting in the economical production of PHA. [This work was supported by the Korean Systems Biology Research Program from the Ministry of Science and Technology. Further supports by LG Chem Chair Professorship and Center for Ultramicrochemical Process Systems (Korea Science and Engineering Foundation) are appreciated.]

SYSTEMATIC ANALYSIS OF ADA-DEPENDENT REGULATION IN *ESCHERICHIA COLI*

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Various alkylating agents in the environment or generated by cellular metabolisms are toxic and mutagenic to cells. It is important for cells to cope with the alkylation damages. In the adaptive response to alkylating agents, cells acquire increased resistance to the alkylation damages by inducing DNA repair systems. It has been known that the repair systems are conserved from bacteria to human. In *Escherichia coli*, the adaptive response system regulated by Ada is induced by the alkylation damages. The Ada protein has the dual function of transcriptional regulator for four genes (*ada*, *alkA*, *alkB* and *aidB*) involved in the adaptive response and a methyltransferase that demethylates two methylated bases (O^6meG and O^4meT) and methylphosphotriesters produced by methylating agents in the sugar phosphate backbone. Although this form of regulation and structure was described more than two decades ago, the physiological characteristics and transcriptional regulations relating to Ada-dependent adaptive response to the alkylation damages remains poorly understood. In this study, the physiological differences between *E. coli* K-12 W3110 and its *ada*-disrupted mutant strains were compared using integrated transcriptome and proteome analyses. From this systematic analysis, the time- and strain-dependent adaptive response to alkylating agents was revealed. For example, the expressions of the genes involved in energy metabolism, transport, chemotaxis and flagellar biosynthesis were different due to the Ada-dependent adaptive response system. Specifically, the extended role of this regulatory system and the additional role of Ada as a transcription factor for other metabolisms could be suggested. This study helps to understand the cellular physiology and get more complete picture of the gene regulatory system relating to the Ada-dependent regulations in *E. coli*. [This work was supported by the Korean Systems Biology Research Program from the Ministry of Science and Technology. Further supports by LG Chem Chair Professorship and Center for Ultramicrochemical Process Systems (Korea Science and Engineering Foundation) are appreciated.]

GENOME WIDE ANALYSIS OF *A. NIGER* METABOLISM DURING INDUSTRIAL FED-BATCH FERMENTATIONS

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The filamentous fungus *Aspergillus niger* is widely used in industry for the production of enzymes and organic acids. The reason for the widespread use of this fungus lies in its ability to produce high amounts of extracellular enzymes and primary metabolites. However, the fungus also produces a considerable amount of unwanted by-products such as polyols and a mixture of organic acids. The by-product formation is problematic both in relation to downstream processing and fermentation process efficiency.

A. niger has been studied for many years and extensive knowledge of central enzymes and pathways has been generated. Even though processes have been improved substantially there is still room for gaining additional information about the metabolism of the organism both regarding the evolutionary rationale or metabolic constraints leading to both product (in the case of organic acids) and by-product formation and strategies for rational engineering of metabolism. Furthermore, the processes employed by industry are in many cases not well described in literature making it difficult for researchers to direct their efforts towards phenomena relevant to the industrial reality.

This project aims at providing systems wide information about the metabolism during oxygen limited fed-batch fermentations resembling the industrial process for glycoamylase production. To this end laboratory scale fed-batch fermentations at several different conditions will be subjected to microarray analysis and vigorous characterization with regard to all compounds relevant to the carbon balance and this data will be combined with a genome wide metabolic model to yield a detailed picture of metabolism useful in designing better processes and strains.

Fed-batch fermentations investigating the impact of glucose concentration/feed control on process performance has been carried out. These fermentations show that by lowering the glucose concentration the production of by-products is lowered. This is not only true for glycerol known to be involved in osmo-regulation, but also for other metabolites. Furthermore, an improved HPLC method revealed new and interesting aspects of the by-product spectrum regarding the identity of key metabolites. Identification and quantification of these compounds are on-going.

The project is financed by The Novozymes Bioprocess Academy.

