Production of D-xylonate and organic acid tolerance in yeast

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VTT

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Doctoral dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the School of Chemical Technology for public examination and debate in Auditorium KE2 (Komppa Auditorium) at the Aalto University School of Chemical Technology (Espoo, Finland) on the 6th of June, 2014, at 12 noon.

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Abstract

Various organic acids have huge potential as industrial platform chemicals. Bio-technological routes of organic acid production are currently being sought, so that fossil resources and petrochemistry could be replaced with renewable resources. Microbial production of organic acids can provide an environmentally sound, sustainable way of producing industrial chemicals, and efficient processes are needed to produce large quantities of acids which are often novel to the production organism. Production of such acids imposes stresses on the organism. These stresses affect the vitality, viability and productivity of the cells in a bioprocess. Understanding the physiology of micro-organisms which have been genetically engineered to produce an organic acid, can make valuable contributions to the development of production organisms for biorefineries, which provide means to convert agricultural and forestry waste into these useful chemicals.

Production of D-xylonate, an industrial platform chemical with high application potential, was successfully demonstrated in various yeast species. D-xylonate is produced from D-xylose via D-xylono-γ-lactone that can be hydrolysed to D-xylonate spontaneously or with the aid of a lactonase enzyme. Various ways to improve production of D-xylonate in the yeast *Saccharomyces cerevisiae*, *Kluyveromyces lactis* or *Pichia kudriavzevii* as production organisms were successfully applied. The best D-xylonate production was obtained by expression of the D-xylose dehydrogenase encoding gene *xylB* from *Caulobacter crescentus* and the highest D-xylonate titre was achieved with *P. kudriavzevii* that produced 171 and 146 g D-xylonate l^{-1}, at a rate of 1.4 or 1.2 g l^{-1} h^{-1}, at pH 5.5 and pH 3, respectively. Production at low pH is desirable as this would make product recovery and process operations more economically feasible.

The consequences of D-xylonate production on the physiology of *S. cerevisiae* were studied in detail, both at population and single-cell level. D-xylonate and D-xylono-γ-lactone were produced and also exported from the cells from the very start of cultivation in D-xylose, even in the presence of D-glucose. There was no apparent preference for export of either compound. However, great amounts of D-xylono-γ-lactone and/or D-xylonate was accumulated inside the cells during the production.

The D-xylonolactone lactonase encoding gene *xylC* was co-expressed with the D-xylose dehydrogenase encoding gene *xylB* (both genes from *C. crescentus*). This lead to a significant increase in the D-xylonate production rate compared to cells expressing only *xylB* and showed that accumulation of D-xylonate and protons releases during hydrolysis, was harmful for the cells. The accumulation of

D-xylonate led to acidification of the cytosol, as determined by loss of pHluorin (a pH dependent fluorescent protein) fluorescence, and this loss of fluorescence was faster in cells co-expressing *xylC* with *xylB* compared to cells expressing *xylB* alone. Acidification of the cytosol was shown to correlate with decreased viability of the D-xylonate producing cells and the rate of loss of pHluorin fluorescence and loss in viability was highly dependent on the pH of the production medium. The decrease in vitality and challenges in export of D-xylonate are major obstacles for D-xylonate production by *S. cerevisiae*. The excellent D-xylonate producer, *P. kudriavzevii* also accumulated large amounts of D-xylonate and suffered decreased vitality, especially when D-xylonate was produced at low pH.

The stress response to weak organic acids is highly dependent on the properties of the acids and the presence of high concentrations of weak organic acids may lead to lost viability. The role of Pdr12, a membrane transporter, in resistance to weak organic acids was studied and found to be highly dependent on the acid. Deletion of *PDR12* led to improved tolerance to formic and acetic acids, a feature that makes this modification interesting for micro-organisms used in biorefining of lignocellulosic hydrolysates that commonly contain these acids.

Biotechnological production of D-xylonic acid with yeast clearly has the potential of becoming an industrially applicable process. In order for biotechnological production processes to become economically feasible, biorefinery approaches in which lignocellulosic hydrolysates or other biomass side- or waste streams are used as raw materials need to be employed. This thesis provides new understanding on how production of an organic acid affects the production host and presents novel approaches for studying and increasing the production.

D-xylonat produktion och tolerans mot organiska syror i jäster

Abstrakt

Organiska syror har en enorm potential som industriella plattformskemikalier. En bioteknisk produktion av organiska syror kunde ersätta produktionen av motsvarande, oljebaserade kemikalier. En mikrobiell produktion av organiska syror kan utgöra ett miljövänligt, hållbart sätt att producera kemikalier för industrin. För detta behövs effektiva processer och mikroorganismer med kapacitet att producera stora mängder syror. Dessvärre är syrorna ofta okända för produktionsorganismen och därmed medför produktionen av syra stora påfrestningar, vilket leder till stress. Denna stress påverkar vitaliteten, livskraften och produktiviteten hos cellerna i en bioprocess. Genom att förstå fysiologin hos mikroorganismer som är genetiskt manipulerade för att producera en organisk syra, kan nya produktionsorganismer för bioraffinaderier utvecklas. I ett bioraffinaderi kan jord-och skogsbruksavfall omvandlas till användbara kemikalier.

D-xylonat, en industriell prekursorkemikalie med stor potential, kan produceras med hjälp av olika jästsvampar. D-xylonat framställs från D-xylos via D-xylono-γ-lakton, som kan hydrolyseras till linjär D-xylonat, spontan eller med hjälp av ett laktonas enzym. I denna studie förbättrades produktionen av D-xylonat märkbart med hjälp av jästerna *Saccharomyces cerevisiae*, *Kluyveromyces lactis* eller *Pichia kudriavzevii* som produktionsorganismer. Den bästa produktionen av D-xylonat erhölls genom att uttrycka *xylB*, en gen från *Caulobacter crescentus* som kodar för ett D-xylos dehydrogenas enzym. Den största D-xylonatproduktionen uppnåddes med *P. kudriavzevii*, som var kapabel att producera 171 eller 146 g D-xylonat l^{-1}, med en hastighet av 1.4 eller 1.2 g l^{-1} h^{-1}, vid pH 5.5 respektive pH 3. Det är fördelaktigt att producera syra vid ett lågt pH-värde, eftersom det gör uppsamlandet av syran enklare och därmed processen mer ekonomiskt lönsam.

Konsekvenserna av D-xylonatproduktionen på *S. cerevisiae* jästens fysiologi studerades i detalj, både på populations- och encellsnivå. Under produktionen samlades stora mängder av D-xylonat och D-xylono-γ-lakton inuti cellerna. Ändå producerades och exporterades D-xylonat från cellerna från början av produktionsprocessen, även i närvaro av D-glukos. Både D-xylonat och D-xylono-γ-lakton exporterades från *S. cerevisiae* cellerna och det fanns ingen uppenbar preferens för någondera molekylen.

Genom att uttrycka genen som kodar för D-xylonolakton laktonas enzymet, *xylC*, tillsammans med genen som kodar för D-xylos dehydrogenas enzymet, *xylB*, fastställdes att ackumulering av linjärt D-xylonat och i hydrolysen frigjorda protoner, var skadligt för cellerna. D-xylonatproduktionen skedde märkbart snabbare i celler som uttryckte både *xylB* och *xylC* jämfört med celler som uttryckte endast

xylB. Ackumuleringen av D-xylonat ledde till att fluorescensen från pHluorin, ett pH-känsligt fluorescerande protein, försvann. Detta antyder att cellens cytosol försurnade då cellen producerade D-xylonat. Fluorescensen från pHluorin proteinet försvann snabbare i de celler som uttryckte både *xylC* och *xylB*, jämfört med de celler som uttryckte endast *xylB*. Denna försurning av cytosolen visade sig korrelera med en minskad livskraft bland cellerna som producerade D-xylonat och graden av försurning och förminskningen i viabiliteten var starkt beroende av pH-värdet i produktionsunderlaget. En förminskad livskraft och utmaningar i exporten av D-xylonat utgör stora hinder för D-xylonatproduktion med *S. cerevisiae*. Även i *P. kudriavzevii* cellerna samlades det stora mängder av D-xylonat och livskraften hos dessa var minskad, speciellt då D-xylonatet producerades vid lågt pH.

Stressreaktionerna gentemot svaga organiska syror är starkt beroende av egenskaperna hos syrorna och höga koncentrationer av svaga organiska syror leder till en förlorad livskraft. Vid studier av den roll transportproteinet Pdr12 har i resistensen mot svaga organiska syror, framkom att syrans egenskaper har stor inverkan på cellernas syratolerans. Mikroorganismer med en deleterad *PDR12* gen uppvisade en förbättrad tolerans mot myr- och ättiksyra, vilket kan utnyttjas vid bioraffineringen av lignocelluloshydrolysat, som oftast innehåller dessa syror.

En bioteknisk produktion av D-xylonsyra med hjälp av jästceller har stor potential att bli en industriellt användbar process. För att biotekniska produktionsprocesser skall kunna bli ekonomiskt möjliga, måste man utveckla bioraffinaderier där lignocellulosahydrolysat eller andra sido- eller avfallsströmmar används som råvaror. Denna avhandling ger ny förståelse för hur produktionen av en organisk syra påverkar produktionsorganismen och presenterar nya metoder för att studera och öka produktionen.

Preface

This study was carried out at the VTT Technical Research Center of Finland in the Metabolic Engineering team, during the years 2010-2013. Part of the work was done at VTT/MSI Molecular Sciences Institute in Berkeley, USA, during research visits in 2012 and 2013. Financial support was provided by the Academy of Finland (Center of Excellence, White Biotechnology – Green Chemistry 2008–2013; project number 118573) and by the VTT Graduate School. Travel funding by the Academy of Finland Graduate School for Biomass Refining (BIOREGS) and Svenska Tekniska Vetenskapsakademien were greatly appreciated. The financial support of the European Commission through the Sixth Framework Programme Integrated Project BioSynergy (038994-SES6) and the Seventh Framework Programme (FP7/2007-2013) under grant agreement No. FP7-241566 BIOCORE are also gratefully acknowledged.

I sincerely want to thank Vice President, Prof. Anu Kaukovirta-Norja and my current and former Technology Managers, Doc. Kirsi-Marja Oksman-Caldentey and Dr. Tiina Nakari-Setälä for providing me with excellent working facilities. I wish to thank Doc. Kristiina Poppius-Levlin, the coordinator of VTT Graduate School, for her support and encouraging attitude.

I had the privilege to have a bunch of supervisors (thesis advisors) during my work for this thesis. I am forever thankful for all the time they gave me and all the efforts they put into guiding me through this journey leading to me finding my passion in science. Research Prof. Merja Penttilä has been a scientific mentor for me. I am truly amazed by her capability to see the big pictures while simultaneously focusing on important details. I admire how Merja gets excited about new ideas and concepts and thinks everything is possible. Dr. Marilyn G. Wiebe introduced me to the wonderful world of bioreactors and taught me the fundamentals of critical scientific questioning. I admire Marilyn's devotion to her work and her profound knowledge and enthusiasm. In addition, Marilyn has helped me improve my English skills and I am grateful for her efforts in revising the language of this thesis and my manuscripts. My current and former Team Leaders, Dr. Mervi Toivari and Dr. Laura Ruohonen were also actively involved in supervising the work for my thesis. Mervi has an incredible capacity of always looking at things from the bright side and she always has a few encouraging words to spare. Laura taught me that devotion and determination pays off. Laura allowed me to choose my own path

towards this thesis, which I am very grateful for. Last, but not least, Dr. Dominik Mojzita has been an endless source of ideas and inspiration for my work. Dominik's enthusiasm and interest in everything and nothing is contagious.

I want to express my deepest gratitude to my supervising professor, Associate Prof. Alexander Frey. Alex showed a very supportive and positive attitude towards my work and put in a lot of time and effort in helping me finish this thesis. I also wish to thank my former supervising professor, Emeritus Prof. Matti Leisola and Assistant Prof. Harri Lähdesmäki, the supervisor of my minor studies. I wish to thank Prof. Annele Hatakka and Prof. Elke Nevoigt for careful pre-examination of the thesis and for their useful comments on how to improve it.

I also want to acknowledge all other co-authors of my publications: Docent Hannu Maaheimo, Dr. Orna Resnekov, Dr. C. Gustavo Pesce, Dr. Martina Andberg, Dr. Anu Koivula, Dr. Mari Valkonen, Dr. Mojca Benčina, MSc. Maija-Leena Vehkomäki and MSc. Esa-Pekka Kumpula.

It has been a privilege to get introduced to NMR spectroscopy by Hannu and to get exposed to his in-depth knowledge and enthusiasm. I wish to thank Orna and Gustavo for introducing me to the wonderful world of fluorescent microscopy and single cell analysis. It was a great opportunity for me to conduct part of my studies in Berkeley and to get exposed to the scientific environment in the Bay area. Also, I really enjoyed biking up the Berkeley hills for admiring the Golden Gate Bridge in the mornings.

It has been a privilege to work with so many skilful, enthusiastic, hardworking and motivated people in the former Cell Factory. The support, advice and help I have been given throughout the years is invaluable. I sincerely wish to thank all of you who have made my days at VTT inspiring, enjoyable and so much fun!

I warmly wish to thank my colleagues and friends, Outi, Joosu, Kiira, Mari, Eero, Pekka, Mira and Stefan. Over the years we have had a lot of fun also outside the lab. Outi is very bright, most diligent and helpful and I will miss all our coffee breaks, discussing things larger or smaller than life. Joosu is not only an excellent scientist but also a great sportsman. With Joosu we discuss skiing and running on a daily basis.

Finally I would like to thank all my friends and my family for endless support and patience with me. You remind me that there is more to life than science. You remind me that hiking in the wilderness, kayaking in the sea or partying like a rock star are also important activities to fit into my busy schedule. Annette, Emilia, Jenny, Kia and Janina, I don't know what I would do without you and I am so grateful for your friendship. Larissa, I value your advice and friendship. Travelling with you is always so much fun and I am happy that you'll always visit me, wherever I move.

My family has always supported me in all my endeavours and means to world to me. Our family is full of love and laughter and I cherish all the times we have to spend together. My father taught me to believe in myself and work hard for getting what I want, and not to let anyone pull me down. I admire my father's courage and strength. My mother is the most caring, helpful and supportive mother one could have. My precious little brother reminds me that dreaming and working for reach-

ing our dreams really is what life is about. Dennis is a multi-talent and also great company for skiing, snowboarding and biking or simply wondering about all and nothing. My older brother, Robin, is hard-working, helpful, skilful, calm and conservative, mostly in a good way. The family farm is in good hands. I also wish to thank my sisters-in-law, Jonna and Minna, for their friendship. I admire their capacity the fit this crazy family, they have become a part of. I deeply thank my family for all the love and encouragements given to me.

Espoo, May 2014

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List of publications

This thesis is based on the following original publications which are referred to in the text as I–VI. The publications are reproduced with kind permission from the publishers.

I   Nygård Y., Toivari M.H., Penttilä M., Ruohonen L., Wiebe M.G. 2011. Bioconversion of D-xylose to D-xylonate with *Kluyveromyces lactis*. Metabolic Engineering 13: 383–391.

II   Toivari M., Nygård Y., Kumpula E., Vehkomäki M., Benčina M., Valkonen M., Maaheimo H., Andberg M., Koivula A., Ruohonen L., Penttilä M., Wiebe M.G. 2012. Metabolic engineering of *Saccharomyces cerevisiae* for bioconversion of D-xylose to D-xylonate. Metabolic Engineering 14: 427–436.

III   Toivari M., Nygård Y., Penttilä M., Ruohonen L., Wiebe M.G. 2012. Microbial D-xylonate production. Applied Microbiology and Biotechnology 96: 1–8.

IV   Toivari M., Vehkomäki M., Nygård Y., Penttilä M., Ruohonen L., Wiebe M.G. 2013. Low pH D-xylonate Production with *Pichia kudriavzevii*. Bioresoure Technology 133: 555–562.

V   Nygård Y., Mojzita D., Toivari M.H., Penttilä M., Wiebe M.G., Ruohonen L. The diverse role of Pdr12 in resistance to weak organic acids. Accepted for publication in Yeast. doi: 10.1002/yea.3011.

VI   Nygård Y., Maaheimo H., Mojzita D., Toivari M.H., Wiebe M.G., Resnekov O., Pesce G.C., Ruohonen L., Penttilä M. Single cell and *in vivo* analyses elucidate the effect of xylC lactonase during production of D-xylonate in *Saccharomyces cerevisiae*. Under revision, Metabolic Engineering.

Author's contributions

Publication I
Yvonne Nygård participated in the designing of the experimental work and carried out most of the laboratory work (most bioreactor cultivations, sample analyses, enzyme assays and intracellular sample preparation), except for strain constructions, analysed and interpreted the results, and collaborated with the other authors to write the article. Yvonne Nygård is the corresponding author of the article.

Publication II
Yvonne Nygård participated in the designing of the experimental work and carried out most of the laboratory work (most bioreactor cultivations, sample analyses, enzyme assays, intracellular sample preparation and part of the strain construction), except for enzyme purification and characterization, ^{1}H NMR spectroscopy and intracellular pH measurement, and collaborated with the other authors to analyse and interpret the results and write the article.

Publication III
Yvonne Nygård participated in writing this review article together with the other authors.

Publication IV
Yvonne Nygård carried out the pH tolerance experiments, and collaborated to analyse the data and write the article together with the other authors.

Publication V
Yvonne Nygård designed the work, carried out all the experimental work and analysed and interpreted the results. Yvonne Nygård drafted the article and is the corresponding author of the paper.

Publication VI
Yvonne Nygård designed the work, carried out all the experimental work except for the ^{1}H NMR spectroscopy measurements, and analysed and interpreted the results. Yvonne Nygård drafted the article and is the corresponding author of the paper.

Contents

Appendices

Publications I–VI

Publication VI of this publication is not included in the PDF version.

List of abbreviations

ABC   ATP-Binding Cassette

ATP   Adenosine triphosphate

bp   Base pair

CFU   Colony forming unit

CoA   Coenzyme A

DHAP   Dihydroxyacetone phosphate

DNA   Deoxyribonucleic acid

FP   Fluorescent protein

GADPH   Glyceraldehyde-3-phosphate dehydrogenase

GFP   Green fluorescent protein

GRE3   Gene encoding Gre3 aldose reductase in *Saccharomyces cerevisiae*

G protein   Guanosine nucleotide-binding protein

HPLC   High performance liquid chromatography

HR   Homologous recombination

K_{cat} Catalytic constant

K_m Michaelis-Menten constant

*LAC4*p   β-Galactosidase promoter of *Kluyveromyces lactis*

leu	Leucine
MCT	Monocarboxylate transporter
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
ORF	Open reading frame
PI	Propidium iodide
PLA	Poly-lactic acid
PPP	Pentose phosphate pathway
RNA	Ribonucleic acid
rpm	Revolutions per minute
SC	Synthetic complete
SCD	Synthetic complete medium with 10 g D-glucose l^{-1}
SCD-leu	Synthetic complete medium with D-glucose lacking leucine
SCDX	Synthetic complete medium with 10 g D-glucose l^{-1} and 20 g D-xylose l^{-1}
SCX	Synthetic complete medium with 20 g D-xylose l^{-1}
SEM	Standard error of the mean
TCA	Tricarboxylic acid
Trp1	Phosphoribosylanthranilate isomerase; catalyzes the third step in tryptophan biosynthesis in *S. cerevisiae*
ura	Uracil
V_{max}	Maximum velocity
vvm	Volume per volume per minute

XDH	Xylitol dehydrogenase
XI	D-xylose isomerase
XK	D-xylulokinase
XKS1	D-xylulokinase encoding gene in *S. cerevisiae*
XR	D-xylose reductase
XYD	D-xylose dehydrogenase
xylB	D-xylose dehydrogenase (xylB) encoding gene in Caulobacter crescentus
xylC	D-xylonolactone lactonase (xylC) encoding gene in *C. crescentus*
xyd1	D-xylose dehydrogenase (XYD1) encoding gene in *Trichoderma reesei*
XYL1	D-xylose reductase (XR) encoding gene in yeast
XYL2	Xylitol dehydrogenase (XDH) encoding gene in yeast
YAC	Yeast artificial chromosome
YP	Yeast peptone
Δ	Deletion
5-HMF	5-hydroxymethyl furfural

1. Introduction

Organic acids are currently generating considerable interest as platform chemicals applicable as building blocks in polymers as well as in food, detergent and pharmaceutical industries. Sugar acids, such as D-xylonic acid, can also be applicable as precursors in the manufacture of biomass derived plastics. Lately climate change and instability of fossil fuel prices have increased the interest in development of new biomass based products and production possibilities and there is a huge potential in the creation of surplus value commodities from renewable sources, especially from biomass unfit for food production.