TRANSCRIPTIONAL AND METABOLIC ANALYSIS OF SCALE-DOWN STUDIES FOR BIOPROCESS IMPROVEMENT: THE CASE OF RECOMBINANT PROTEIN PRODUCTION INDUCED BY TEMPERATURE

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Process scale-up can be costly and slow as different performances can result between small- and industrial-scale cultures. Understanding cell responses on different scales contributes to improving cellular performance through metabolic engineering, and increases the success of bioprocess development and scale-up. The lambdaPL/cI857 is a convenient expression system because fine-tuning of heterologous gene expression can be attained by simple induction schemes of temperature increases. Yet, differences in the heating rates as scale increases, and detrimental consequences of increasing culture temperature on E. coli physiology, must be considered. In this contribution, we present a case of bioprocess improvement by transcriptional and metabolic analysis of scale-down studies. Transcriptional and metabolic responses of E. coli to temperature-induced synthesis of preproinsulin (PPI) in high-cell density fed-batch cultures were examined during temperature increase at heating rates of 6, 1.7, 0.8 and 0.4 °C/min, in a scale-down approach for mimicking fermentors of 0.1, 5, 20, and 100 m3, respectively. As the heating rate increased, the maximum production of PPI decreased, whereas more carbon was lost as byproducts. Slow heating rates diminished stress and reduced the negative effects of biosynthetic activities, as reveled from the transcriptional response of distinctive genes from heat-shock, stress regulation and transcriptional/translational machinery. Such behavior indicates that compared to small-scale, conventional large-scale conditions favor PPI production. However, even under the best induction conditions at higher temperatures, PPI production and growth ceases around 2 to 3 h after induction. To avoid such a problem and based on the transcriptional results, an alternative induction scheme, consisting of temperature oscillations between 42 °C and 30 or 37 °C, was developed. With such induction strategy, the initial PPI production rate was between 1.4- and 2-times lower than at 42 °C. However, longer production periods could be attained compared to conventional induction conditions, which resulted in a 1.2- to 1.6-fold increase in maximum PPI concentration using the novel induction strategy. Maximum biomass accumulation during post-induction phase was between 4.0 to 10.0 gDCW compared with 16.0 to 30.0 gDCW at 42 °C. Acetate accumulated between 3.3 to 4.2 g/L at 42 °C, whereas it only reached 1.5 to 2.9 g/L in oscillated cultures. Lactate accumulated between 3.1- to -3.7 g/L and between 1.5- to -3.1 g/L for cultures induced at constant and oscillated temperature, respectively. Succinate accumulated up to 1.0 g/L in cultures at 42 °C, whereas it was not detected during induction by temperature oscillation. The results obtained here show the potential of physiological studies for process development and scale-up to overcome process limitations. We are using the results of this study to improve cells performance using metabolic engineering strategies.

PROTEOMIC AND PHYSIOLOGICAL CHARACTERISTICS OF SUCCINIC ACID-OVERPRODUCING MANNHEIMIA SUCCINICIPRODUCENS AND ITS STRAIN IMPROVEMENT

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The well-known succinic acid producer, *Mannheimia succiniciproducens* MBEL55E is isolated from a Korean cow. With the full genome sequence, we analyzed the *M. succiniciproducens* proteome using two-dimensional gel electrophoresis (2-DE) and tandem mass spectrometry. Proteome reference maps of *M. succiniciproducens* were established in the first place by analyzing whole cellular proteins, membrane proteins and secreted proteins. One hundred twenty-nine proteins from the whole cell proteome, 48 proteins from membrane proteome, and 30 proteins from secreted proteome were identified and characterized by various bioinformatic tools. The presence of proteins on the 2-D gels previously annotated as hypothetical proteins or proteins having putative functions were also confirmed. Based on the maps, cells in the different growth phases were analyzed at the proteome level. Many consistent relationships between the enzyme levels and metabolite profiles were found. Several changes of enzymes associated with amino acid biosynthesis, organic acids formation, stress response, and membrane protein synthesis could be seen.

In addition, we also inspect the proteome alteration of a genetically engineered succinic acid overproducing mutant, *M. succiniciproducens* LPK7. Proteome of the mutant cells at the exponential and stationary phases was compared with those of the wild type strain (*M. succiniciproducens* MBEL55E) to elucidate the global physiological and metabolic changes responsible for succinic acid overproduction. Comparative proteomic analyses between the wild type and the mutant showed the apparent differences in 87 and 69 protein spots at the exponential and stationary phases, respectively. In a similar manner, we found that the mutant strain varied the expression levels of 58 proteins as the cells grow. Through the statistical analysis, ATP associated enzymes, glycolytic enzymes, NADH-dependent enzymes, and pyruvate metabolizing enzymes were changed significantly in expression level. Above all, two distinctively and exclusively expressed proteins associated with NADH were further characterized by gene deletion experiment.

In conclusion, comparative proteome profiling revealed valuable information to understand physiological changes during growth and succinic acid overproduction, and subsequently suggested target genes to be manipulated for the strain improvement. These results clearly suggest that further comparative proteome profiling under various genetic and/or environmental perturbations will reveal more new insights into the physiology and metabolic characteristics of *M. succiniciproducens*.

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ENGINEERING OF SPHINGOLIPID BIOSYNTHESIS IN THE NON-CONVENTIONAL YEAST *PICHIA CIFERRII*

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Sphingolipids are ubiquitous lipid compounds in eukaryotic cells and tissues. They serve important structural functions as constituents of cellular membranes and the permeability barrier of mammalian skin. As such they also attracted commercial interest, because their topical application as active ingredients in cosmetic formulations improves barrier function. Ceramides containing phytosphingosine as sphingoid base are synthesized at commercial scale based on the acetylated sphingoid base produced via a fermentation process, employing classically improved strains of the non-conventional yeast *Pichia ciferrii*. Because barrier function of human *strateum corneum*, the outermost layer of mammalian skin, also depends on the presence of sphingosine (1,3-dihydroxy-2-amino-4-octadecen)-based ceramides, we started a strain engineering program, employing a combination of metabolic engineering as well as classical strain improvement methods to develop a biotechnological process for production of sphingosine.

TRANSLATION OF GENOMICS DATA INTO USEFUL METABOLIC ENGINEERING STRATEGIES: CONSTRUCTION OF A 3-HYDROXYPROPIONIC ACID PRODUCING E. COLI.

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With the limited supply and increased demand of non-renewable resources, biologically produced products from renewable feedstocks, including 3-hydroxypropionic acid (3-HP) have become an attractive alternative to petroleum based products. 3-HP is a bio-product with several large market applications including acrylic acid, 1,3-propanediol and acrylamide, that is currently being pursued as a product of a genetically engineered microbial fermentations.

We have developed several new high-resolution and comprehensive genomics tools that can be used to understand and optimize complex phenotypes needed in industrial organisms. We have used these strategies to inform directed strain engineering efforts. In particular, we have been able to confirm both toxicity and tolerance mechanisms of 3-HP in E. coli that were identified by the genomics based SCALEs approach (multi-SCale Analysis of Library Enrichments). Utilizing this global knowledge of 3-HP toxicity we have constructed E. coli strains for both tolerance to and production of 3-HP.