Chemicals can be produced from biomass through chemical or biotechnological conversions. Microbial production of organic acids provides an environmentally friendly, sustainable way of producing industrial chemicals. Biological processes can be highly selective and stereospecific, they work in mild conditions (lower temperatures compared to chemical processes and neutral or low pH) and the final products are potentially biodegradable. However, for a biotechnological process to be economically feasible and to compete with the existing processes, the production organism must meet challenging demands in terms of production yield, productivity and robustness. To meet such criteria, fundamental understanding of cellular physiology of the production organisms is needed.

Industrial biotechnology is the use of living organisms or enzymes for production of compounds for e.g. food, pharmaceuticals or chemical applications. Biotechnological processes such as ethanol fermentation in beer or wine production by yeast or preservation of milk products or soy sauce by lactic acid produced by bacteria, to name a few, have been employed since the early civilisations. In the last century, several bacteria and fungi have been successfully genetically mutated or modified to overproduce a wide range of native and non-native compounds, including biofuels and pharmaceuticals as well as commodity and speciality chemicals. Fungi are widely used in the production of organic acids and several organic acids are already produced via large-scale bioprocesses. Metabolic engineering is the practice of introducing new genetic and regulatory processes within cells in order to change metabolic activities of the cells towards a desired activity such as the production of a certain substance. Well known examples of modified cell factories include human insulin production by *Saccharomyces cerevisiae*, citric acid production by *Aspergillus niger* and bioethanol production from D-xylose by *S. cerevisiae*.

The effects of genetically modifying an organism by introducing new metabolic pathways may be greater than just introducing a new product. Product tolerance is essential in engineered as well as natural microbial cell factories. The impact of acid production on the physiology of the production host is not well known and the understanding of how fungi respond to changes in pH and regulate intracellular pH is very limited. Redox and energy balances of the cell are of greatest importance for cell vitality, and altering these balances, through metabolic engineering, may limit production. Yeast and other cells are complex systems, in which even small alterations may have drastic effects on the fitness of the cell and the production of a compound of interest.

1.1 Yeast as cell factories

Yeast offer several advantages as production hosts, including simple nutritional requirements and high tolerance to stress and low pH. Bacteria often demand expensive nutritional supplements, complex growth media and high pH for good growth, making both production and product recovery expensive. When producing organic acids, tolerance to low pH is beneficial since keeping the pH high by addition of a neutralizing agent increases costs and makes recovery of the product more laborious, as the acid is converted into a salt that then subsequently has to be reprocessed to obtain the free acid. In order to have an economically viable process, high titres are needed and therefore the production organisms must also have great tolerance to the organic acid itself, especially as even small concentrations of some organic acids are inhibitory to most microorganisms.

1.1.1 *Saccharomyces cerevisiae* – an eukaryotic model organism and an industrial production host

S. cerevisiae, also called baker's yeast, is the most well-characterized eukaryotic model organism. It is widely used in molecular biology and medicine, as well as food and beverage manufacturing processes including winemaking, brewing and baking. *S. cerevisiae* can grow both in aerobic and anaerobic conditions and it can produce ethanol even in the presence of oxygen; features which have made *S. cerevisiae* an important industrial production organism. *S. cerevisiae* grows fast in many different surroundings and it is robust and quite tolerant to low pH and toxic compounds. Many processes using *S. cerevisiae* have GRAS (Generally Recognised As Safe) status. The *S. cerevisiae* genome was the first eukaryotic genome to be sequenced (Goffeau et al. 1996). *S. cerevisiae* cells have successfully been engineered to produce a wide range of compounds, including heterologous peptides such as insulin and Hepatitis B vaccine, pharmaceuticals such as antibiotics, industrial platform chemicals including lactic acid and n-Butanol or biofuels (e.g. 2nd generation bioethanol). The wide range of molecular biology tools and the high capacity for homologous recombination make genetic manipulations in *S. cerevisiae* relatively easy. The large amount of data collected and large variety of studies done with *S. cerevisiae* make the design of experiments and

process parameters fairly rational. Genome-scale metabolic networks (Förster et al. 2003), functional profiling of open reading frames (ORFs) by gene-deletion mutants (Shoemaker et al. 1996, Giaever et al. 2002) as well as a great amount of transcriptomic and proteomic data is publicly available for *S. cerevisiae*.

1.1.2 Non-conventional yeast as production organisms

While *S. cerevisiae* is without doubt the most studied and most utilized yeast, other species are becoming increasingly important. Recent advances in the development of molecular biological tools and procedures for genetic engineering of so-called non-conventional (non *S. cerevisiae*) yeast have increased the development of new production processes. Non-conventional yeast are used due to their specific properties, such as the capacity to effectively metabolize or produce certain compounds. Industrially important non-conventional yeast include *Yarrowia lipolytica (Candida lioplytica), Pichia* spp., *Kluyveromyces* spp. and recently also a few *Candida* spp. Table 1 lists the synonyms of yeast commonly refered to in this thesis.

Table 1. Yeast commonly refered to in this thesis and the synonyms for these.

Saccharomyces cerevisiae		
Yarrowia lipolytica	*Candida lipolytica*	
Pichia pastoris	*Komagataella pastoris*	
Pichia kudriavzevii	*Issatschenkia orientalis*	*Candida krusei*
Kluyveromyces lactis	*Candida sphaerica*	
Kluyveromyces marxianus	*Candida kefyr*	
Pichia stipitis	*Scheffersomyces stipitis*	
Trichoderma reesei	*Hypocrea jecorina*	

Y. lipolytica is an oleaginous yeast capable of degrading hydrocarbons and producing important metabolites such as organic and fatty acids. *Pichia pastoris*, *Pichia methanolica* (*Pichia pinus*) and *Pichia angusta* (*Hansenula polymorpha*) are methylotrophic yeast that are capable of growing on methanol and a number of *Pichia* spp. are used for production of heterologous proteins (Papanikolaou and Aggelis 2011).

Several recent studies recognize *Pichia kudriavzevii* (also called *Issatschenkia orientalis* or *Candida krusei*) as a robust production organism, e.g. for ethanol production (Kitagawa et al. 2010, Dhaliwal et al. 2011, Kwon et al. 2011, Isono et al. 2012). *Kluyveromyces lactis* and *Kluyveromyces marxianus* are interesting production hosts due to their native ß-galactosidase activity, capacity for ethanol production and capability of utilizing a great variety of substrates, including lactose (Spencer et al. 2002) and D-xylose (Margaritis and Bajpai 1982, Billard et al. 1995). *K. lactis* is considered a model organism in the *Kluyveromyces* genus, whereas *K. marxianus* is reported to be more robust in terms of temperature tolerance and substrate spectrum. Both species have been demonstrated to naturally

produce of a wide range of compounds, including lactic acid, ethanol, aroma compounds such as fruit esters and monoterpene alcohols and industrial enzymes like laccase, β−galactosidase, glucoamylase, inulinase, and polygalacturonases, among others (reviewed by e.g. Micolonghi et al. 2007 and Fonseca et al. 2008). These yeast have also been used for expression of heterologous proteins and in bioremediation.

1.1.3 Fungal production of organic acids

Yeast and other fungi naturally produce various organic acids, and production of a large number of organic acids has been achieved by genetic engineering. Examples of organic acids produced naturally include citric, gluconic and oxalic acid production by *A. niger* and itaconic acid by *Aspergillus terreus* (reviewed by Magnuson and Lasure 2004). α-Ketoglutaric, pyruvic, isocitric, and citric acids can all be produced by *Y. lipolytica* (reviewed by Finogenova et al. 2005).

Organic acids are generating increased interest, since they can be used as building-block chemicals and can be derived from renewable carbon sources. Today, only a few organic acids are commercially produced with micro-organisms, even though there are bioprocesses available for several of the acids which are currently produced chemically (for a review, see e.g. Magnuson and Lasure 2004, Sauer et al. 2008 or Nita et al. 2013). The quantities produced and product spectrum is however expected to greatly increase in the near future since organic acids, as commercially important platform chemicals, are identified as priority targets both in the US (Werpy et al. 2004) and in EU (de Jong 2011, Nita et al. 2013).

Commercial, microbial production of lactic acid has been employed since the 1990s (Sauer et al. 2010). Lactic acid is the pre-cursor for polylactic acid (PLA), one of the materials most commonly used in bio-based plastics today. Lactic acid has been produced by several engineered yeast species, including *S. cerevisiae* (Skory 2003), *K. lactis* (Porro et al. 1999), *Pichia stipitis* (Ilmén et al. 2007), *Candida sonorensis* (Ilmén et al. 2013), and *Pichia kudriavzevii* (Suominen et al. 2007). For a review on industrial production of lactic acid, see Miller et al. (2011).

The production of D-glycolic acid, another platform chemical of high potential for biopolymer synthesis, was recently demonstrated in *S. cerevisiae* and *K. lactis* (Koivistoinen et al. 2013).

1.2 Yeast physiology

1.2.1 Growth of yeast

In liquid culture, yeast cells typically grow exponentially (Fig. 1), beginning with a lag phase, when cells adjust to the environment before growth. After the lag phase, the cells grow exponentially, at a maximal growth rate for the specific environmental condition. After the exponential growth phase, as cell growth becomes limited as a consequence of the lack of nutrients, the cells decrease their specific

growth rate and enter the stationary phase. When cells grow on a mix of sub-strates, the preferred carbon source is used first during the exponential growth phase. When the preferred carbon source is depleted the cells enter a diauxic shift, during which they adapt to growth on the alternative/less preferred carbon source. In the stationary phase the cell number and mass is constant, and there is little cell division. Cells can remain viable for a long time in stationary phase. Viability is a term used to describe if a cell is alive or dead, defined as capable of reproduc-tion. Vitality, another commonly used term to describe the cell condition, describes the physiological condition, or metabolic activity of a cell. For a review on methods for determination of viability and vitality in yeast, see Heggart et al. (2000).

Exponential growth curve
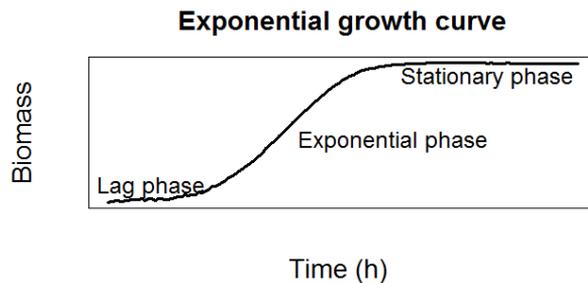
Figure 1. A typical exponential growth curve.

Yeast generally grow at pH values from 2.5–8.5, but grow best in medium at pH 3.5–6.0 (Matthews and Webb 1991). Yeast generally prefer acidic external pH, since the uptake of various nutrients depends on the proton gradient across the plasma membrane.

1.2.2 Metabolism and redox balance

Metabolic reactions can be divided into anabolic pathways, i.e. reductive process-es leading to the production of new cellular materials and compounds, and cata-bolic pathways, i.e. oxidative processes which remove electrons from substrates that are used to generate energy in the form of ATP. Together the reductive and oxidative reactions in the cell form a redox balance. ATP is the energy currency of the cell: the capacity to move energy around in the cell via coupling of ATP dephosphory-lation to thermodynamically unfavourable reactions is crucial for cell maintenance.

The major source of energy for yeast is D-glucose and other hexose sugars. In glycolysis (Embden–Meyerhof–Parnas (EMP) pathway in yeast), the predominant D-glucose catabolic route in *S. cerevisiae*, one D-glucose molecule is converted to 2 pyruvate molecules, resulting in a net production of 2 ATPs and 2 NADH mole-cules. (Fig. 2). In general, pyruvate produced during glycolysis can be further converted to acetyl coenzyme A (acetyl-CoA) during respiratory dissimilation in the mitochondria or to ethanol and CO_2 via fermentation.
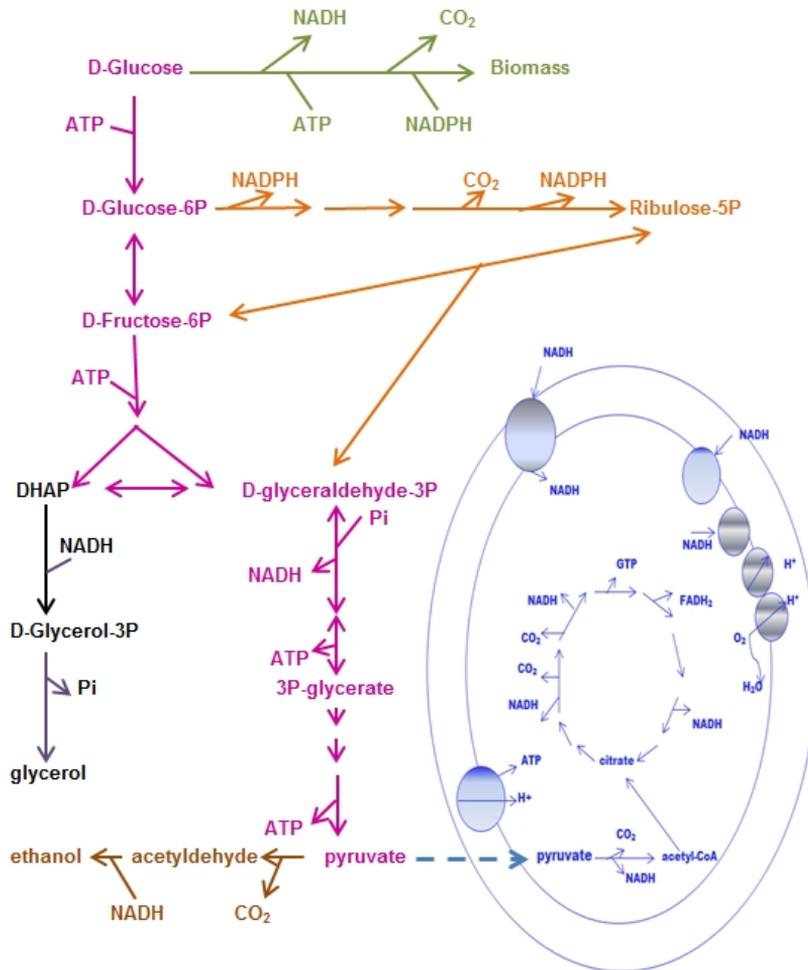
Figure 2. Central metabolism in *Saccharomyces cerevisiae*, showing NADH, NADPH, FADH$_2$ and ATP production and utilization. NADPH is produced by the oxidative part of the pentose phosphate pathway (PPP, in orange), while glycolysis (in pink) provides ATP and NADH. Ethanol is produced by pyruvate reduction (in brown) with NADH as a co-factor. Glycerol can be formed as a by-product to re-store redox balance via the consumption of NADH (in black). In the precence of oxygen (in blue), pyruvate can be oxidized to carbon dioxide and water by the TCA cycle, and the NADH/FADH$_2$ formed are reoxidized by the electron transport chain (presented as grey circles in the mitochondrial inner membrane) and ATP-synthase (blue circle in the mitochondrial inner membrane), generating ATP. In the presence of oxygen, cytosolic NADH can also be reoxidized by NADH dehydro-genases or transferred via different shuttle mechanisms into the mitochondria. *S. cerevisiae* has external NADH dehydrogenases (Nad1/Nad2) at the matrix side of the inner mitochondrial membrane. Biomass generation is summarized in green. DHAP = dihydroxyacetone phosphate. Figure modified from Guadalupe Medina (2013).

The pentose phosphate pathway (PPP, Fig. 2) converts D-glucose to CO_2 and pentose sugars. The latter are needed for synthesis of nucleotides and aromatic amino acids. Moreover, this pathway it is important for generating NADPH. Many anabolic reactions, such as the fatty and amino acid, sterol and purine synthesis, require NADPH.

In the Tricarboxylic acid (TCA) cycle, acetyl-CoA is converted to CO_2, H_2O (Fig. 2) and intermediates for the synthesis of amino acids and other molecules important for the cell through a series of altogether ten different chemical reactions that also generate ATP, NADH and $FADH_2$. The NADH and $FADH_2$ produced in the TCA cycle or previously in glycolysis are under aerobic conditions oxidized to NAD^+ and FAD by the electron transport chain. The electron transfer chain is a series of proteins in the mitochondrial inner membrane that transfers the electrons to oxygen. The transfer of electrons is coupled to export of protons across the inner mitochondrial membrane, creating an electrochemical potential that is used in chemiosmosis for generation of ATP from ADP and P_i (inorganic phosphate) by the ATP synthase. The electron transfer chain and chemiosmosis are referred to as oxidative phosphorylation. The malate-aspartate shuttle translocates electrons produced during glycolysis across the semipermeable inner membrane of the mitochondrion for oxidative phosphorylation. In addition, *S. cerevisiae* has several different shuttle mechanisms for transferring cytosolic NADH into the mitochondria (for a review on NADH metabolism, see Bakker et al. 2001).

Fermentation is the only anaerobic mode of sugar dissimilation in *S. cerevisiae*. Crabtree positive yeast, including *S. cerevisiae,* produce ethanol in the presence of excess sugar and oxygen as well as in the absence of oxygen, rather than directing all pyruvate to the TCA cycle for biomass and energy generation. Intracellular pyruvate accumulation, due to a high glycolytic rate, exceeding that of the pyruvate dehydrogenase and/or limited capacity of the respiratory system to oxidise mitochondrial NADH is assumed to lead to the Crabtree effect (van Dijken et al. 1993). The extent to which fermentation and respiration occur in this respiro-fermentative metabolism varies with D-glucose concentrations and the specific growth rate of the culture (Guadalupe Medina 2013). D-Glucose dissimilation in *S. cerevisiae* is fully respiratory only at low D-glucose concentrations and low specific growth rates (Guadalupe Medina 2013). The NADH formed during glycolysis, is re-oxidized during fermentation (van Dijken and Scheffers 1986), and NAD^+ serves as an electron acceptor in many other pathways and reactions. Under anaerobic conditions, when excess NADH cannot be oxidized in the TCA cycle, glycerol (the main by-product in fermentation) serves as an electron acceptor, to maintain redox balance.

Enzymes involved in redox balance maintenance, oxidoreductases, catalyse the transfer of electrons from one molecule, the reductant, to another molecule, the oxidant, while using NADP(H) or NAD(H) as a cofactor. Transhydrogenases that are found in many bacteria and also in some eukaryotes, are used in co-factor generation, switching oxidised and reduced forms of NADP(H) and NAD(H). Verho et al. (2003) identified the first fungal NADPH-glyceraldehyde-3-phosphate dehydrogenase (GADPH) in *K. lactis* and they suggested that this enzyme has transhy-

drogenase activity (NADP$^+$ + NADH $\leftrightarrow$ NADPH + NAD$^+$), which can regenerate both NADPH and NAD$^+$. Several bacterial transhydrogenases, including the *Escherichia coli* transhydrogenase, encoded by *udhA*, and other enzymes used in recycling co-factors such as the *Bacillus subtilis* glyceraldehyde 3-phosphate dehydrogen-ase encoded by *gapB,* and also the *K. lactis* GADPH encoding gene, have been successfully expressed in *S. cerevisiae* (Verho et al. 2002, Toivari et al. 2010).

1.2.3   pH homeostasis and organic acid stress in yeast

The maintenance of a constant intracellular pH is crucial for all cells. The redox state of the cell (Goffeau and Slayman 1981, Veine et al. 1998), the pH gradient across intracellular membranes needed for cross-membrane transport (Goffeau and Slayman 1981, Wohlrab and Flowers 1982), and metabolic reactions such as glycolysis (Vojinović and von Stockar 2009) are all highly influenced by intracellu-lar pH. Intracellular pH is not dependent on the extracellular pH of the growth medium (Orij et al. 2009, Valkonen et al. 2013), but can be altered by addition of certain weak acids to the medium, as some undissocited acids can permeate the cell membrane and dissociate in the cytosol leading to release and accumulation of protons that decrease the pH of the intracellular space (Ullah et al. 2012). Weak organic acids do not dissociate fully while strong acids can dissociate completely.

The intracellular pH varies between the cell compartments as a function of the environment of the cells and the availability of nutrients. Moreover, the pH of the different compartments depends on the pH of the other compartments of the cell (Martínez et al. 2008, Martínez-Muñoz and Kane 2008). Upon D-glucose addition, the intracellular pH of starved or D-glucose limited cells transiently decreases and then rapidly increases (Martínez-Muñoz and Kane 2008, Orij et al. 2009). Orij et al. (2012) claimed that intracellular pH controls the growth rate of yeast.

Yeast vacuoles have an acidic pH, whereas the pH of the mitochondria usually is slightly higher compared to the cytoplasmic pH (Preston et al. 1989, Martínez-Muñoz and Kane 2008, Orij et al. 2009). The ionization state of a compound is dependent on the pH; therefore proteins can be affected by changes in pH. Charged acidic or basic amino acid side chains can alter the conformation or solu-bility of a protein and as a result, the activity of an enzyme and interaction be-tween proteins.

The recent development of fluorescent protein based pH sensors (described in section 1.3.2) has enabled the determination of pH in live cells and in the orga-nelles of unperturbed cells. Orij et al. (2012) studied the intracellular pH of yeast cultures growing on D-glucose; during the exponential growth phase the yeast cells had neutral pH (7.2 ± 0.2), but after D-glucose depletion the pH of the cells decreased to 5.5 (Orij et al. 2009, Orij et al. 2012). Zdraljevic et al. (2013) showed that the cytoplasmic pH of D-xylonate producing *S. cerevisiae* cells decreased below pH 5.

Pma1 is the major plasma membrane H$^+$-ATPase that pumps protons out of the cell (Serrano et al. 1986) and the major regulator of cytoplasmic pH and plasma

membrane potential. Plasma membrane potential is required for the activity of multiple secondary transporters (for a review on Pma1, see Ambesi et al. 2000). *PMA1* is an essential gene, and Pma1 activity and abundance in the cell is regulated both at transcriptional and post-translational levels (Serrano et al. 1986). Pma1 is activated by D-glucose and by decrease in extracellular pH, through changes in the kinetic parameters of the enzyme (Serrano et al. 1986). In low pH the V_{max} of Pma1 is increased, resulting in more active proton pumping (Perlin et al. 1989).

The vacuolar H^+-ATPase in *S. cerevisiae*, the V-ATPase (Puopolo and Forgac 1990), is responsible for maintaining the pH of the vacuoles and other cellular organelles (for a review on the V-ATPase, see Graham et al. 2003 or Kane 2006) and it is also important in the control of cytosolic pH through removal of cytosolic protons and maintenance of energy in the form of membrane potential, by storage of protons (Martínez-Muñoz and Kane 2008, Diakov and Kane 2010, Orij et al. 2012). Protons are stored in the vacuoles and the membrane potential created between the vacuole and the cytosol provides energy for various cellular functions. Furthermore, the V-ATPase is important for proper localization of Pma1 to the cell membrane (Martínez-Muñoz and Kane 2008). Yeast mutants lacking V-ATPase activity were viable but did not grow in ambient pH above 6.5 (Nelson and Nelson, 1990). The regulation of the V-ATPase is very complex and involves regulation of V1 subunit assembly and reversible V1 and V0 disassembly (Kane 1995).

The mechanism for pH sensing and regulation, particularly in acidic conditions, remains elusive despite extensive studies. The Rim101 pathway (Li and Mitchell 1997) is responsible for pH regulation and adaptation to alkaline conditions (reviewed by e.g. Peñalva et al. 2008 and Maeda 2012) and it is also upregulated in conditions of weak organic acid stress, even though not induced by low pH per se (Mira et al. 2009). The Rim101 pathway is activated by proteolytic processing of the Rim101 transcription factor, as a response to alkaline conditions (Li and Mitchell 1997). The Rim101 pathway is linked to the ESCRT (endosomal sorting complex required for transport) components responsible for further pH signaling and also protein sorting (Hayashi et al. 2005), and reviewed by Henne et al. (2011). In addition, the Snf1 pathway, which is primarily involved in the adaptation of cells to D-glucose limitation and for growth on carbon sources other than D-glucose, is known to be involved in adaptation to alkaline pH (Hayashi et al. 2005). For a review on the Snf1 pathway, see Hedbacker and Carlson (2008). A very recent study reported that G protein-coupled receptor (GPCR) signaling decreased in response to reduced pH through the binding of protons to Gα subunits (Isom et al. 2013). Isom et al. (2013) predicted that 10% of non-redundant protein structures contain proton-binding regions and concluded that the Gα protein, a subunit of the G-protein complex, functions as a pH sensor and as a transducer of GPCR signaling.

Many organic acids are inhibitory to yeast and other microorganisms, a feature that makes these attractive preservatives in the food and beverage industries. The most commonly used and therefore most studied acids, utilized as preservatives are acetic, sorbic and propionic acid (Piper et al. 2001). These acids are consid-

ered to be safe for human consumption, while food spoilage microbes are inhibited by their presence. Nevertheless, there are many spoilage yeast and mould species that are able to grow in the presence of the maximum permitted levels of weak organic acids (Holyoak et al. 1999). Recently, tolerance to organic acids has gained attention due to the increased interest in the development of 2nd generation bioprocesses. Lignocellulosic hydrolysates can contain weak organic acids like acetic, formic and levulinic acid up to concentrations of several grams per litre (reviewed by e.g. Almeida et al. 2007, Mills et al. 2009).

In aqueous solution, acids exist in a pH dependent equilibrium between the undissociated (uncharged) and dissociated (charged anions) forms. The pKa (dissociation constant) of the acid determines the degree of dissociation. The weak organic acids discussed in this thesis have pKa values below 5, which means that at low pH, the majority of the acids are undissociated. Many undissociated weak organic acids can freely pass through the plasma membrane (Abbott et al. 2009), and lipophilic acids, such as sorbic acid may enter and retain within the cell membrane (Ray 1996, Holyoak et al. 1999). The extent to which an acid is capable of diffusing through the membrane is dependent on its properties; the greater the lipophilicity the better the acids can dissolve into the membrane, and also negatively affect the membrane structure. Acids that enter the cytosol may cause intracellular acidification, as the acids dissociate in the cytosol and release protons.

Stress responses which are upregulated in *S. cerevisiae* during weak organic acid stress include a general stress response, regulated by transcription factors Msn2/Msn4 (Schüller et al. 2004), the RIM101 pathway (Mira et al. 2009), and genes induced by the Haa1 and War1 transcription factors (Kren et al. 2003, Fernandes et al. 2005, Gregori et al. 2008). War1 activates the expression of *PDR12* (Kren et al. 2003), which is known to be involved in resistance to several weak organic acids (Piper et al. 1998, Holyoak et al. 1999, Bauer et al. 2003). Pdr12 exports anions from the cytoplasm and is a major component in resistance to weak organic acids.

Even though widely studied, the inhibitory mechanisms of weak organic acids are not yet fully understood, especially as the effect of one acid may differ significantly from the effect of another. In addition to decreased intracellular pH and challenges to keep the cellular energy balance while trying to maintain the intracellular pH, organic acids may cause alterations in the plasma membrane structure (Ray 1996, Bracey et al. 1998, Piper et al. 2001), or cause oxidative stress and increased free radical formation (Piper 1999). High concentrations of acid have also been shown to reduce cell viability (Ludovico et al. 2001, Lourenço et al. 2011, Semchyshyn et al. 2011). The ability to adapt to weak organic acids has been suggested to be dependent on limiting the entry of the acid to the cell, e.g. by changes in plasma membrane or cell wall composition (Piper et al. 2001, Ullah et al. 2013).

1.2.4 Transport of organic acids

The yeast plasma membrane accommodates a large variety of transporters for nutrient molecules, including carbohydrates, amino acids and phospho-organic compounds, etc (see YTPdb online: A wiki database of yeast membrane transporters, described in Brohée et al. 2010). The mechanism(s) by which organic acids (or their lactone forms) are excreted from cells is not well understood, although most transport systems for weak acids belong to primary or secondary transport mechanisms (for a review, see van Maris et al. 2004). Primary transport demands ATP, whereas secondary transport systems use energy stored in (electro-) chemical gradients. The primary transport mechanisms include ATP-Binding Cassette (ABC) transporters and ATPases, whereas the secondary transporters include uniporters, symporters and antiporters (Konings et al. 1997). The export of organic acid by microorganisms engineered to produce large quantities of these is crucial for maintenance of pH homeostasis. Acid export can be accomplished via uniport of the undissociated acid or via symport of the anion with a proton (van Maris et al. 2004).

Many anion transporters belong to the ABC transporter family. Pdr12 is a membrane transporter that was shown to actively extrude benzoate (Piper et al. 1998) and fluorescein (Holyoak et al. 1999). Deletion of Pdr12 was shown to render cells more resistant to for instance sorbic and propionic acid (Piper et al. 1998, Holyoak et al. 1999, Bauer et al. 2003, Ullah et al. 2012) and *PDR12* expression can be induced by some moderately lipophilic carboxylic acids (Hatzixanthis et al. 2003). Yor1 is a multidrug transporter that mediates export of many different organic anions, including acetate and propionate (Cui et al. 1996).

Examples of monocarboxylate transporter (MCT) family transporters include the Jen1 symporter that mediates transport of lactate (Casal et al. 1999, Pacheco et al. 2012), pyruvate (Akita et al. 2000), acetate and propionate (Casal et al. 1996) and Ady2, demonstrated to transport acetate, propionate, formate and lactate (Paiva et al. 2004). The aquaglyceroporin Fps1 is reported to be involved in uptake of acetate and efflux of glycerol (Mollapour and Piper 2007).

1.3 Tools for metabolic engineering and synthetic biology

Metabolic engineering and synthetic biology are rapidly accelerating fields. Increased availability of sequenced genomes, variants of enzymes from many different organisms, and well established genetic tools for transforming a large range of organisms allow rapid construction of new metabolic pathways and synthetic genetic elements. Extensive databases of gene expression, metabolic reactions and networks, as well as enzyme structures and functions, allow one to search for desired reactions and design or evolve novel enzymes for reactions that do not exist. New analytical tools enable the measurement of RNA, protein, and metabolites even at the single cell level and detailed models aid in the design of enzymes and metabolic pathways. The cost of *de novo* synthesis of genes and of nucleic

acid sequencing have decreased with the establishment of new technologies, enabling construction of new, artificial elements which can be used to control cellular metabolism.

The field of designing and constructing new biological entities such as enzymes, genetic circuits, or even complete cells, and the artificial redesign of existing biological systems is referred to as synthetic biology (Fig. 3). Synthetic biology combines molecular and systems biology. By applying engineering principles it seeks to transform biology using a component based approach for engineering of integrated circuits and cellular systems, etc. (definition according to the Synthetic biology engineering research centre, Synberc: http://www.synberc.org/what-is-synbio, cited 5.3.2014). Synthetic biology seeks inspiration from traditional engineering disciplines to design and build engineered systems with standardized and well characterized parts. The focus is on the design and construction of core components or parts that can be combined, modelled, understood, and tuned in a standardized way to meet specific performance criteria. Parts, such as genes or part of genes, promoters, terminators or artificial control elements, can easily be assembled into larger integrated pathways and systems for different purposes such as the production of chemicals. For recent reviews on metabolic engineering and synthetic biology, see e.g. Stephanopoulos (2012), Keasling (2012) or Singh (2014).
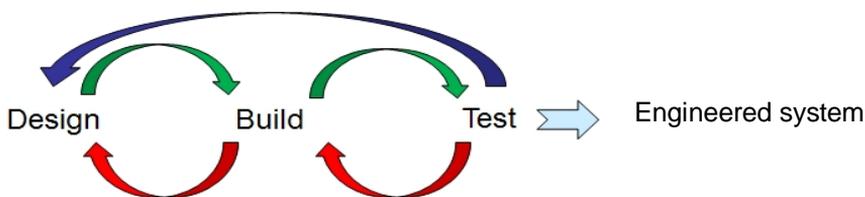
Figure 3. A scheme for synthetic biology as iterative circles of designing, building and testing. The figure is modified from a figure by Lynn Andrea Stein, in Interactive Programming in Java, available at http://www.cs101.org/ipij/design, cited 5.3.2014.

High-throughput methods, namely, methods that perform a great number of simultaneous measurements and automation of strain generation and screening, have rapidly transformed the landscape of biological research. Array-based high-throughput methods, including microarrays for analysis of DNA or RNA and assays for protein/DNA, protein/protein, and cell-level interactions are today routinely used in metabolic engineering research. These tools allow the behaviour of thousands of genes, transcripts, and proteins to be studied, and enable quantitative analysis of gene networks and metabolic fluxes.

1.3.1 Molecular biological tools for genetic engineering of yeast

Molecular biology enables characterization, isolation and manipulation of the molecular components of cells. Molecular biology tools are used to study and modify gene expression and protein function. Expression plasmids and cassettes are often constructed and manipulated in *E. coli* before they are introduced to yeast, where manipulations are more time-consuming. Recently developed technologies for faster and more advanced cloning include Golden Gate cloning developed by Engler et al. (2009) and Gibson Assembly by Gibson et al. (2009). The most widely used yeast transformation protocol of today is the lithium acetate method by Gietz et al. (1992).

S. cerevisiae is known for the outstanding efficiency of homologous recombination (HR), a feature which is frequently utilized during genetic engineering of it. In HR, nucleotide sequences are exchanged between two similar or identical molecules of DNA. In nature this mechanism is used by cells to accurately repair harmful breaks of DNA and to produce new combinations of DNA sequences during meiosis. The RAD52 group of proteins are responsible for the occurrence of HR events and Rad51 plays a key role in finding the homology and initiating the strand invasion (Shinohara et al. 1992). Transcriptional activity may induce HR (Keil and Roeder 1984). Ku-dependent non-homologous end-joining is another mechanism for DNA repair that functions in parallel with HR. For a recent review on homologous recombination in yeast, see Karpenshif and Bernstein (2012).

Homologous recombination is the most commonly used method for creating modifications such as deletions, replacements or insertions in the yeast chromosome. In this method, the target ORF is replaced with a genetic marker. For example a gene encoding a protein to make an auxotrophic strain prototrophic i.e. capable of synthesising a specific amino acid and thus able to grow in medium lacking this amino acid, may be used as a marker. Deletions are commonly constructed by transformation with a linearized plasmid or DNA fragment with the marker gene and sequences homologous to the regions flanking the target ORF. The Cre-Lox recombination system, first described in *S. cerevisiae* by Sauer (1987), allows genetic marker recycling by removing a marker gene flanked with LoxP sites. The Cre recombinase recombines the DNA sequence of two proximate LoxP sequences, cleaving out the sequence between the loxP sites. Another system similar to the Cre-lox is the Flp-FRT recombination technology based on the Flp recombinase (Schlake and Bode 1994).

Ma et al. (1987) first described plasmid construction by HR in yeast. In this method, yeast cells are transformed with a linearized plasmid and a DNA fragment containing sequences which are homologous to allow HR to occur with the linear plasmid and to create a circular plasmid. HR in *S. cerevisiae* is very efficient, allowing for several recombination events to occur concurrently and yeast HR has even been used to assemble a *de novo* synthesized genome, the *Mycoplasma mycoides* JCVI-syn1.0 genome (Gibson et al. 2010).

Yeast artificial chromosomes (YACs) are large vectors for cloning megabase-sized DNA fragments (Murray and Szostak 1983). YACs can be maintained as small circular plasmids in *E. coli*, but they are linearized and ligated together before being used for transformation of yeast. YACs contain an ARS (autonomously replicating sequence), CEN (centromere) and two TEL (telomers) elements and behave as natural yeast chromosomes. YACs can be modified by homologous recombination and retrofitted (modified) for a variety of different organisms. YACs are well established instruments for the study of eukaryotic genomes and valuable tools for synthetic biology (Partow 2012).

1.3.2 Fluorescent biosensors for monitoring bioprocesses – new tools for cell biology

Fluorescence is the emission of light by a substance that has previously absorbed light or other electromagnetic radiation. Fluorescence can be used for analysing and tracking biological components such as molecules, proteins or cells. Many compounds are autofluorescent; they naturally emit fluorescence. Other compounds can be visualized using light-emitting stains such as fluorescently labelled antibodies or fluorescent molecules that bind to certain structures. Examples of commonly used fluorescent stains include propidium iodide that binds to nucleic acids, and cyanine dyes, like Cy3 and Cy5 that can be used to label proteins.

Fluorescent proteins (FPs) have the unique property of being able to form a chromophore (called fluorophore) and thus being detectable by light illumination. The most studied FP, the green fluorescent protein (GFP) was first purified from *Aequorea Victoria* in the 1960s (Shimomura et al. 1962) but was not cloned until 1992 (Prasher et al. 1992). The first studies with GFP fused to other proteins, in which GFP was used as a molecular probe, were published in the late 1990s (Chalfie et al. 1994). After this, FPs and fluorescent microscopy have revolutionized and also become routine tools of cell biology.

When FPs are excited with light at a for the FP specific wavelength, the FP absorbs photons from the light and an electron in the fluorophore is raised to an excited state. After this, part of the energy of the electron is dissipated by molecular collisions or transferred to a proximal molecule, while the rest of the energy is emitted as a photon, with lower energy wavelength light. The fluorescence of the photon can be measured after it is distinguished from the excitation light with an emission filter that attenuates all of the light transmitted by the excitation filter. The excitation filter transmits only those wavelengths of the illumination light that efficiently excites the photon of the fluorophore in use. FPs have been genetically enhanced to be diversely coloured, more stable or faster folding. Today, many FPs from various organisms have been identified and isolated and simultaneous monitoring of several proteins fused to different FP's proteins (yellow, cyan or red FPs being the most common) is possible if they emit light at different wavelengths. Microscopic imaging allows cells to be studied during cultivation by measuring the

fluorescent signals from FP sensors. For a review on the use of FPs in yeast, see Bermejo et al. (2011).