MULTIPLE APPROACHES TO IMPROVING HETEROLOGOUS POLYKETIDE PRODUCTION FROM E. COLI

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In this work, computational, genetic, and process engineering themes were applied to study and improve the heterologous production of the erythromycin polyketide precursor 6-deoxyerythronolide B (6dEB) from E. coli. First, a genome-scale flux balance analysis was conducted, together with minimization of metabolic adjustment optimization, to better profile the heterologous system and suggest experimental process and genetic changes to improve 6dEB biosynthesis. In this study, several gene deletions were predicted to improve 6dEB biosynthesis. Many of these deletions were connected to the succinate metabolic node and suggested options to be experimentally tested. In addition, a robustness analysis identified optimal cell growth and 6dEB production as a function of the propionate substrate uptake rate. Next, based in part on the computational analysis, genetic manipulations of the E. coli cell were performed in an effort to rationally improve 6dEB production and substrate-to-product conversion. Both native and heterologous components were varied through either gene deletion or dosage to gauge the relative impact on final 6dEB levels. Of the heterologous components tested, the biosynthetic and substrate provision enzymes had the greatest impact on 6dEB biosynthesis. Finally, a separate analysis systematically varied media components with the goal of identifying optimized final compositions best suited to support E. coli-derived 6dEB biosynthesis. The results from this study revealed that tryptone has a positive impact on 6dEB production and could substitute for exogenous propionate feeding. Using this approach, 6dEB titers of 160 mg per L 6dEB were achieved (a 22-fold improvement over current culture media). At one level, these approaches aim to better understand the heterologous production of complex natural products highlighted, in this case, by the E. coli 6dEB polyketide product. Furthermore, insight gained from these approaches was either redirected or will be redirected towards improving the production of 6dEB and other complex natural products to be heterologously produced.

METABOLIC PROSTHESIS FOR OXYGENATION OF ISCHEMIC TISSUE

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This presentation discloses new ideas and preliminary results on the development of a "metabolic prosthesis" for local oxygenation of ischemic tissue under physiologically neutral conditions. We report for the first time selective electrolysis of physiological saline by repetitively pulsed charge-limited electrolysis for the production of oxygen and suppression of free chlorine. Using 800 µA amplitude current pulses and <200 µsec pulse durations, we demonstrate prompt oxygen production and delayed chlorine production at the surface of a fused 0.85 mm diameter spherical platinum electrode. The data, interpreted in terms of the ionic structure of the electric double layer, suggest a strategy for in situ production of metabolic oxygen via a new class of "smart" prosthetic implants for ischemic disease such as diabetic retinopathy. We also present data indicating that collateral pH drift, if any, can be held constant using a feedback-controlled three electrode electrolysis system that chooses anode and cathode pair based on pH data provided by local a sensor.

All physiological fluids contain significant levels of Cl–. Repetitively pulsed charge-limited electrolysis of this fluid may have application in the treatment of ischemic disease such as diabetic retinopathy via oxygenation of ischemic retinal tissue with minimal co-production of free chlorine. As reviewed by Ameri et al. (2007), the surgical techniques for intraocular retinal electrode prosthetic implants have now been demonstrated. Of course, real-world physiological fluids are complex solutions that contain more than buffered Na+ and Cl–. For example, human aqueous and vitreous humors contain millimolar levels of lactate, glucose and ascorbate. Experiments presently underway indicate that these organic compounds alter the chlorine and oxygen yields, but do not change the basic results of this presentation. For example, the organic constituents, especially ascorbate, react with free chlorine so that the restriction on the 200 µsec pulse duration is lifted. At the same time, however, they compete, with varying degrees of success, for electrochemical oxidizing equivalents at the anode and lower the Faradaic efficiency of oxygen evolution. These are research issues of electrode materials, fabrication techniques, and kinetic strategies that will be the focus of future investigations.

METABOLITE ESSENTIALITY OF *VIBRIO VULNIFICUS* CMCP6 FOR DRUG TARGETING

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Vibrio vulnificus is a halophilic and highly human-pathogenic bacterium, showing very high mortality rate when infected. In order to facilitate the drug development process for this, we undertook *in silico* analysis to identify specific drug targets in the genome-scale metabolism of *V. vulnificus*. With a newly sequenced and annotated genome of *V. vulnificus*, we first reconstructed its genome-scale metabolic model consisting of 945 reactions and 765 metabolites. Subsequently, we employed constraints-based flux analysis to validate the model in comparison with experimental data, and subsequently to predict drug targets. Briefly, constraints-based flux analysis is an optimization-based simulation technique that calculates metabolic fluxes based on the mass balance of metabolites with assumption of pseudo-steady state.

Metabolite essentiality herein refers to the cell phenotype as a consequence of zero consumption of a metabolite. If removal of a certain metabolite leads to cell death, it is deemed ¡®essential metabolite¡¯. Its lethal effects are comparable to simultaneous deletion of outgoing metabolic reactions around that removed metabolite. Therefore, from essential metabolites, it is possible to easily identify various combinations of reactions that suppress cell survival, thereby enabling ¡®multi-targeting¡¯. Multi-targeting is now considered critical factor for antibacterials, and, in fact, currently available effective antibacterials aim multiple sites of the pathogen.