Recent developments in biosensors include fluorescent reporters which indicate cellular ATP (Berg et al. 2009, Imamura et al. 2009) and NAD(H) (Hung et al. 2011, Zhao et al. 2011) levels. The pH sensitive GFP mutant, called pHluorin was developed to assess cytosolic pH in single cells (Miesenböck et al. 1998, Maresová et al. 2010) and has been used to measure intracellular pH in yeast (Orij et al. 2009, Orij et al. 2012, Ullah et al. 2012, Zdraljevic et al. 2013, Valkonen et al. 2013), filamentous fungi (Bagar et al. 2009), bacteria (van Beilen and Brul 2013) and mammalian cells (Miesenböck et al. 1998).

Zdraljevic and co-workers (2013) have shown that production of D-xylonate led to a progressive acidification of the cytosol. When the intracellular pH is below 5 the fluorescence from pHluorin is lost and the fluorescence of the cells cannot be distinguished from autofluorescence (Zdraljevic et al. 2013), probably due to unfolding of the protein and loss of fluorescence, as was shown for GFP and many of its derivatives, at a pH < 5.5 (Ward 2005).

Many studies have used fluorescent markers on population level, but a number of studies have demonstrated great differences between genetically identical cells that share a common environment (Colman-Lerner et al. 2005, Sachs et al. 2005, Lin et al. 2012, Zdraljevic et al. 2013, Valkonen et al. 2013). In a population of genetically identical cells, individual cells exhibit a range of responses to stress, and the average for the population may be decided by rare cells which have large responses (Shah et al. 2013). Population-averaged data are subject to systematic errors: although one can reliably infer qualitative trends, it is difficult to generate precise, quantitative conclusions from such experiments (Warren 2008), and even apparent sub-groups may remain unnoticed. Therefore, it is valuable to study cellular physiology at the single cell, as well as at the population level.

1.4 Biomass and D-xylose as a raw material for chemicals

Lignocellulosic biomass such as straw, corn stover, sugarcane bagasse or wood, typically contains approximately 30–45% cellulose, 20–30% hemicellulose and 15–25% lignin. In contrast, many fruit, and in particular citrus peel, contains large amounts of pectin and only small amounts of lignin (for a review on plant biomass composition, see Edwards and Doran-Peterson 2012).

Cellulose is nature's most common renewable organic compound and it is widely used in paper and pulp production. Cellulose is a polysaccharide of β-(1,4)-linked D-glucose units. Hemicellulose is a heteropolymer that contains various portions of branched sugar monomers, the most abundant being the pentoses D-xylose and L-arabinose. Hemicellulose also contains various amounts of hexoses, mainly D-glucose, D-mannose and D-galactose. Lignin is a complex and heterogeneous, aromatic biopolymer that gives plants their strength. Due to its high energy value, lignin is most commonly burned, even though a wide range of applications for the valorisation of lignin have been developed.

The hemicellulosic portion of biomass has not been as widely used as cellulose, but current interest in biorefinery concepts that integrate different biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass has led to a substantial increase in processes developed to include hemicellulose as a raw material. In order to achieve commercially viable biotechnological processes for conversion of lignocellulose waste, the utilization of both the hemicellulosic as well as the cellulosic portion of the biomass must be achieved. For recent reviews on (enzymatic) hydrolysis of lignocellulosic biomass, see Wahlström (2014) or Rahikainen (2013).

Hydrolysis of hemicellulose leads to release of D-xylose, L-arabinose, D-mannose, acetic acid, D-galactose, and D-glucose. D-xylose can be produced from hemicellulose by several different techniques, such as steam treatment and acid or alkaline hydrolysis, or by chemical or enzymatic means (Olsson and Hahn-Hägerdal 1996, Lachke 2002, Rahikainen, 2013). Many processes employ a combination of these methods and the preferred hydrolysis method is dependent on both the raw material and the production method or host. A considerable obstacle in bioconversions of lignocellulosic hydrolysates is that they do not only contain fermentable sugars, but also a wide range of compounds that may have inhibitory effects on the process (Olsson and Hahn-Hägerdal 1996, Mills et al. 2009). The inhibiting compounds include chemicals, like acids, added to the biomass for hydrolysis or compounds formed from the biomass when it is degraded during the hydrolysis. The composition of these inhibitory compounds and sugars released during the hydrolysis depends strongly on the raw material and hydrolysis process employed.

During acid hydrolysis, D-xylose can be further degraded to furfural under high temperature and pressure (Dunlop 1948), whereas D-glucose, D-galactose and D-mannose can be degraded to 5-hydroxymethyl furfural (5-HMF) (Ulbricht et al. 1984). When furfural is broken down in the hydrolysis, formic acid is formed, whereas 5-HMF breaks down to levulinic acid (Dunlop 1948, Ulbricht et al. 1984). The presence of 5-HMF and furfural may reduce the intracellular concentrations of NADH and NADPH, thus altering metabolism (Ask et al. 2013).

Formic, acetic and levulinic acid are weak organic acids that are inhibitory to yeast due to the stress of maintaining the cytosolic pH and the accumulation of anions. In addition, lignin breakdown generates inhibitory compounds, including a variety of phenolic compounds. Phenolic compounds can enter and break membranes in cells (Heipieper et al. 1994). For a review on the compounds present in lignocellulosic hydrolysates and inhibition of bioprocesses, see Palmqvist and Hahn-Hägerdal (2000) or Mills et al. (2009).

1.4.1 D-xylose – a renewable raw material for biorefineries

D-xylose is the second most abundant monosaccharide in the world, being found in lignocellulose, within most plant cell walls, and thus in large amounts in hydrolysates made in agricultural and industrial process waste streams. The amount of D-xylose in agricultural residues such as cereal straws and husks can be as high

as 31% of the dry weight. In hardwoods such as birch and oak, D-xylose content is about 17% of the total dry weight (reviewed by Jeffries and Shi 1999).

D-xylose is not yet widely used as a substrate in industrial processes, but developments of applications for D-xylose utilization have been substantial. Many bacteria, archaea and yeast are naturally able to metabolise D-xylose, and metabolic engineering has enabled D-xylose conversion by many host organisms, which do not metabolise D-xylose naturally, including *S. cerevisiae*. D-xylose can be converted to biomass or products such as organic acids or alcohols. However, even after extensive research and great improvements, the conversion rates and yields of e.g. ethanol from D-xylose are still often low, compared to those obtained when using D-glucose as a substrate. With respect to the yield, theoretically the most advantageous products from D-xylose are xylitol, D-xylonic acid and lactic acid (Buchert and Viikari 1988, Ilmén et al. 2007).

The conversion of D-xylose into ethanol has received a lot of attention because of the incentives and regulations which have been implemented to incorporate bioethanol in transportation fuel. The second generation ethanol production processes utilize biomass unfit for food production and the fermentation of pentoses has been significantly improved both in terms of rates and yields. Both evolved and genetically engineered *S. cerevisiae* can metabolize D-xylose to ethanol (for reviews, see Toivola et al. 1984 and Cai et al. 2012).

1.4.2 D-xylose metabolism

There are several different microbial D-xylose utilization pathways (Fig. 4). Bacteria and also some fungi can convert D-xylose to D-xylulose using D-xylose isomerases (Lawlis et al. 1984, Harhangi et al. 2003). After phosphorylation D-xylulose enters the pentose phosphate pathway (PPP) and is further converted to pentoses which are used in the synthesis of nucleotides, nucleic acids and aromatic amino acids. The oxidative part of the PPP is the major source of NADPH in cells and growth on D-xylose requires oxygen, for regeneration of NAD^+ through oxidative phosphorylation. NADPH is used in reductive biosynthesis reactions.
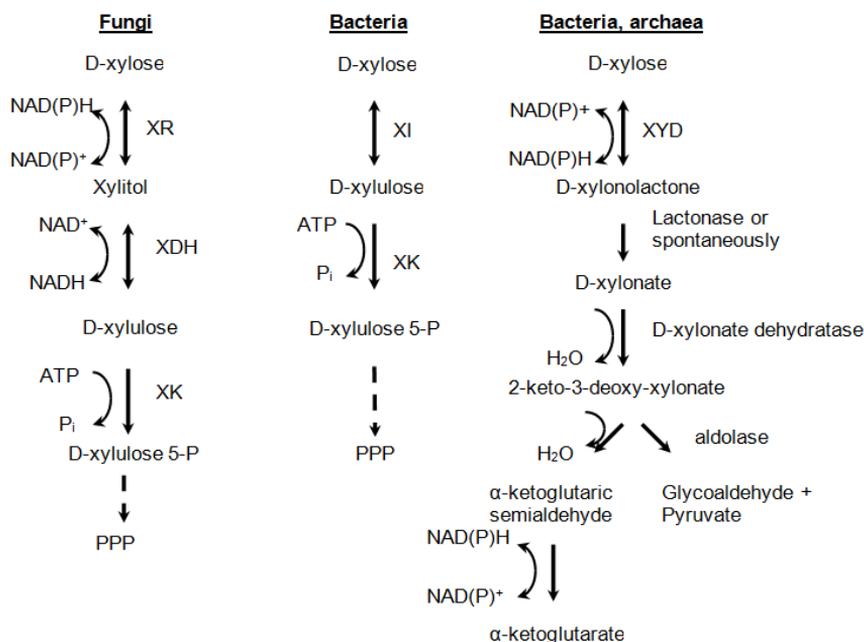
Figure 4. D-xylose metabolic pathways of fungi, bacteria or archaea. Figure is based on Figures shown by Hahn-Hägerdal et al. (2007) and Meijnen et al. (2009). XR = D-xylose reductase; XDH = xylitol dehydrogenase; XK = D-xylulokinase; XI = D-xylose isomerase; XYD = D-xylose dehydrogenase.

Many ascomycetous fungi, such as species belonging to the genera *Kluyveromyces*, *Candida* and *Trichoderma* (*Hypocrea*), aerobically convert D-xylose into xylitol and this reduction is most commonly carried out by a NADPH-linked D-xylose reductase (XR). The xylitol is then oxidized to D-xylulose by a NAD$^+$ linked xylitol dehydrogenase (XDH) and the D-xylulose is subsequently phosphorylated by D-xylulokinase (XK) before it enters the PPP (Fig. 4, Billard et al. 1995, Lachke 2002, Hahn-Hägerdal et al. 2007).

Some bacteria and archaea such as *Haloarcula marismortui* (Johnsen and Schönheit 2004), *Caulobacter crescentus* (Stephens et al. 2006) and *Haloferax volcanii* (Johnsen et al. 2009) break down D-xylose using the oxidative Weimberg pathway (Fig. 4). In this pathways, D-xylose is converted into D-xylono-γ-lactone by an NAD(P)$^+$ dependent D-xylose dehydrogenase (XYD) and the D-xylono-γ-lactone is converted into D-xylonate spontaneously (at neutral pH) or by a lactonase (Buchert and Viikari 1988). Both *C. crescentus* (Stephens et al. 2006) and *H. volcanii* (Johnsen et al. 2009) have a lactonase in the same operon with their D-xylose dehydrogenase encoding genes.

D-xylonate is converted into 3-deoxy-D-glycero-pentulosonic acid (2-keto-3-deoxy-D-xylonic acid) by a dehydratase (Dahms 1974). In the Dahms pathway, the 2-keto-3-deoxy-D-xylonic acid is split by an aldolase into glycoaldehyde and

pyruvate (Dahms 1974), whereas in the Weimberg pathway the 2-keto-3-deoxy-D-xylonic acid is converted into α-ketoglutaric semialdehyde by a second dehydratase (Weimberg 1961). This α-ketoglutaric semialdehyde is then further oxidized by a NAD^+ dependent dehydrogenase into α-ketoglutarate.

1.4.3   D-xylose transport in fungi

Yeast that are naturally capable of D-xylose utilization typically have two different transport systems: one of which is specific for D-xylose and another which has greater affinity for D-glucose (reviewed by Jeffries and Jin 2004). D-xylose specific transport, by which D-xylose is transported against a concentration gradient, requires energy (Jeffries and Jin 2004).

  S. cerevisiae transports D-xylose through hexose transporters that have much higher affinities for D-glucose than for D-xylose (Saloheimo et al. 2007). It was previously thought that only trace amounts of D-xylose gets into a cell when D-glucose is present, but later studies have shown that the D-xylose uptake rate is dependent on the D-glucose concentration of the medium (Bertilsson et al. 2008). At D-glucose concentrations below 5 g l^{-1}, the D-xylose uptake rate of *S. cerevisiae* increases exponentially, relative to the decreasing D-glucose concentration of the medium (Bertilsson et al. 2008). Since D-xylose is transported into the cell most efficiently using D-glucose inducible transporters, optimal D-xylose uptake occurs at low, but non-zero, D-glucose concentrations (Bertilsson et al. 2008).

1.4.4   *S. cerevisiae* strains engineered to utilize D-xylose

The desire for using the non-food part of plant biomass as a raw material for bio-fuels and bio-based chemicals has led to the development of several D-xylose utilizing *S. cerevisiae* strains. Introduction of XI (Kuyper et al. 2003) or XDH and XR encoding genes (Kötter et al. 1990, Kötter and Ciriacy 1993) has enabled *S. cerevisiae* to grow on D-xylose. Either of these modifications, together with the overexpression of the endogenous *XKS1* gene encoding D-xylulokinase (Ho et al. 1998, Toivari et al. 2001) has enabled *S. cerevisiae* to ferment D-xylose to ethanol. Adaptation and random mutagenesis approaches have also been employed in order to improve D-xylose utilization in *S. cerevisiae*.

  Co-factor imbalance (Bruinenberg et al. 1983), insufficient capacity of the PPP for D-xylulose conversion (Walfridsson et al. 1996, Karhumaa et al. 2005), a limited rate of ATP production (Sonderegger et al. 2004), as well as poor D-xylose uptake (Gárdonyi et al. 2003) have been suggested to be limiting factors in efficient D-xylose conversion by *S. cerevisiae*. Overexpression of heterologous D-xylose transporters has so far not greatly improved cell growth on D-xylose or specific ethanol productivity in D-xylose-utilizing strains. For a recent review on metabolic engineering of *S. cerevisiae* for D-xylose fermentation, see Cai et al. (2012).

1.5   D-xylonate – an industrial platform chemical with the potential to be produced by microorganisms

D-xylonic acid, derived from the hemicellulose sugar D-xylose, has applications similar to D-gluconic acid that is produced by oxidation of D-glucose. D-gluconic acid serves a wide range of applications in the food, chemical, construction and pharmaceutical industries (reviewed by Ramachandran et al. 2006). D-xylonic acid could serve as a non-food carbohydrate D-gluconic acid substitute. In addition, there are several reported and patented applications for D-xylonate, including in dispersal of concrete (Chun et al. 2006), in the production of co-polyamides (Zamora et al. 2000, Chun et al. 2006) and as a precursor for 1,2,4-butanetriol synthesis (Niu et al. 2003), though currently there is only limited commercial production of D-xylonate.

D-xylose can be oxidized to D-xylono-γ-lactone by D-xylose dehydrogenases or glucose oxidases (Fig. 5), found in a wide range of species across the kingdoms of life. Glucose oxidases are oxido-reductases that catalyses the oxidation of D-glucose to D-glucono-γ-lactone. The D-xylono-γ-lactone hydrolyses spontaneously in aqueous solution or is hydrolysed to D-xylonate by a lactonase (Buchert and Viikari 1988).
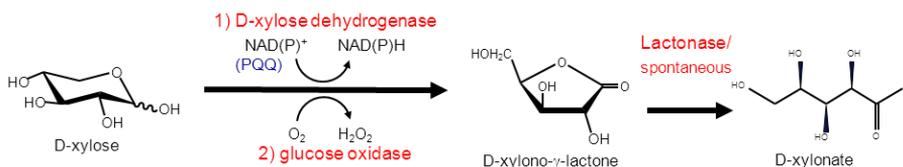
Figure 5. The reaction from D-xylose via D-xylono-γ-lactone to D-xylonate.

XYD encoding genes have been identified in the freshwater bacterium *C. crescentus* (Stephens et al. 2006), the filamentous fungus *Hypocrea jecorina* (*Trichoderma reesei*), (Berghäll et al. 2007), the archaea *H. morismortui* (Johnsen and Schönheit 2004) and *H. volcanii* (Johnsen et al. 2009) and from *Sus domesticus* (pig liver, Zepeda et al. 1990). Moreover, D-xylose dehydrogenase activity has been observed in *Trichoderma viride* (Kanauchi and Bamforth 2003).

D-xylonate is produced by various bacteria in particular *Gluconobacter oxydans* and *Pseudomonas putida* (reviewed by Buchert 1990) and also by some yeast (Kiesling et al. 1962, Suzuki and Onishi 1973). Toivari et al. (2010) were the first to describe D-xylonate production as a result of metabolic engineering. In this study, *S. cerevisiae* expressing the *xyd1* D-xylose dehydrogenase encoding gene from *T. reesei* produced 3.8 g D-xylonate l^{-1}, at rates between 25 and 36 mg D-xylonate l^{-1} h^{-1} (Toivari et al. 2010). In addition, production of D-xylonate by genetically engineered *E. coli* was also recently reported (Liu et al. 2012, Cao et al. 2013).

Obstacles to using *E. coli* or other bacteria for D-xylonate production are the requirement of these species for neutral pH and their inhibition by high concentrations of inhibitory compounds found in lignocellulosic hydrolysates (reviewed by Buchert et al. 1988). In *Pseudomonas* and *Gluconobacter* species, the enzymes responsible for D-xylonate production have wide substrate specificity, and therefore convert not only D-xylose but many sugars and sugar alcohols possibly present in hydrolysates to the corresponding acids (Buchert 1990).

Enzymatic conversion of D-xylose to D-xylonate by D-glucose oxidases from *A. niger* (Pezzotti and Therisod 2006) or *G. oxydans* (Chun et al. 2006) has also been described.

1.6   Aims of study

The aims of this thesis were to increase D-xylonate production by yeast, to better understand the stress responses related to the production of a weak organic acid and to determine pH tolerance and resistance to weak organic acids. The aim of the study on the role of Pdr12 in weak organic acis tolerance was to determine how the tolerance to acetic, formic, sorbic, propionic, glycolic, lactic and levulinic acid was altered when Pdr12 was deleted or overexpressed in *S. cerevisiae*.

Another goal was to determine the role of a lactonase in the production of D-xylonic acid in yeast. In relation to this, new techniques in single cell analysis of D-xylonate production and *in vivo* measurement of intracellular D-xylonate and D-xylono-γ-lactone concentrations were applied and further developed in this work.

In order to further increase D-xylonate production, an industrial *S. cerevisiae* strain, *Kluyveromyces lactis*, and *Pichia kudriavzevii* were engineered to produce D-xylonate.

2.   Materials and methods

2.1   Yeast strains used in this work

Sequences of reference strains, for strains (Table 2) used in this work are publicly available: for *S. cerevisiae* at Saccharomyces Genome Database www.yeastgenome.org, for *K. lactis* at the Genolevures database: http://genolevures.org/klla.html and for *P. kudriavzevii* at DDBJ/EMBL/GenBank under the accession ALNQ00000000 (Chan et al. 2012).

Table 2. Strains used and referred to in this thesis.

Strain name	Strain number; parental strain (+ plasmid (B-number))	Gene over-expressed, origin of gene	Publi-cation
Kluyveromyces lactis			
reference strain	H3632		I, IV
xyd1	H3677*; xyd1*	*xyd1, Trichoderma reesei*	I
xyd1 ∆XYL2	H3763; H3632∆*xyl2::KMX, xyd1*	*xyd1, Trichoderma reesei*	I
xyd1 ∆XYL1	H3765; H3632∆*xyl1::KMX, xyd1*	*xyd1, Trichoderma reesei*	I
Saccharomyces cerevisiae			
parent, reference strain, CEN.PK 113-17A	H2802; MATα, *ura3*-52 *HIS3 leu2*-3, 112 *TRP1 MAL2*-8c *SUC2*		II
reference strain	H3680; H2802 + B1181 (empty vector)		II
xylB, xylB	H3698; H2802 + B3441 (*xylB*)	*xylB, Caulobacter crescentus*	II
SUS2DD	H3700; H2802 + B3443 (*SUS2DD*)	*SUS2DD, Sus domesticus*	II
xyd1	H3725; H2802 + B2871 (*xyd1*)	*xyd1, Trichoderma reesei*	II, *ud*

	H3734; H2802 + B3575 (*rrnAC3034*)	rrnAC3034, *Haloarcula marismortui*	*ud*
	H3779; H2802 + B3694 (xylB)	N-Strep-tag- *xylB, Caulobacter crescentus*	II
xylB xylC	H3938; H2802 + B3441 + B3574	*xylB, xylC, Caulobacter crescentus*	II
parent, reference strain, CEN.PK 2-1D	H1346; MATα, *leu2*-3/112 *ura3*-52 *trpl*-289 *his3*Δ1 *MAL2*-8c *SUC2*		V
Δ*cmk1* + p*PDR12*	H4129; H1346Δ*cmk1*::*TRP1* + pPDR12	*PDR12, Saccharomyces cerevisiae*	V
R + p*PDR12* (reference + pPDR12)	H4130; H1346, *TRP1* + pPDR12	*PDR12, Saccharomyces cerevisiae*	V
Reference (R)	H4131; H1346, *TRP1* + B2158		V
Δ*cmk1*	H4132; H1346Δ*cmk1*::*TRP1* + B2158		V
Δ*pdr12*	H4133; H1346Δ*pdr12*::*LEU2*, TRP1		V
Δ*pdr12*Δ*cmk1*	H4134; H1346Δ*pdr12*::*LEU2*; Δ*cmk1*::*TRP1*		V
xylB	H3817; H2802Δ*gre3*::*xylB*	*xylB, Caulobacter crescentus*	VI
xylB xylC	H3862; H2802Δ*gre3*::*xylB*, Δxks1*::*xylC	*xylB, xylC, Caulobacter crescentus*	VI
xylB	H4221; H3817*, LEU2*:mCherry, *URA3*::pHluorin	*xylB, Caulobacter crescentus*	VI
xylB xylC	H4306; H3862, *LEU2*:mCherry, *URA3*:pHluorin	*xylB, xylC, Caulobacter crescentus*	VI
xylC	H4306; H3900Δ*xks1:KanMX*	*xylC, Caulobacter crescentus*	VI
B-67002 *xylB*, B-67002 xylB	H3935; VTT B-67002, *Gre3*::*xylB*, *Gre3*::*xylB*	*xylB, Caulobacter crescentus*	II,IV
B-67002	VTT- B-67002 (industrial origin)		
Pichia kudriavzevii			
	VTT C-79090T		IV
	VTT C-05705		IV
	VTT C-75010		IV
	VTT-C-12903; VTT C-79090T, xylB	*xylB, Caulobacter crescentus*	IV

* ud = unpublished data

2.2 Media and culture conditions

Yeast peptone (YP), modified yeast synthetic complete (SC, Sherman et al. (1983)) or the defined medium described by Verduyn et al. (1992) was used in this work, as indicated in the results.

For small scale cultures, yeast were grown in either 20 or 50 ml medium in 100 or 250 ml Erlenmeyer flasks, respectively, with 250 rpm shaking at 30°C or in glass tubes (height: 30 cm, diameter: 4 cm), rotated at ~30° angle. For larger scale cultures, yeast were grown in in Multifors bioreactors (max. working volume 500 ml, Infors HT, Switzerland). The maximum specific growth rate of the strains was calculated from optical cell density measurements of the cultures during growth on D-glucose. Growth curves were automatically measured with the Bioscreen analyser (Bioscreen C MBR automated turbidometric analyser, Growth Curves Ltd., Finland) or as increase in CO_2 in bioreactor cultures.

2.3 Metabolite analyses

Intra- and extracellular metabolites (D-xylose, D-glucose, ethanol, D-xylonate, D-xylono-γ-lactone and xylitol) were analysed by HPLC, ^{1}H NMR spectroscopy and by the hydroxamate method described by Lien (1959). When D-xylose was present in the sample, D-xylonate could not be accurately determined using HPLC, therefore the D-xylonate concentrations were also measured using the colorimetric hydroxamate method. ^{1}H NMR spectroscopy is the only method that distinguished between D-xylonate and D-xylono-γ-lactone.

For intracellular measurements, 10–20 ml of cells were collected by centrifugation, and washed twice with 20 ml 50 mM Na-acetate buffer, pH 6.8. When concentrations were measured by HPLC, the washed pellets were freeze-dried and re-suspended in 1 ml 5 mM H_2SO_4. The cell membranes were broken by freezing the cells. When concentrations were measured by ^{1}H NMR spectroscopy, the cell extracts were prepared with glass beads, as described by Berghäll et al. (2007).

2.4 Determination of vitality and viability

Viability was determined by comparing the number of viable colony forming units (CFU) on SCDX (SC with 20 g D-xylose l^{-1} and 10 g D-glucose l^{-1}), SCD (SC with 10 g D-glucose l^{-1}) or YPD (YP with 20 g D- glucose l^{-1}) medium to the total cell number determined microscopically or to the number of viable CFU in the control condition. The number of metabolically active (vital) cells was determined microscopically by methylene blue (0.25 g l^{-1} in 0.04 M Na-citrate buffer, pH 8.3 or in 0.1 M Na-phosphate buffer, pH 4.6, according to Painting and Kirshop 1990), or by propidium iodide (PI) (1 µg ml^{-1} (Invitrogen, UK)) staining as described by Zdraljevic et al. (2013).

2.5   Enzyme activity measurements

D-xylose dehydrogenase (XYD) activity was measured from crude cell extracts of cells washed twice in 25 ml 10 mM Na-phosphate buffer, pH 7 and broken with glass beads in either 10 mM Na-phosphate buffer, pH 7 or 50 mM Na-acetate buffer, pH 5. Buffers for cell lysis were supplemented with protease inhibitor (cOmplete Mini EDTA-free, Roche) according to the manufacturer's instructions. Spectrophotometric activity was determined according to Berghäll et al. (2007), in 100 mM Tris/HCl, pH 8 or pH 6.8, in the presence of 2 mM $MgCl_2$ using either 1 mM NAD^+ or $NADP^+$ as cofactor and 100 mM D-xylose as substrate. Protein concentration from crude cell extracts was determined using a Bio-Rad protein assay kit, based on the method developed by Bradford (1976).

The production of D-xylono-γ-lactone by xylB and subsequent hydrolysis of the lactone by xylC was monitored over time *in vitro* by ^{1}H NMR spectroscopy in 100 mM Na-phosphate buffer at pH 6.8 or 50 mM Na-acetate buffer at pH 5, using 400 ml cell extract, 3 mM D-xylose, 5 mM NAD^+, 1.5 mM $MgCl_2$ and 60 ml D_2O.

2.6   pHluorin as a tool for determining acidified cells

The decrease in cytosolic pH below pH 5 causes loss of fluorescence from pHluorin (Zdraljevic et al. 2013), and this feature was used to determine acidification of D-xylonate producing cells. For this application the method is very reliable, and relative changes in pHluorin intensity and ratio of pHluorin fluorescence measured at 410/470 nm can be correlated to a change in pH. When pHluorin is expressed constitutively, the level of probe expression due to different amount of integrated pHluorin copies, does not affect measurement of intracellular pH or acidification due to loss of fluorescence of the probe. An "acidified" cell (pH < 5) was defined as a cell the fluorescence of which measured at 410 nm, was similar to the auto-fluorescence of corresponding cells expressing no fluorescent protein, incubated in the same condition.

For fluorescent imaging, cells from pre-cultures were mounted to concanavalin A coated glass bottom 384 well plates, and washed repeatedly to remove un-bound cells, as described by Zdraljevic et al. (2013). The cells were imaged manually using an inverted fluorescent microscope with controlled stage and shutters, in a room maintained at 30C°.

3.   Results and discussion

The feasibility of D-xylonate production in yeast was demonstrated by Toivari et al. (2010). In this study *S. cerevisiae* expressing *T. reesei xyd1* produced 3.8 g D-xylonate l^{-1}.

The aim of this thesis was to increase D-xylonate and to deepen the understanding of D-xylonate production, pH homeostasis and acid resistance (section 1.6). This was done by constructing new strains that expressed four different genes encoding D-xylose dehydrogenases and evaluating their ability to produce D-xylonate. The physiological consequences of D-xylonate production on the host, as well as pH homeostasis and acid resistance were studied in detail in the *S. cerevisiae* CEN.PK2 strain, expressing the *xylB* D-xylose dehydrogenase encoding gene from *C. crescentus*. D-xylonate production was also studied in alternative yeast strains: *K. lactis*, *P. kudriavzevii* and an industrial *S. cerevisiae* strain.

3.1   Production of D-xylonate with *S. cerevisiae* CEN.PK lab strains

High D-xylonate yields were obtained by choosing an efficient D-xylose dehydrogenase (XYD) and with minor process optimizations (II). However, during production, D-xylonate accumulated intracellularly and led to a decrease in vitality and viability of the production hosts (I–IV, VI). In order to understand the consequences of D-xylonate production on the physiology of the host, D-xylonate production and pH homeostasis was studied at the single cell as well as population level in *S. cerevisiae* (II, VI).

D-xylose is converted to xylitol by the endogenous Gre3 aldose reductase in *S. cerevisiae*. However, deletion of *GRE3,* did not substantially affect D-xylonate production in *S. cerevisiae* expressing *xylB* (II), therefore *GRE3* was not generally deleted in strains expressing a D-xylose dehydrogenase encoding gene from a plasmid (II). However, xylB was integrated to the GRE3 locus of integrant strains (VI).

3.1.1 Selection and activity of D-xylose dehydrogenase enzymes

Only a few XYDs have been characterised (Zepeda et al. 1990, Johnsen and Schönheit 2004, Berghäll et al. 2007, Johnsen et al. 2009, Stephens et al. 2006) – these are primarily from bacteria or archaea, with known oxidative D-xylose metabolism. The activities of four NAD(P)$^+$-dependent XYDs in *S. cerevisiae* CEN.PK 113-17A strain were compared. The activity of *T. reesei* xyd1 in *S. cerevisiae* was compared with the activity of this enzyme in *K. lactis* (Table 3). The activity of *C. crescentus* xylB was measured from *S. cerevisiae* and *P. kudriavzevii* cell extracts.

Table 3. Specific activity of different heterologous D-xylose dehydrogenases in different yeast species (*n* = 3–6).

MC = gene expressed from a multicopy plasmid, I = gene integrated to genome.
+ = 0-5 g l^{-1}, ++ = 5-20 g l^{-1}, +++ = 20+ g l^{-1}

Source organism and gene	Expression host	Expression	Co-factor	Activity (nkat/ mg protein)	D-xylonate production
T. reesei xyd1[1]	*S. cerevisiae*	MC	NADP$^+$	2.0 ± 0.8	+
T. reesei xyd1[1]	*K. lactis*	I	NADP$^+$	4.2 ± 0.2	+
Sus domesticus SUS2DD[2]	*S. cerevisiae*	MC	NADP$^+$	1.3 ± 0.1	+
H. marismortui rrnAC3034[3]	*S. cerevisiae*	MC	NADP$^+$	0.00	-
C. crescentus xylB[4]	*S. cerevisiae*	MC	NAD$^+$	45.4 ± 3.0	++
C. crescentus xylB[4]	*S. cerevisiae*	I	NAD$^+$	33.6 ± 2.5	++
C. crescentus xylB[4]	*P. kudriavzevii*	I	NAD$^+$	2.0 ± 0.2	+++

Superscripts indicate the reference for the XYD encoding gene:
[1] Berghäll et al. (2007)
[2] Zepeda et al. (1990)
[3] Johnsen and Schönheit (2004)
[4] Stephens et al. (2006)

In *S. cerevisiae*, the activity of the NAD$^+$ dependent xylB was much higher when compared to the NADP$^+$ dependent enzymes. It may be that NAD$^+$ was a more favourable co-factor, in *S. cerevisiae*, or that this enzyme was more stable and thus gave a higher activity (Table 3). The activity of xyd1 was slightly higher in *K. lactis* than in *S. cerevisiae*, even though *xyd1* was expressed in *K. lactis* from a single integrated gene and not from a multicopy plasmid, as in *S. cerevisiae*. The

activity of xylB in *S. cerevisiae* was ~35% higher when *xylB* was expressed from a multicopy plasmid compared to when a single copy of *xylB* was integrated to the genome (Table 3). Notably, the activity of *C. crescentus* xylB was much lower in *P. kudriavzevii* when compared to *S. cerevisiae* CEN.PK 113-17A. This may reflect differences in promoter strength, or a limited suitability of *S. cerevisiae* codons for high-level expression in *P. kudriavzevii*.

XylB was purified from *S. cerevisiae* using an N-terminal Strep-tag, and its kinetic properties and pH optimum were determined. *In vitro*, xylB was most active at pH 9, and a high activity was detected from pH 7 to 10. At pH 5, the activity was reduced to 30% of the maximal. The purified enzyme had activity only with the pentose sugars D-xylose, L-arabinose and to a much lesser extent D-lyxose (measured at pH 9), but not with D-ribose, D-glucose, D-galactose or D-mannose. High specificity of xylB for D-xylose would be an important feature if lignocellulosic hydrolysates were to be used as raw material for D-xylonate production. Aldose reductases often have a broad substrate specificity (reviewed by Buchert 1990) and conversion of other biomass sugars into acids (mainly D-glucose into D-gluconate) would make separation of the product difficult.

D-xylose was clearly the best substrate for xylB and the specificity constant (k_{cat}/K_m) was 400–600 fold higher for D-xylose than for L-arabinose. The affinity of xylB for D-xylose ($K_m = 0.08$–0.40 mM at pH 6.8–9) was noticeably higher than that of xyd1 ($K_m = 43$ mM at pH 8.1, when purified from *S. cerevisiae*; Berghäll et al. 2007).

In vitro enzyme activity measurements are useful for determining that an enzyme can be successfully expressed and folded in a heterologous host. Co-factor and substrate availability as well as differences in pH, or concentration of salt or other compounds between *in vivo* and *in vitro* surroundings of the enzyme all have an effect on the specific activity of an enzyme.

Genetic features, such as promoter strength and regulation, and genomic localization also influence the expression level of an enzyme. The activity of an enzyme expressed from a multicopy plasmid is expected to be higher compared to the activity of an enzyme expressed from an integrated gene. However, optimal *in vivo* activity does not necessarily occur at the highest *in vitro* activity. Too high *in vivo* activity may have detrimental effects on the metabolism of the host and a lower activity may therefore improve fitness of the cells and thus productivity. Zdraljevic et al. (2013) showed that expression of *xylB* from a chromosomal locus led to a ~2-fold increase in D-xylonate production, compared to expression from a multicopy plasmid.

Introducing the *xyd1*, *xylB* or *SUS2DD* XYD encoding genes in *S. cerevisiae* led to conversion of D-xylose to D-xylonate (Table 3). In contrast, expressing the *Haloarcula marismortui* XYD encoding gene (rrnAC3034) led to neither measureable XYD activity nor D-xylonate production. It may be that this enzyme was not properly folded or that it was unstable.

3.1.2   Production of D-xylonate with different XYDs

S. cerevisiae strains expressing *xylB* and *SUS2DD* genes encoding XYDs were grown in bioreactor cultures at pH 5.5. The D-xylonate production was ~5 fold higher with the NAD^+ dependent xylB, compared to the $NADP^+$ dependent SUS2DD or xyd1 (Table 4).

Table 4. D-xylonate and xylitol production and D-xylonate production rate by *S. cerevisiae* expressing *xylB*, *xyd1* (Toivari et al. 2010), *SUS2DD* or containing an empty vector. Cells were grown in bioreactors with 23 ± 1 g D-xylose l^{-1} and 9 ± 1 g D-glucose l^{-1}, supplemented with 5 ± 1 g ethanol l^{-1} after ~54 h. Cultures were agitated at 500 rpm with 1 vvm aeration. Values are mean ± SEM (n = 2 or 3).

Strain	XYD	D-xylonate (g l^{-1})	xylitol (g l^{-1})	D-xylonate production rate (g l^{-1} h^{-1})
H3698	xylB	16.7 ± 1.8	2.7 ± 0.1	0.23 ± 0.02
H3725	xyd1	3.4 ± 0.3	4.8 ± 0.2	0.03 ± 0.00
H3700	SUS2DD	2.7 ± 0.1	4.5 ± 0.2	0.02 ± 0.00
H3680	-	0	4.4 ± 0.4	0

The reference strain produced no D-xylonate but similar amounts of xylitol as the strains expressing *SUS2DD* or *xyd1*, whereas the *xylB* expressing strain produced less xylitol compared to the other strains (Table 4). The *xylB* expressing strain produced D-xylonate at an approximately 10 times higher rate than observed with the strains with the $NADP^+$-dependent XYDs, xyd1 or SUS2DD (Table 4). All strains produced similar amounts of biomass, ~5 g l^{-1}.

The much higher production of D-xylonate, with the NAD^+ dependent xylB, compared to the $NADP^+$ dependent XYDs may reflect NAD^+ being more abundant in the cytosol compared to $NADP^+$. Yeast have a wide range of mechanisms for NAD^+ regeneration, including ethanol or glycerol formation or respiration of cytosolic NADH, via oxidative phosphorylation. $NADP^+$ is mainly formed in anabolic reactions, but NADPH can also be oxidized via the electron transport chain (Bruinenberg et al. 1983). However, the dissimilation of NADPH is subject to respiratory control (Bruinenberg et al. 1983) and $NADP^+$ concentrations in the cell are usually much lower than intracellular NAD^+ concentrations (Pollak et al. 2007). The cytoplasmic aldose reductase Ald6, encoded by *ALD6* which is constitutively expressed in *S. cerevisiae*, also uses $NADP^+$ as co-factor and thus competes with xyd1 (Toivari et al. 2010). Nevertheless, $NADP^+$ depletion was not found to be a main constraint in the production of D-xylonate with the $NADP^+$ dependent xyd1 enzyme from *T. reesei*, as expression of NADPH utilizing enzymes *udhA*, *gapB* or *GDH2* did not improve D-xylonate production in *S. cerevisiae* (Toivari et al. 2010).