Using constraints-based flux analysis, each metabolite was removed from the model one by one, and its effect on the cell phenotype was examined. It was assumed that various nutrients are available for the pathogen inside the human body, and thus, the simulation was conducted under the condition of defined rich medium. From this, a total of 107 metabolites were predicted to be essential for the cell survival. These include various energy-carrying compounds (e.g. ATP), amino acids, cofactors and so forth.

These candidate targets still need to be subjected to further *in silico* analyses in order to narrow them down. In particular, it is necessary to compare the sequence of enzymes that are associated with the essential metabolite with the human genome sequence using BLAST to exclude candidates that might cause side-effects in the human body. This study demonstrates that drug targeting using *in silico* approaches facilitates not only the systems-level analysis of the bacterial metabolism, but also a rational design of experiments applicable to biomedical science.

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PRODUCTION OF ARTEMISINIC ACID, A PRECURSOR TO THE ANTI-MALARIAL PRE-API ARTEMISININ, IN YEAST CAUSES OXIDATIVE STRESS

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Artemisinin is an effective antimalarial component used in artemisinin-based combination therapies (ACTs). Current production of this sesquiterpene lactone endoperoxide, via extraction from *Artemisinin annua* plants, is insufficient to meet global demand. Combined microbial metabolic engineering and chemical synthesis to develop a semi-synthetic manufacturing process can help incease supply, improve quality and stabilize price fluctuations. *Saccharomyces cerevisiae* has been engineered to produce artemisinic acid (AA), which can be converted chemically to artemisinin and related compounds [Ro, Paradise, et al. (2006) Nature 440:940-943]. The engineered strains include high expression of three genes from the plant: amorphodiene synthase (ADS), a cytochrome P450 (CYP71AV1), and its cognate reductase (A.a.CPR1). It is well known that P450/CPR pairs sometimes act in an "uncoupled" manner that consumes NADPH and generates H_20_2 and other reactive oxygen species (ROS) without oxidizing the substrate. Published work has shown the degree of uncoupling differs among P450s and CPRs, and changes with reaction conditions. We have co-expressed CYP71AV1 and A.a.CPR1 in several engineered strains of yeast. We examined cellular ROS content, cell viability, product titer, and gene expression. Our data are consistent with a substantial amount of uncoupling. We will present results suggesting that some strategies for ameliorating uncoupling are promising, while others have little positive effect.

HETEROLOGOUS EXPRESSION OF POLYKETIDES IN FUNGI AND OPTIMIZATION BY USING *IN SILICO* ANALYSIS

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Polyketides are the source of some of the most potent antibiotics and anticancer agents available today. They constitute a large group of natural compounds produced primarily by fungi and bacteria. The productivity is often very low in the native producer and therefore the construction of super hosts via *in silico* predictions and gene targeting presents a real advantage. Three species well suited as hosts for polyketide production are *S. cerevisiae*, *A. niger* and *A. nidulans*. *Aspergilli* are of special interest as they are similar to the natural producers in various aspects.

Metabolic networks in filamentous fungi are considerably more difficult to model as compared with bacteria or simple eukaryotes (such as *S. cerevisiae*), mainly due to the increased physiological and genomic complexity, and hence a larger metabolic and regulatory network. Stoichiometric metabolic models of *S. cerevisiae*, *A. niger*, and *A. nidulans* have already been constructed [Förster, 2003, Pel, 2007, David, 2006]. We have used the in house developed tools for gene targeting in *Aspergilli* for metabolic engineering of the strains [Nielsen, 2007]. An iterative model-building cycle consisting of predictions, verifications and model improvement, was exploited for creating environment-friendly and highly productive microbial cell factories.