3.1.3   Effect of xylC on D-xylonate production – *in vitro* activity measurements

D-xylose is oxidized to D-xylonate via D-xylono-γ-lactone. The D-xylono-γ-lactone hydrolyses either spontaneously or with the help of a lactonase (Buchert and Viikari 1988). The *xylXABCD* operon of *C. crescentus* (Stephens et al. 2006), from which the *xylB* XYD encoding gene originates also contains a D-xylonolactone lactonase encoding gene, *xylC* (Stephens et al. 2006). Co-expression of *xylB* with *xylC* was evaluated and XYD activities of the strains expressing *xylB* or *xylB* together with *xylC* were measured by ^{1}H NMR spectroscopy and spectrophotometer. D-xylonate and D-xylono-γ-lactone could be distinguished by ^{1}H NMR spectroscopy, thus this method allowed measurement of activity of both xylB and xylC, whereas the spectrophotometric assay only measures the formation of NADH, hence only the activity of xylB.

The results of both methods of measurement were qualitatively similar. At pH 6.8, the xylB and the xylB xylC strain had similar XYD activity, in all conditions and times tested (SCD (SC medium with 10 g D-glucose l^{-1}), SCDX (SC medium with 20 g D-xylose l^{-1} and 10 g D-glucose l^{-1}) and SCX (SC medium with 20 g D-xylose l^{-1})): 26 ± 1 nkat mg protein^{-1} in the xylB strain and 30 ± 1 nkat mg protein^{-1} in the xylB xylC strain, as determined spectrophotometricly. However, when measured by ^{1}H NMR spectroscopy, the conversion of D-xylose into D-xylono-γ-lactone was slightly faster in the xylB xylC strain compared to the strain with only xylB (Fig. 6). Moreover, D-xylono-γ-lactone was rapidly hydrolysed to D-xylonate in the xylB xylC strain. After 40 min incubation, very little D-xylono-γ-lactone remained, but D-xylonate was detected. In the xylB strain, 3 mM D-xylose was consumed before the sample was taken after ~7 h of reaction, but complete conversion of D-xylono-γ-lactone to D-xylonate required 48 h. Expression of xylC together with xylB led to a significantly increased rate of production of D-xylonate.
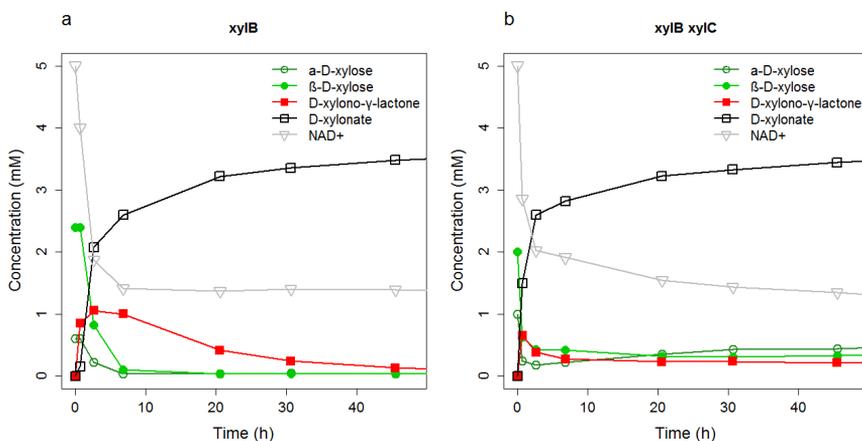
Figure 6. Conversion of D-xylose (green open (α-D-xylose) or closed (β-D-xylose) circles) to D-xylono-γ-lactone (red solid squares) by cell extracts of *S. cerevisiae* expressing *xylB* (a) or *xylB* and *xylC* (b) from *C. crescentus*, monitored by ^{1}H NMR spectroscopy. The production of D-xylono-γ-lactone by xylB and subsequent hydrolysis of the lactone to D-xylonate (black open squares), spontaneously or by xylC and consumption of NAD^{+} (grey triangles) was also monitored *in vitro* over time in 100 mM Na-Phosphate at pH 6.8, using 400 µl cell extract, 3 mM D-xylose, 5 mM NAD^{+}, 1.5 mM $MgCl_2$ and 60 ml D_2O.

The difference in NAD^{+} consumption seen between the the spectrophotometric and ^{1}H NMR assays could be explained by the different natures of the assays. Product inhibition probably didn't occur in the spectrometric assay, since the amount of D-xylono-γ-lactone produced in the time frame of the assay (6 min 36 s), measuring formation of NADH (max. 1 mM), was very small, too small to inhibit or compete with D-xylose (100 mM) for xylB. In the ^{1}H NMR spectroscopy assay, only 3 mM of D-xylose, but 5 mM NAD^{+} was provided, so that D-xylose was limiting and therefore competitive inhibition by D-xylono-γ-lactone could occur. In the xylB xylC extract, D-xylono-γ-lactone was rapidly hydrolysed to D-xylonate, eliminating product inhibition, enabling the activity of xylB in the xylB xylC strain to be greater compared to the activity in the strain lacking xylC lactonase. For technical reasons it was not possible to use the same substrate and co-factor concentrations in the ^{1}H NMR spectroscopy and spectrophotometric assays.

At pH 5, the difference between the D-xylose conversion rate between the xylB and xylB xylC strain was much more pronounced than at pH 6.8 (data not shown). Only D-xylonate but no D-xylono-γ-lactone was seen at pH 6.8 with the xylB xylC strain, but both were present at pH 5. Equal amounts of D-xylonate and D-xylono-γ-lactone were detected in the xylB xylC extract after 24 h. This suggested that the activity of xylC was more affected by the decreased pH, compared to the activity of xylB.

The higher *in vitro* XYD activity of the xylB xylC strain as determined by ^{1}H NMR spectroscopy, together with data on the intracellular accumulation and initial production with the xylB and xylB xylC strains (section 3.1.5) indicated that xylB may be inhibited by D-xylono-γ-lactone, but not by D-xylonate. However, when D-xylono-γ-lactone is converted to D-xylonate by the lactonase, the thermodynamic equilibrium is altered, and the reverse reaction by xylB, back to D-xylose is less favourable when D-xylonate concentrations are low. Proper characterization of xylC together with xylB is needed for making accurate conclusions on the features of these enzymes.

3.1.4 Growth on D-glucose in the presence of D-xylose is impaired in the *xylB* and *xylC* expressing strain

The co-expression of *xylC* with *xylB* significantly decreased the growth rate on D-glucose, in the presence of D-xylose (SCDX), compared to the strain expressing only *xylB*, only *xylC* or without any genetic modifications (Fig. 7a). In SCDX, the xylC and parent strains had specific growth rates of 0.19 h^{-1}, the xylB strain grew with a specific rate of 0.16 h^{-1} and the xylB xylC strain had a specific growth rate of only 0.05 h^{-1}. When grown on D-glucose, in medium lacking D-xylose, (SCD), all strains grew at a similar specific growth rate of 0.19 h^{-1}.
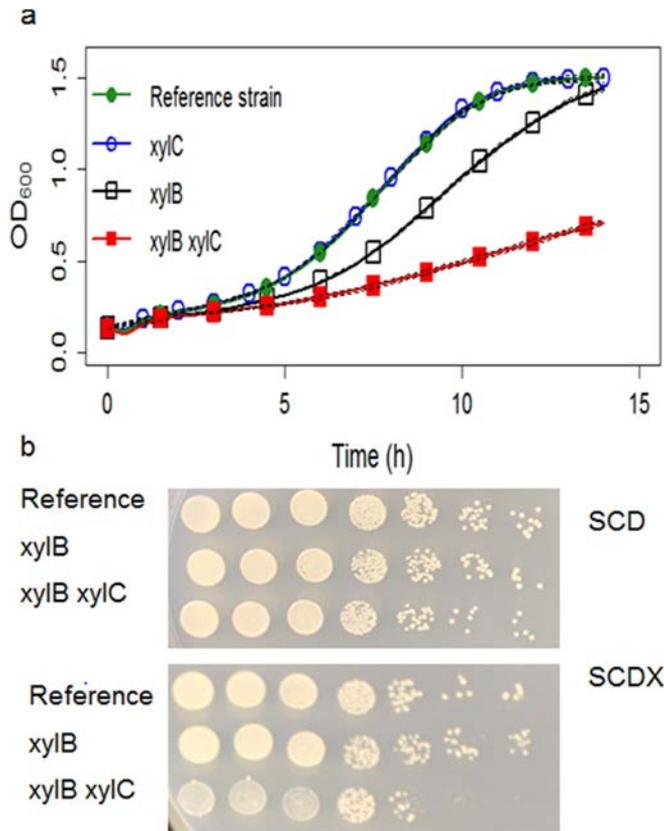
Figure 7. a) Growth curves of of *S. cerevisiae* expressing *xylB* (black open squeres), *xylB* and *xylC* (red solid squares) or *xylC* (blue open circles) from *C. crescentus* or without any genetic modification (reference strain, green solid circles), grown in SC medium containing 10 g D-glucose l^{-1} and 20 D-xylose l^{-1} (SCDX). Dashed lines represent mean ± SEM for 4 biological replicates. b) Serial dilutions of the reference, xylB and xylB xylC cells grown on agar solidified agar solidified SC medium containing 10 g D-glucose l^{-1} (SCD, above) or on SCDX (below) for 48 h.

The decrease in growth on D-glucose in medium with D-xylose showed that D-xylose was metabolized already while D-glucose was abundant (see section 3.1.6 for data on intracellular accumulation of D-xylonate during D-glucose con-sumption). Several studies show that D-xylose uptake and utilization is inhibited by the presence of D-glucose (Kötter and Ciriacy 1993, Boles and Hollenberg 1997, Krahulec et al. 2010, Subtil and Boles 2012), but it has also been shown that the uptake of D-xylose is dependent on the D-glucose concentration of the medium, being highest at low but non-zero D-glucose concentrations (Pitkänen et al. 2003, Bertilsson et al. 2008, Krahulec et al. 2010) and that the D-xylose uptake rate

would not be the limiting factor in D-xylose utilization, unless D-xylose utilization would be dramatically improved (Gárdonyi et al. 2003).

In order to test viability, comparable amounts of cells, measured as OD_{600}, were taken from overnight cultures in SCD, and inoculated as a dilution serie on agar solidified SCD or SCDX. When cells were transferred from SCD to SCDX agar, fewer xylB xylC colonies grew (76%) as compared to xylB and reference strains (100% growth, Fig. 7b). On SCD plates, all strains grew well (Fig. 7b). Thus, it appears that some of the xylB xylC cells were unable to divide when exposed to D-xylose. There was a great heterogeneity within the xylB xylC cell population, with many small colonies and a few normal sized colonies. All reference and xylB cells produced colonies of approximately similar size. The xylB cells grew well on SCDX plates (Fig. 4b). Taken together, the decreased growth rate of the xylB and xylB xylC strains was likely due to the decrease in vitality and viability of the D-xylonate producing cells (see section 3.1.6).

3.1.5 Production of D-xylonate by *xylB* and *xylB xylC* expressing strains

Production of acid at low pH is desirable, as this would reduce the need for neutralizing agents and make product recovery easier, thus lowering the production costs. Therefore, D-xylonate production at pH 3 was evaluated in addition to pH 5.5 that was previously used for D-xylonate production with *S. cerevisiae* (Toivari et al. 2010). D-xylonate was produced in bioreactors using medium with ~20 g D-xylose and 10 g D-glucose l^{-1}, with an addition of ~5 g ethanol l^{-1}, as described before (Toivari et al. 2010). The ethanol was added in order to provide additional energy for the D-xylose oxidation without inhibiting D-xylose uptake.

Expression of *xylB* D-xylose dehydrogenase encoding gene in *S. cerevisiae* led to a production of 16.7 ± 1.8 or 5.5 ± 0.9 g D-xylonate l^{-1} at pH 5.5 or 3, respectively. Co-expression of the *C. crescentus* D-xylono-γ-lactone lactonase encoding gene, *xylC*, along with *xylB* led to an increase in initial production of D-xylonate, both at pH 5.5 and at pH 3, compared to the strain lacking the lactonase. However, at pH 5.5, the total D-xylonate production by the xylB xylC strain was 21% lower than that of the parent strain expressing xylB alone (13.2 ± 2 g l^{-1} and 16.7 ± 1.8 g l^{-1}, respectively), even though the initial D-xylonate production (during the first 50 h of cultivation) had been greater in the xylB xylC strain compared to the xylB strain (Fig. 8a). After ~50 h, D-xylonate production by xylB xylC essentially stopped, although D-xylose was still available.

At pH 3, the xylB xylC strain produced more D-xylonate (6.7 ± 1.1 g l^{-1}) and less xylitol (1.5 ± 0.2 g l^{-1}) than the xylB strain (5.5 ± 0.9 and 2.2 ± 0.1 g l^{-1}, respectively) at higher initial volumetric and specific production rates (Fig. 8b). The xylB xylC strain produced slightly less biomass and xylitol compared to the xylB strain, both at pH 5.5 and at pH 3 (II). As expected, production of D-xylonate, xylitol and biomass were reduced for both strains at pH 3, compared to pH 5.5 (II).
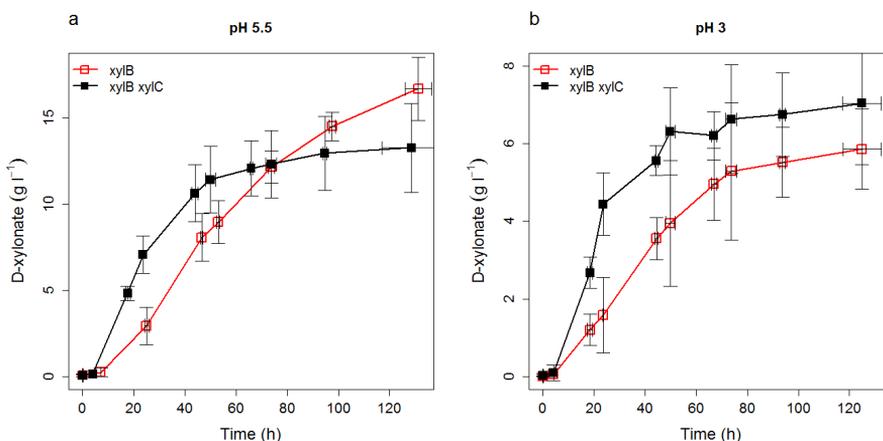
Figure 8. Production of D-xylonate at pH 5.5 (a) or at pH 3.0 (b) by *S. cerevisiae* expressing *xylB* (red open symbols) or *xylB* and *xylC* (black solid symbols) from *C. crescentus* in bioreactors, in SC medium with 9 ± 1 g D-glucose l^{-1} and 23 ± 1 g D-xylose l^{-1}, supplemented with 5 ± 1 g ethanol l^{-1} after ~50 h. Cells were maintained at 30 °C, at 500 rpm and 1.0 vvm aeration. Error bars show $\pm$ SEM ($n = 2$ to 4). D-xylonate was measured by the hydroxamate method and by HPLC.

D-xylonate production was also studied in shake flask cultures without pH control, in SCX (SC medium with 20 g D-xylose l^{-1}) or SCDX (SC medium with 10 g D-glucose l^{-1} and 10 g D-xylose l^{-1}). The initial pH of both media was 6.0, but in 48 h the pH had dropped to 3.6 or 2.8, for SCX and SCDX respectively. In addition to the standard condition, SCDX, medium without D-glucose as a co-substrate (SCX) was used, in order to study the influence of energy supply on the production of D-xylonate.

In the shake flask cultures, the xylB xylC strain initially produced much more D-xylonate, both in SCX and SCDX, when compared to the xylB strain (Fig. 9a). The initial production rate in SCDX (during the first 8 h) was 0.42 ± 0.1 g D-xylonate l^{-1} h^{-1} for the xylB xylC and 0.26 ± 0.4 g D-xylonate l^{-1} h^{-1} for the xylB strain. However, already after this sampling at 8 h, production essentially stopped in the xylB xylC culture, whereas the xylB strain continued producing D-xylonate for at least 4 more h, which resulted in equal final D-xylonate concentrations, 3.8 ± 0.1 g D-xylonate l^{-1}, for both strains produced, (Fig. 9a).
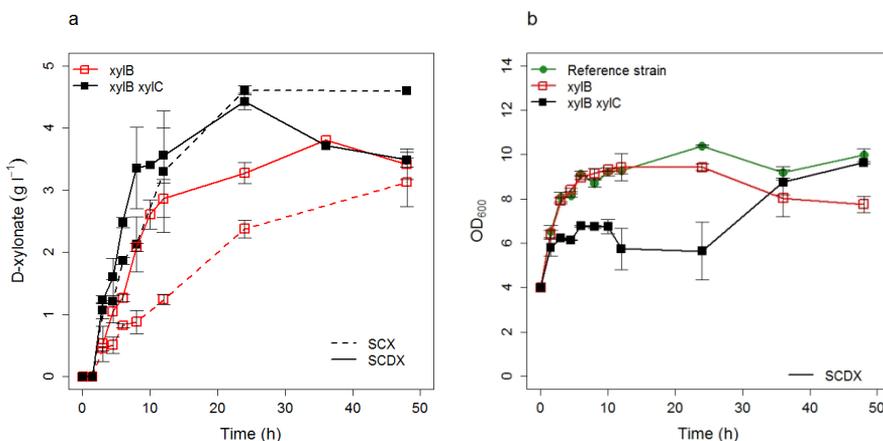
Figure 9. a) Production of D-xylonate by *S. cerevisiae* expressing *xylB* (red open symbols) or *xylB* and *xylC* (black solid symbols) from *C. crescentus* in SC medium and b) growth (as OD_{600}) of these strains and *S. cerevisiae* without gentic modifications (reference strain, green solid circles) in SC medium with 18 g D-xylose l^{-1} (SCX, dashed lines) or 9 ± 1 g D-glucose l^{-1} and 18 g D-xylose l^{-1} (SCDX, solid lines) in shake flasks (250 rpm, 30°C). Error bars show ± SEM for duplicate cultures. D-xylonate was measured by the hydroxamate method and by HPLC.

None of the strains grew in SCX. The main growth of the strains in SCDX, as measured by OD_{600}, occurred much later for the xylB xylC strain when compared to the xylB strain, which grew as fast as the reference strain when starting from an OD_{600} of 4 (Fig. 9b). The maximum OD_{600}, measured after 36 h of cultivation in SCDX, was similar for all strains. This suggests that although the xylB strain initially used energy for production of biomass, the xylB xylC strain used the energy for production and export of D-xylonate and NAD^+ generation. D-xylonate production per biomass of the xylB xylC strain after 24 h, as measured by OD_{600}, was approximately 2 times higher (~1.2 and ~0.8 g l^{-1} OD_{600}^{-1}) compared to the xylB strain (~0.6 and ~0.3 g l^{-1} OD_{600}^{-1}, in SCX or SCDX, respectively). In SCX, total D-xylonate production was 4.6 ± 0.1 g D-xylonate l^{-1} for the xylB xylC strain and 3.1 ± 0.1 g D-xylonate l^{-1} for the xylB strain (Fig. 9a).

Interestingly, the total D-xylonate produced by the xylB xylC strain was higher in SCX compared to SCDX (Fig. 9). The activity of xylB was similar in both strains in both media. Even though both the xylB and the xylB xylC strain produced D-xylonate much faster when D-glucose was provided as a co-substrate, this led to a more rapid arrest in D-xylonate production when compared to production in SCX. The D-glucose of the SCDX medium was utilized during the first ~5 or 7 h of cultivation (for the xylB or the xylB xylC strain, respectively) and ethanol remained in the medium for 30 h or until the end of the experiment.

The extracellular accumulation of D-xylonate was also measured after 60 and 180 min incubation in SCX or SCDX (Fig. 10). The ^{1}H NMR spectroscopy measure-

ments distinguish between D-xylono-γ-lactone and D-xylonate. The xylB xylC strain produced mainly D-xylonate, whereas for the xylB strain both D-xylono-γ-lactone and D-xylonate were detected in the production medium (Fig. 10). These results showed that both D-xylono-γ-lactone and D-xylonate could be exported from the cells.
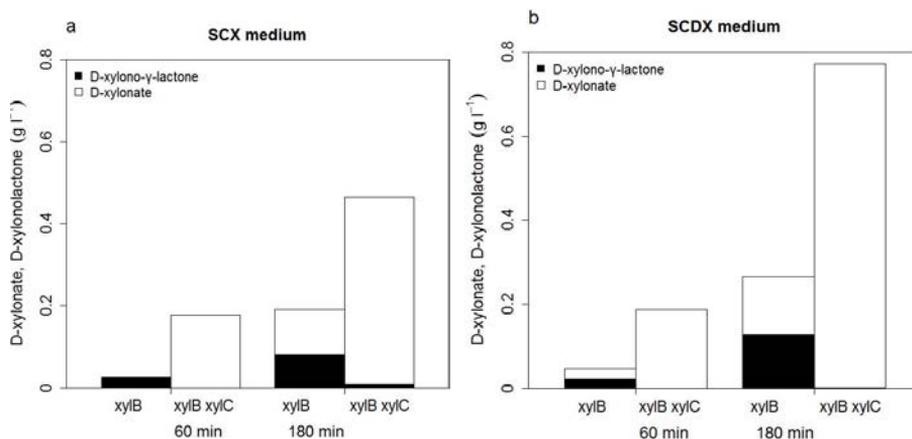
Figure 10. Concentrations of D-xylono-γ-lactone (black) and D-xylonate (white) in SC medium with a) 20 g D-xylose l^{-1} (SCX) or b) 10 g D-glucose l^{-1} and 20 g D-xylose l^{-1} (SCDX) in shake flasks by *S. cerevisiae* expressing *xylB* or *xylB* and *xylC* from *C. crescentus*. The 60 min samples were derived from replicate cultures and values shown are averages (SEM was negligible), whereas the 180 min samples were from a single culture. D-xylonate and D-xylono-γ-lactone was measured by ^{1}H NMR spectroscopy.

The ^{1}H NMR spectroscopy showed that D-xylonate was produced already while there was still plenty of D-glucose in the medium. D-xylonate was produced and exported in the presence of D-glucose. Importantly, the presence of D-glucose (up to 10 g l^{-1}) did not completely inhibit the uptake of D-xylose to the cells, even though *S. cerevisiae* transports D-xylose through hexose transporters with a much higher affinity for D-glucose than D-xylose (Saloheimo et al. 2007).

3.1.6 Intracellular accumulation of D-xylonate leads to decreased vitality and viability

During D-xylonate production in bioreactors, a proportion of the cells lost vitality and accumulated large amounts of intracellular D-xylonate. The initial concentration of D-xylonate was higher in the xylB xylC strain compared to the xylB strain at pH 3. In the cells grown at pH 3, the intracellular D-xylonate concentration was ~110 mg D-xylonate [g biomass]$^{-1}$ in the xylB xylC strain after 8 h incubation and ~40 mg D-xylonate [g biomass]$^{-1}$ in the xylB strain. When the cells were grown at pH 5.5, the accumulation was measured only after 24 h growth. At this time point,

the xylB xylC strain had ~55 mg D-xylonate [g biomass]$^{-1}$ and the intracellular concentration in the xylB strain was ~60 mg g^{-1} (Fig. 11a). In this study, D-xylonate and D-xylono-γ-lactone was not measured separately.

Production of D-xylonate resulted in 15–60% loss of vitality, already during the first ~25 h cultivation. Approximately 30% of the xylB xylC and ~15% of the xylB cells became metabolically inactive during this time, when grown at pH 5.5 (Fig. 11b). When the pH was 3, ~60% of the xylB xylC and ~40% of the xylB cells were metabolically inactive after 24 h. At pH 3, the proportion of inactive cells increased until only ~20% of the cells remained active, whereas the proportion of metabolically active cells at pH 5.5 remained quite stable after the first 24 h. More inactive cells were measured in lactonase expressing cells than in the strain without xylC and the loss of vitality was more rapid in the xylB xylC strain compared to the xylB strain (Fig. 11b). The decrease in vitality of the xylB and xylC strain was also studied at the single cell level (section 3.1.7).
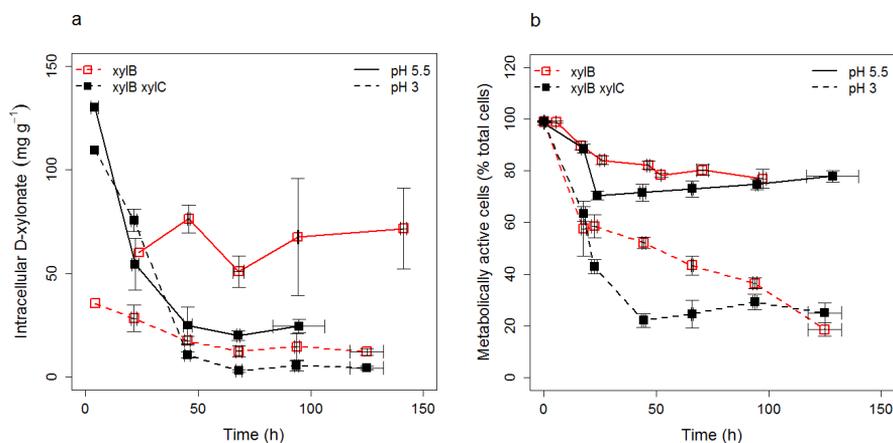
Figure 11. a) Intracellular D-xylonate (expressed as mg [g dry biomass]$^{-1}$) of and b) percentage of metabolically active cells (as determined by methylene blue staining) in populations of *S. cerevisiae* expressing *xylB* (red open squares) or *xylB* and *xylC* (black solid squares) from *C. crescentuS. ce*lls were grown in bioreactors at pH 5.5 (solid lines) or pH 3.0 (dashed lines) in SC medium with 9 ± 1 g D-glucose l^{-1} and 23 ± 1 g D-xylose l^{-1}, supplemented with 5 ± 1 g ethanol l^{-1} after ~50 h. Cells were maintained at pH 5.5 or at pH 3.0, 30 °C, with 500 rpm and 1.0 vvm aeration. Error bars show SEM (*n* = 1–3). D-xylonate was measured from cell extracts by HPLC.

Intracellular D-xylono-γ-lactone and D-xylonate concentrations were measured from cell extracts of cells grown in shake flask cultures, after 30, 60 and 180 min incubation in SCX or SCDX (Fig. 12). The xylB xylC cells contained almost only D-xylonate, whereas the percentage of D-xylono-γ-lactone, compared with the total amount of D-xylono-γ-lactone and D-xylonate, in the xylB cells was 38 ± 2%, in both media.
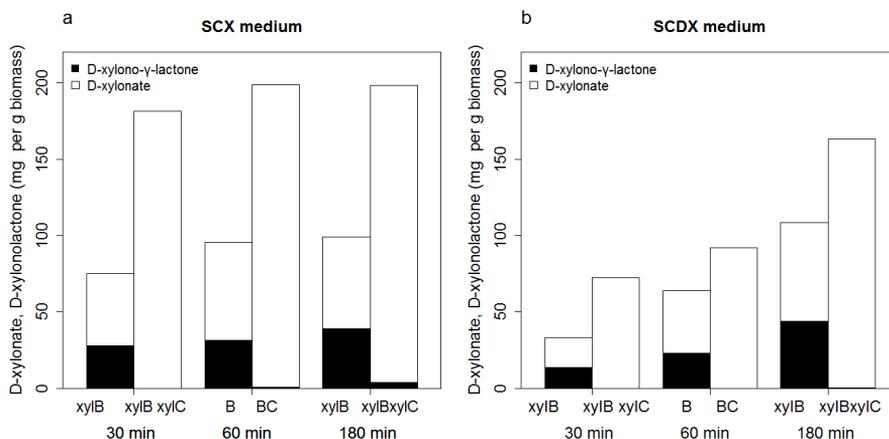
Figure 12. a) Intracellular concentrations of D-xylonate and D-xylono-γ-lactone (expressed as mg [g dry biomass]$^{-1}$, calculated from OD_{600} values, 1 OD l^{-1} estimated to correspond to 0.25 g l^{-1}) in *S. cerevisiae* expressing *xylB* or *xylB* and *xylC* (black solid squares) from *C. crescentus* (measured from cell extracts), after 30, 60 and 180 min incubation in SC medium with a) 20 g D-xylose l^{-1} (SCX) or b) 10 g D-glucose l^{-1} and 20 g D-xylose l^{-1} (SCDX) in shake flasks, shaking at 250 rpm, at 30°C, with initial pH of 6.0. The 60 min samples were derived from replicate cultures and values shown are averages (SEM was negligible whereas the other samples were from single cultures. D-xylonate and of D-xylono-γ-lactone was measured from cell extracts by ^{1}H NMR spectroscopy.

In SCDX, the intracellular concentrations of D-xylonate or D-xylonate and D-xylono-γ-lactone increased with time in both the xylB and xylB xylC strain, whereas the intracellular concentrations in xylB and xylB xylC cells in SCX were constant at all times studied. This might indicate that D-glucose induced a transporter(s) that was involved in export of D-xylonate and D-xylono-γ-lactone from the cells or simply that energy was needed for transport and was therefore more efficient in SCDX than in SCX. Initial extracellular D-xylonate production was also somewhat higher in SCDX for both strains (Figs. 10a-b) possibly due to enhanced transport of D-xylose, or increased pools of co-factors and energy carriers in cells growing in the presence of the preferred energy source D-glucose. D-xylose is transported into *S. cerevisiae* cells using D-glucose inducible transporters (Bertilsson et al. 2008).

The amounts of D-xylonate and D-xylono-γ-lactone detected in by ^{1}H NMR spectroscopy after 30–180 min cultivation in shake flasks were somewhat higher than when measured after ~4 h incubation in bioreactor cultures, but the measurements with both methods were of the same order of magnitude. At later times in bioreactor cultures, intracellular amounts of metabolites (measured as mg g^{-1} dry weight) were generally higher in the xylB strain compared to the xylB xylC

strain, but this might be simply caused by the higher proportion of metabolically inactive cells in the xylB xylC cultures.

The *in vivo* measurement of D-xylonate and D-xylono-γ-lactone in intact cells showed that there was a strong positive correlation between the concentrations of intracellular and extracellular D-xylono-γ-lactone (R^2 = 0.89) and D-xylonate (R^2 = 0.72) in xylB cells during 4 h of incubation in SCX, whereas there was no comparable correlation in the xylB xylC strain within this time (VI: data not shown). The intracellular D-xylonate accumulation in xylB xylC cells levelled off after approx. 15 min incubation in SCX and remained constant. The intracellular D-xylono-γ-lactone and D-xylonate amounts levelled off in the xylB cells after approx. 1 h incubation (VI: data not shown). Thus, the *in vivo* measurements were well in line with the *in vitro* measurements described above.

Intracellular volume has been reported to range between 1.5 and 2 ml [g biomass]$^{-1}$ for *S. cerevisiae* (de Koning and van Dam 1992, Gancedo and Serrano 1989), thus the intracellular D-xylonate concentrations in the xylB xylC cells were up to > 100 g l^{-1}. The intracellular concentrations did not correlate with the extracellular concentrations of D-xylonate, except during the very initial phase, for the xylB strain. However, the proportions of intracellular D-xylonate and D-xylono-γ-lactone correlated well with the proportions of extracellular D-xylonate and D-xylono-γ-lactone in both strains. Therefore it seems that the transport mechanism(s) for export of D-xylonate and D-xylono-γ-lactone had no preference for either compound.

The intracellular concentration of D-xylonate when incubated in either SCX or SCDX was higher in the xylB xylC strain when compared to the total amount of D-xylonate and D-xylono-γ-lactone in the xylB strain. The *in vitro* enzyme activity measurements (section 3.1.3) may indicate that xylB was inhibited by D-xylono-γ-lactone. This could explain why intracellular concentrations were lower in xylB cells: less D-xylose could be converted by xylB, compared to the xylB xylC cells, in which the D-xylono-γ-lactone is hydrolysed to D-xylonate that apparently did not inhibit the xylB enzyme.

Toivari et al. (2010) suggested that the D-xylose uptake capacity of *S. cerevisiae* limited the production of D-xylonate. If this was the case, only negligible amounts of D-xylose would be found inside the cells during D-xylonate production. The concentration of intracellular D-xylose during 180 min incubation in SCX or SCDX was 9–22 mg [g biomass]$^{-1}$ in the reference strain expressing no XYD encoding gene, whereas only up to 8 mg D-xylose [g biomass]$^{-1}$ was detected in the xylB and xylB xylC strains. There was no clear difference in D-xylose concentrations between the xylB and xylB xylC strains, at any time measured or between the media, even though the D-xylonate production rate was much higher in the xylB xylC strain. Intracellular D-xylose concentrations varied from 0.2–7 mg g^{-1} in the cell extracts; small amounts of D-xylose were detected in all samples. Therefore, it is unlikely that D-xylose uptake was the main limiting factor in D-xylonate production in these strains.

3.1.7 Loss of pHluorin fluorescence and subsequent loss of vitality during production of D-xylonate

The relation between cytosolic pH and viability during D-xylonate production was studied at the single cell level in *S. cerevisiae* expressing *xylB* and *xylB* together with *xylC*. Zdraljevic et al. (2013) found that production of D-xylonate led to acidification of the cytosol. pHluorin fluorescence is lost when the intracellular pH is below 5 (referred to as "acidified" cells, Zdraljevic et al. 2013).

Co-expression of *xylC* and *xylB* (xylB xylC) led to a much faster loss in pHluorin fluorescence during incubation in medium with D-xylose (SCX), compared to cells expressing *xylB* alone (xylB) (Fig. 13). Practically all, 99%, of the xylB xylC, but only 9% of the xylB cells lost pHluorin fluorescence in SCX, after 4.5 h incubation (Fig. 13). When no D-xylose was provided to the cells, the intracellular pH of the cells remained unchanged in almost all cells; less than 1% of the xylB cells, and only about 3% of the xylB xylC cells lost pHluorin fluorescence during 4.5 h of incubation in SC medium (VI).
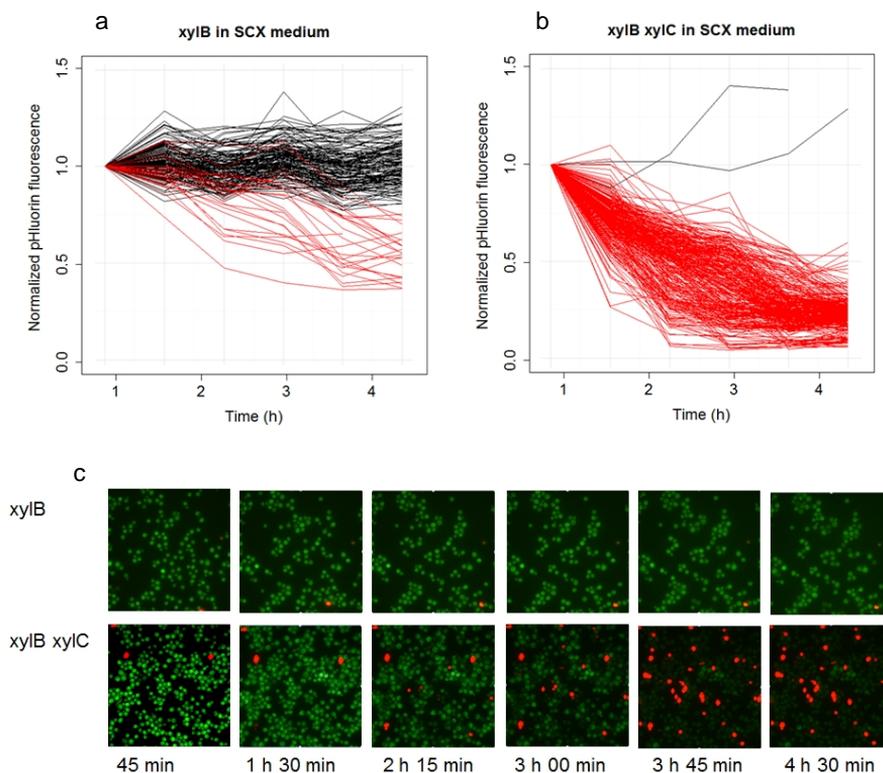
Figure 13. Fluorescent intensity, measured at 410 nm, of a) xylB and b) xylB xylC cells incubated in SC medium with 20 g D-xylose l^{-1} (SCX). Cells were attached to wells of glass bottom plates and imaged over time. Each line represents a single cell. Lines in red represent cells which lost pHluorin fluorescence during the period of observation. c) Images of pHluorin (at 410 nm, in green) and propidium iodide (at 610 nm, in red) fluorescence of the cells monitored in parts a) and b).

The direct measurement of cytosolic pH during rapid acid production and concomitant fast cytosolic acidification was not possible using this experimental set-up. Nevertheless, the observation that pHluorin fluorescence was lost during D-xylonate production, as also demonstrated by Zdraljevic et al. (2013), allowed us to use the loss of pHluorin fluorescence as a tool for studying the physiological consequences of acid production and the heterogeneity in the population. The use of pHluorin, together with measurement of intracellular D-xylonate and D-xylono-γ-lactone concentrations demonstrated that the acidification of the cytosol during acid production most likely resulted from hydrolysis of intracellularly accumulated lactone. Even though the extracellular D-xylonate production was higher in the xylB xylC strain compared to the xylB strain, the intracellular accumulation of both D-xylonate and D-xylono-γ-lactone was similar in both strains. This showed that

accumulation of D-xylonate and the during the hydrolysis of D-xylono-γ-lactone released protons caused the acidification of the cytosol. The concentration of D-xylonate was much higher inside the xylB xylC cells when compared to the xylB strain. Therefore, the cytosolic acidification was much more rapid when the D-xylonate producing cells co-expressed the *xylC* and *xylB* genes than when *xylB* was expressed alone and D-xylono-γ-lactone was opened spontaneously or by unspecific lactonase activity. The accumulation of acid and released protons inside the cells led to a constant struggle to maintain the pH of the cells at a level sufficient for metabolic activity.

Cytosolic acidification, as demonstrated by loss of pHluorin fluorescence, was associated with and probably led to decreased viability. The viability of the xylB xylC cell decreased much faster, compared to cells expressing *xylB* alone. After 4.5 h incubation in SCX, 13% of the xylB xylC cells were metabolically inactive as determined by staining with propidium iodide (PI), while the percentage of metabolically inactive xylB cells was only 0.5% (Fig. 13c). When no D-xylose was provided to the cells (SC medium) 2.0% of the xylB xylC cells and 0.5% of the xylB cells stained with PI.

Single cell analysis allows spatial and temporal insight into the variability within a population. During the process of becoming acidified a large proportion of cells lost pHluorin fluorescence, but remained metabolically active. Zdraljevic et al. (2013) found a correlation between the amount of xylB within a cell and the cytosolic acidification. Similarly, it might be that differences in the rates of which cells became acidified in the xylB xylC strain reflect the differences in the amounts of xylB and/or xylC enzyme within each cell. However, since we initiated production of D-xylonate in cells which had been in stationary phase, the differences in the rate at which they became acidified might also reflect other heterogeneity within the culture. Stationary-phase cells were used, because growing cells did not stay attached to the glass at the bottom of the microscope wells and therefore monitoring single cells during growth was not possible. Stationary-phase yeast cultures are physiologically heterogeneous, e.g. in terms of cell cycle phase (Allen et al. 2006) as well as intracellular pH (Weigert et al. 2009, Valkonen et al. 2013). In this study, however, no correlation between the initial pH, when coming from the stationary phase, and the rate of acidification of the cells during D-xylonate production was found.