Production of heterologous enzymes and polyketides in fungi is of great industrial relevance. One model polyketide 6-MSA has already been expressed heterologously in *S. cerevisiae* [Wattanachaisaereekul, 2007]. Optimization of this production can be achieved by using in silico predictions. These predictions have been used to increase productivity of this model polyketide. Furthermore other fungal strains may one day produce these compounds heterologously to give even further insights into regulation of polyketides production.

In this work, physiological response of the fungal strains following the heterologous expression of polyketides is presented. Furthermore metabolic models to predict gene targets to increase production are evaluated. Testing these predictions by flux analysis will enable further improvement of the model and thus lead to improved productivity of the strain.

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DELINEATE A CARBON SOURCE FROM ENERGY SOURCE IN METABOLIC ENGINEERING: AN EXAMPLE WITH *AGROBACTERIUM* SP.

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In a typical bioprocess employing metabolically engineered strains, a sugar (usually glucose) is used as both carbon and energy source. The coupling of carbon metabolism and energy metabolism is one of the most important factors that complicate metabolic engineering efforts. A necessary balance has to be achieved between supplying adequate precursors for target molecule synthesis and providing energy that powers the synthesis and maintains vital cell functions dictated by the process specifics. The ability to delineate the dual roles of a sugar offers a novel strategy to engineer useful strains and design more efficient bioprocesses.

Previously, we have successfully engineered a curdlan-producing *Agrobacterium* strain for oligosaccharide (N-acetyllactosamine) and polysaccharide (hyaluronan) synthesis. These complex carbohydrates are important target molecules for metabolic engineering as they play key roles in many biological processes and are pursued as anti-infective drugs and vaccines. Their synthesis is recognized as both carbon and energy intensive. A uniquely challenging aspect of the metabolic engineering efforts is the requirement of high-energy compounds, in the form of sugar nucleotides, as precursors. The involvement of central carbon metabolism and regulations by high-energy compounds bring a new level of complication in the metabolic engineering efforts.

We discovered that the engineered *Agrobacteium* strain was capable of simultaneously utilizing sucrose and citrate in the synthesis of complex carbohydrates. The presence of citrate, in fact, stimulated the synthesis by as much as seven fold. The stimulation accompanied a significant increase sucrose uptake and consumption. The stimulation was apparently unique with this organism, as the phenomenon was not observed when tested with *Escherichia coli*. The stimulation was much more significant with sucrose (the preferred carbon source) than glucose. Apparently, citrate, consumed through the TCA cycle, provided the energy source necessary to power the synthesis whereas sucrose, metabolized through the glycolysis pathway, provided the sugar skeleton for the synthesis. By delineating the roles played by sucrose from serving as both energy and carbon source to just the carbon source, more precursors were available for the synthesis of the target molecules. Therefore, more products were synthesized. Key enzymes activities in the ED pathway were inhibited by the presence of citrate and ATP, suggesting that the delineation was made possible by the regulatory role of either citrate or ATP.

In this presentation, a model consistent with our experimental results and metabolic flux analysis will be presented to explain the delineation. The possibility of using delineation as a general metabolic engineering strategy for other complex molecules and in other organisms will be discussed.

METABOLIC CONDITIONING BY EXTRACORPOREAL NORMOTHERMIC PERFUSION FOR RECOVERY OF REJECTED DONOR LIVERS

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Liver transplantation is currently the only established treatment for end-stage liver disease, but is limited by a severe shortage of viable donor livers. Livers obtained from donors after cardiac death (DCD) are currently an untapped source that could significantly increase the pool of donor livers. However, preservation of these DCD livers by conventional static cold storage (SCS) is associated with a higher risk of primary non-function and delayed graft failure. Normothermic extracorporeal liver perfusion (NELP) has been suggested as an improvement over SCS. In this study, we developed a small-scale animal system to optimize NELP. To provide proof-of principle data, livers recovered from male Lewis rats were subjected to 1hr of warm ischemia and preserved with 5hrs of SCS or NELP, and transplanted into syngeneic recipients. As additional controls, non-ischemic livers preserved with 6hrs of SCS or NELP, and unpreserved ischemic livers were transplanted. We found that following NELP, ischemically damaged livers could be orthotopically transplanted into syngeneic recipients with 90% survival (N=10) after 4 weeks, which was comparable to control animals which received healthy livers preserved by SCS (N=6) or NELP (N=11) for 6hrs. On the other hand, animals from ischemia/SCS control group all died within 12hrs post-operatively (N=6). Similarly, animals that received ischemic livers without preservation all died within 24hrs after transplantation (N=6). In conclusion, these results suggest that NELP has the potential to reclaim warm ischemic livers that would not be transplantable otherwise. The rat model used in this study is a useful platform to further optimize NELP as a method of recovery and preservation of DCD livers. We are currently studying the metabolic profile of livers during perfusion to determine the mechanisms of action of NELP, as well as to characterize differences between livers that survive vs. those that fail.

INTEGRATED AND THERMODYNAMICALLY CURATED GENOME-SCALE METABOLIC MODEL OF *MYCOBACTERIUM TUBERCULOSIS*

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Mycobacterium Tuberculosis (Mtb) is the leading cause mortality among infectious diseases. The ability of this organism to persist in the macrophages of the host is fundamental in Mtb's effectiveness. Lipid metabolism plays a key role in persistence of Mtb to host-defence and antimicrobial treatment. Jamshidi et al (2007) and Beste et al (2007) constructed genome-scale metabolic models of Mtb. We have integrated and expanded these models to investigate specifically lipid metabolism. Our work covers:

i. ICL/MCL bi-functional enzyme that is jointly required for propionate metabolism and for Mtb growth and survival in mice,
ii. Methyl Citrate Cycle,
iii. Reactions for the biosynthesis of lipids required in pathogenesis,
iv. Preliminary thermodynamic information of the compounds and reactions of the model,

Results obtained with the integrated *in silico* Mtb has been able to reproduce several phenotypic characteristics observed in various experiments done at Laboratory of Bacteriology (LABAC) at EPFL and others. Ongoing and future work will focus on clearing the *in silico* dependence of Mtb on certain carbon sources and assuming different objective functions.

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FLUX BALANCE ANALYSIS OF CHLAMYDOMONAS REINHARDTII

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With rising carbon dioxide levels in the atmosphere, knowledge of how photosynthetic organisms convert atmospheric carbon dioxide into metabolites and other important compounds is becoming increasingly important. Almost half of all inorganic carbon is fixed by aquatic algae and other marine organisms (1). These organisms have great promise to not only be used to sequester carbon dioxide from the air, but also for the economical production of chemicals because the inputs into the system (light and CO2) are essentially free. Another potential use is for the production of renewable energy; it has already been shown that lipids in algae can be converted to biofuels (2, 3) and both algae and cyanobacteria are capable of hydrogen production (4, 5). However a better understanding of photosynthetic metabolism must first be achieved in order to maximize production through metabolic engineering. Flux balance analysis (FBA) has previously been applied to the photosynthetic prokaryote *Synechocystis* (6), which provides insight into the metabolic processes involved in carbon fixation. In order to gain a better understanding of how compartmentation affects metabolic processes involved in photosynthesis without having to model several cell types (i.e. in higher plants), a green algae was chosen as the model organism. The metabolic network of primary and intermediary metabolism for a eukaryotic green alga, *Chlamydomonas reinhardtii*, was reconstructed from genomic and biochemical information. This is the first such model for an algal species. The completed network includes 480 metabolic reactions and 449 intracellular metabolites and includes the reactions for glycolysis, TCA cycle, pentose phosphate pathway, amino acid synthesis, lipid synthesis, nucleotide synthesis, starch synthesis, and chlorophyll synthesis and includes three intracellular compartments (mitochondria, cytosol and chloroplast). Through the reconstruction effort, 16 enzymes were found to be missing from the *C. reinhardtii* database but assumed present in the model to complete the metabolic network. Based on BLAST searches, one newly annotated enzyme (fructose-1,6-bisphosphatase) was added to the *C. reinhardtii* database. FBA was used to predict fluxes for three growth conditions, autotrophic, heterotrophic and mixotrophic growth. The completed model is a valuable tool for further exploration of photosynthetic metabolism and possible future metabolic engineering efforts.

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