3.2   Production of D-xylonate using industrial yeast

While *Saccharomyces cerevisiae* is widely used as a production organism, other yeast species are attracting more attention as new production hosts, due to qualities such as superior robustness or wider substrate spectrum. Therefore, D-xylonate production was studied in the D-xylose metabolizing *Kluyveromyces lactis* and in *Pichia kudriavzevii,* a multi-stress-tolerant organism capable of growing at low pH. D-xylonate was also produced with an industrial *S. cerevisiae* strain, isolated from spent sulphite liquor (VTT B-67002).

K. lactis can naturally use D-xylose as a carbon source and may therefore have efficient uptake mechanisms for D-xylose. In addition to the benefit of D-xylose utilization for energy maintenance, *K. lactis* was chosen as a host for D-xylonate production with xyd1 (a NADP$^+$ dependent D-xylose dehydrogenase from *T. reesei*), as it was expected to have good capacity for generation of NADP$^+$. *K. lactis* has several routes for regeneration of intracellular NADP$^+$, in addition to the pentose phosphate pathway, such as a NAD(P)H-accepting external dehydrogenase (Tarrío et al. 2006), NAD(P)$^+$-accepting glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Verho et al. 2003) and glutathione reductase (KlGLR1, González-Siso et al. 2009).

P. kudriavzevii has previously been shown to have greater tolerance to lactic acid than *S. cerevisiae* (Halm et al. 2004) and the relatively good growth of *P. kudriavzevii* VTT C-79090T at pH 3, suggested that *P. kudriavzevii* is a potential candidate for D-xylonate production.

The industrial *S. cerevisiae* strain used for D-xylonate production was chosen due to its robustness, good tolerance to lignocellulosic hydrolysates, low pH and ethanol.

3.2.1   Effect of pH on specific growth rate of yeast growing on D-glucose

The inhibitory effect of an acid is highly dependent on the pH of the medium. Tolerance to weak organic acids and low pH are thought to be related (Piper et al. 2001). In addition, tolerance to low pH is important as production of D-xylonate at low pH is desirable. Therefore, the effect of pH on the specific growth rate of yeast growing on D-glucose was studied when the different hosts for improving D-xylonate production were chosen.

The specific growth rates on D-glucose of the strains used for D-xylonate production were compared at pH values from 2 to 8 (Fig. 14). All strains had maximal specific growth rates at pH 6, when grown in defined medium in Bioscreen micro titre wells (Fig. 14). *K. lactis* GG799 was the least tolerant of these species to low pH. *S. cerevisiae* CEN.PK2-1D had a higher specific growth rate at pH < 4.5, when compared to the industrial *S. cerevisiae* strain B-67002. The specific growth rates on D-glucose at pH 3–7 of a set of industrial and laboratory *S. cerevisiae* strains (including CEN.PK strains) was reported to be comparable to each other (Albers and Larsson 2009), even though industrial strains are often more robust to inhibitors in a production medium or to other stress factors.
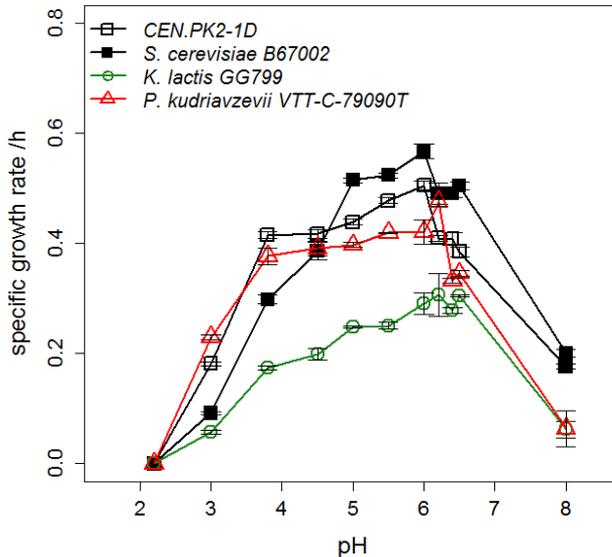
Figure 14. Comparison of specific growth rates of *S. cerevisiae* CEN.PK2-1D (open black squares), B-67002 (solid black squares), *K. lactis* GG799 (open green circles) and *P. kudriavzevii* VTT C-79090T (open red triangles) at pH 2–8 in defined medium, described by Verduyn et al. (1992), with 20 g D-glucose l^{-1} as carbon source, 30 °C. Cells were grown in 300 μL microtitre wells and OD_{600} was measured in a Bioscreen C MBR automated analyser. The specific growth rate of *K. lactis*, VTT B-67002 and VTT C-79090T was also determined from biomass and CO_2 production in a bioreactor at pH 6.2. Error bars represent ± SEM (n = 4–10).

3.2.2 Bioconversion of D-xylose to D-xylonate with *K. lactis*

The *T. reesei xyd1* gene, encoding $NADP^+$-dependent D-xylose dehydrogenase that was previously shown to enable D-xylonate production with *S. cerevisiae* (Toivari et al. 2010) was expressed in an industrial *K. lactis* strain.

Pre-growth on D-xylose increased the D-xylose consumption rate of *K. lactis*, compared to pre-growth on D-glucose by 77% in the reference strain H3632 (to 0.20 ± 0.02 g D-xylose l^{-1} h^{-1}) and by almost 12-fold in the *xyd1*-expressing strains (to 0.27 ± 0.02 g D-xylose l^{-1} h^{-1}). Evolved strains of *S. cerevisiae* engineered to produce ethanol from D-xylose have been shown to take up D-xylose at rates of 0.2–1.1 g D-xylose l^{-1} h^{-1}, whereas non-evolved strains consume D-xylose at rates between 0.05 and 0.1 g l^{-1} h^{-1} (Pitkänen et al. 2005, Van Vleet and Jeffries 2009, Liu and Hu 2010). The D-xylose consumption rate of *S. cerevisiae* expressing *xyd1* was 0.06 g D-xylose l^{-1} h^{-1} (Toivari et al. 2010). In *K. lactis*, D-xylose transport has not yet been studied, but a putative, active sugar transporter belonging to the putative D-xylose proton symporter family) has been identified (Palma et al. 2007).

K. lactis expressing *xyd1* produced 6.3 ± 0.1 g D-xylonate l^{-1} (Fig. 15a), approximately twice the amount as was demonstrated with *S. cerevisiae* expressing the same enzyme. In agreement with this, the activity of xyd1 in *K. lactis* was approximately double compared to *S. cerevisiae* (4.2 ± 0.2 and 2.0 ± 0.8 nkat mg protein^{-1}, respectively). In contrast to *S. cerevisiae*, *K. lactis* has predominantly respiratory metabolism under aerobic conditions. Therefore it might be that the availability NADP$^+$ in *K. lactis* was less limited.

The more efficient uptake of D-xylose by *K. lactis* compared to *S. cerevisiae* may contribute to the higher D-xylonate production. Also increased energy levels due to D-xylose catabolism and thus better tolerance to intracellular acid accumulation and/or better D-xylonate export capacity may contribute to the increased production.

In order to evaluate whether disruption the native D-xylose metabolism in *K. lactis* could improve the production of D-xylonate, the xylose reductase (XR) encoding gene *XYL1* or the xylitol reductase (XDH) encoding gene *XYL2* was deleted from the D-xylonate producing *K. lactis* strain (Fig. 15c). Deletion of *XYL1*, disrupted xylitol production in *K. lactis* H3765, while deletion of the putative *XYL2* gene, disrupted metabolism of xylitol in *K. lactis* H3763.

Deletion of *XYL1* resulted in production of 26% more D-xylonate (8.0 ± 0.8 g D-xylonate l^{-1}) than H3677, containing the endogenous *XYL1* and *XYL2* (6.3 ± 0.1 g D-xylonate l^{-1}; Fig. 15a.), while deletion of the putative *XYL2* resulted in production of 14.1 ± 0.5 g xylitol l^{-1} and production of 69% less D-xylonate (2.0 ± 0.1 g D-xylonate l^{-1}) than the reference strain H3677 (Fig. 15b). Compared to the strain expressing *XYL1* and *XYL2,* the Δ*XYL1* strain continued producing D-xylonate for longer (Fig. 15a).
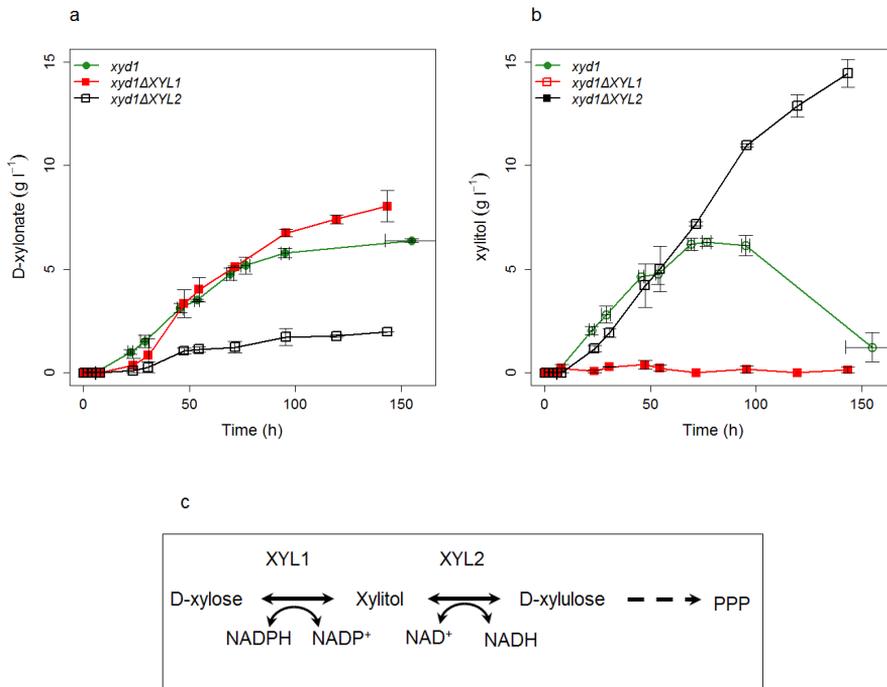
Figure 15. Extracellular accumulation of (a) D-xylonate and (b) xylitol by *K. lactis* xyd1 (solid red squares), xyd1Δ*XYL1* (solid green circles) and xyd1Δ*XYL2* (open black squares) on 10 g D-galactose l^{-1} and 20 g D-xylose l^{-1} in bioreactors, at pH 5.5, 30 °C. Cells were pre-grown on D-xylose. Error bars show ± SEM for duplicate cultures. D-xylonate was measured by the hydroxamate method and by HPLC. c) Depiction of native D-xylose metabolic pathway in *K. lactis*. XYL1 = D-xylose reductase, XYL2 = xylitol dehydrogenase.

Disrupting the native pathway for D-xylose utilisation in order to redirect D-xylose to D-xylonate, rather than to biomass and/or xylitol, was shown to improve D-xylonate production, indicating that the increased energy generation from D-xylose consumption was not the main reason for improved D-xylonate production in *K. lactis*, when compared to *S. cerevisiae*.

K. lactis cells producing D-xylonate accumulated up to > 70 mg D-xylonate [g biomass]$^{-1}$ of intracellular D-xylonate. This level was detected after 31 h of cultivation and this amount is comparable to that seen in D-xylonate producing *S. cerevisiae* cells (Fig. 11a and 12). However, the intracellular D-xylonate concentrations of *K. lactis* generally decreased as the extracellular D-xylonate concentration increased, with a strong negative correlation (R^2 = 0.98), in contrast to what was seen with *S. cerevisiae* cells in which the intracellular D-xylonate amounts generally remained high throughout the cultivations (Fig. 11a). When either *XYL1* or *XYL2* was deleted, intracellular D-xylonate did not accumulate

above 44 mg [g biomass]$^{-1}$, and remained approximately constant at 26 ($\Delta XYL1$) or 31 ($\Delta XYL2$) mg D-xylonate [g biomass]$^{-1}$ between 31 and 143 h.

The viability of *K. lactis* cells producing D-xylonate was not determined. Using a more robust D-xylose utilising yeast, such as *K. marxianus* for D-xylonate production could be more beneficial, as *K. lactis* was very sensitive to low pH (Fig. 14). Significantly ($p < 0.05$) more D-xylonate was produced with *K. lactis* than achieved earlier with *S. cerevisiae* using the same XYD (xyd1). Therefore, it would be interesting to test whether expression of *xylB*, which significantly ($p < 0.05$) improved D-xylonate production in *S. cerevisiae*, would also improve D-xylonate production in *K. lactis*.

3.2.3 D-xylonate production with an industrial *S. cerevisiae* strain

Saccharomyces cerevisiae strain B-67002 was engineered to express *xylB* integrated in two of several *GRE3* loci. This strain was cultured in complex medium at pH 5.5 with 8 g D-glucose l^{-1} and 21 g D-xylose l^{-1} supplemented with 4 g D-glucose l^{-1} and 28 g D-xylose l^{-1} at 47.4 h. This resulted in production of a total of 43 ± 1 g D-xylonate l^{-1}, and 8 g xylitol l^{-1} after 120 h (Fig. 16a), at an initial D-xylonate production rate of 0.44 g l^{-1} h^{-1}.
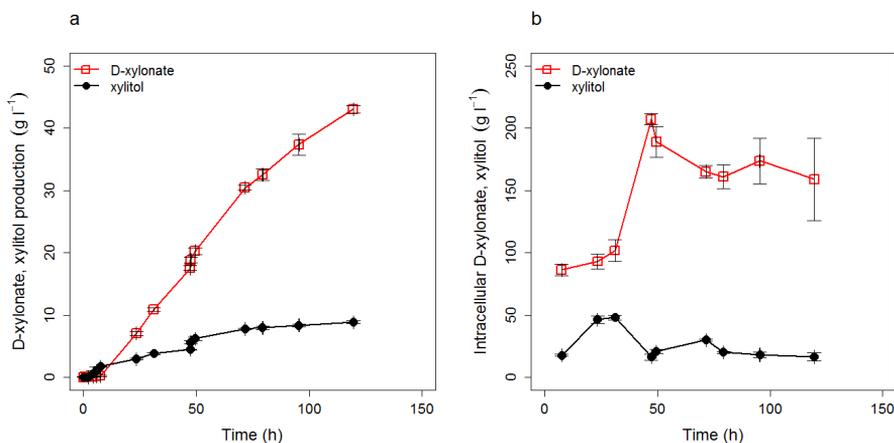
Figure 16. Extracellular (a) and intracellular accumulation (b) of D-xylonate (red open squares) and xylitol (black solid) by *S. cerevisiae* VTT B-67002 *xylB* at pH 5.5 in bioreactors, in YP medium from 49 D-xylose l^{-1}. The cultures initially contained 8 g D-glucose l^{-1} and 21 g D-xylose l^{-1} and were provided ~4 g D-glucose l^{-1} and 28 g D-xylose l^{-1} at 47.4 h. Error bars represent ± SEM for triplicate cultures. D-xylonate and xylitol were measured by the hydroxamate method and/or by HPLC.

At pH 3, B-67002 xylB produced only 13 ± 0.3 g D-xylonic acid l^{-1}, and cell vitality decreased rapidly, so that less than 10% of the population remained metabolically active after 67 h cultivation (II: data not shown). At pH 5.5, no significant loss in

vitality occurred during the first 31 h of D-xylonate production, but as the cultivation proceeded, cells progressively lost vitality and after 120 h, 77 ± 1% of the cells were no longer metabolically active (II: data not shown). B-67002 xylB cells accumulated 80–100 mg D-xylonate [g biomass]$^{-1}$ during the first 50 h of cultivation at pH 5.5, and for the remaining cultivation up to ~170 mg [g biomass]$^{-1}$. At pH 3, the intracellular D-xylonate concentrations were low and decreased with time, likely reflecting the large proportion of non-vital cells and thus release of D-xylonate due to cell lysis. Vitality of B-67002 xylB at pH 3 was negatively correlated to the extracellular D-xylonate concentration (R^2 = 0.94).

Even though use of an industrial *S. cerevisiae* strain improved D-xylonate production 2.5 fold compared to production in the CEN.PK strain, the initial production rate (0.44 ± 0.01) was still ~4 fold lower than the overall productivity observed with *Enterobacter cloacae* (1.6 g l^{-1} h^{-1}, Ishizaki et al. 1973) and a higher production rate would be needed for industrial production. Higher titres would be expected with provision of more D-xylose and optimisation of production conditions might lead to increased production, but D-xylonate export, maintenance energy and maintenance of cell viability would remain bottlenecks for efficient production. The obstacles for D-xylonate production by the industrial *S. cerevisiae* strain were similar to what was seen with the *S. cerevisiae* CEN.PK strain and also with *K. lactis*.

3.2.4   D-xylonate production with *P. kudriavzevii*

In order to achieve higher production of D-xylonate at low pH, *Pichia kudriavzevii* was engineered to express the *C. crescentus xylB*. *P. kudriavzevii* was chosen due to its good tolerance to low pH and reported tolerance to lactic acid.

P. kudriavzevii VTT-C-12903, expressing the *xylB* XYD encoding gene, grown in YP medium at pH 5.5, produced a maximum of 171 ± 5 g D-xylonate l^{-1} from 171 ± 5 D-xylose l^{-1}, when D-xylose was added in pulses every ~24 h (Fig. 17a). Only 17.1 ± 1.5 g xylitol l^{-1} was produced, and the xylitol was partly being consumed after 146 h. Thus, VTT-C-12903 was capable of metabolizing D-xylose via xylitol to biomass.
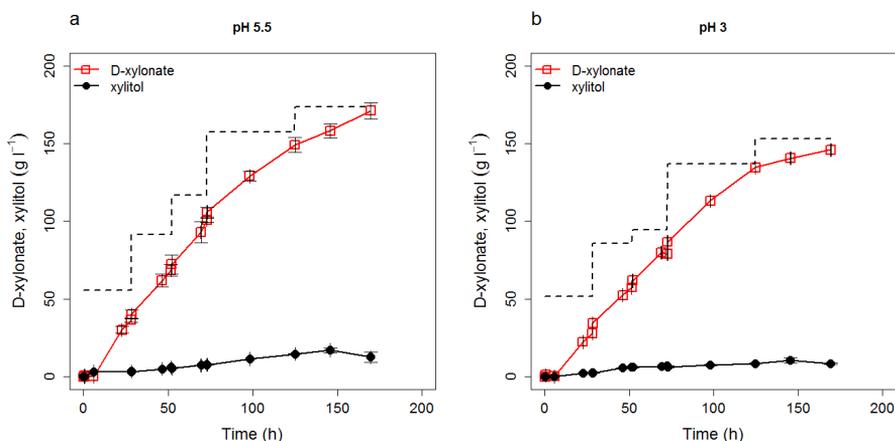
Figure 17. D-xylonate (red open squares) and xylitol (back solid circles) accumulation by *P. kudriavzevii* VTT-C-12903 at a) pH 5.5 b) pH 3 in bioreactor cultures provided with a total of 171 g l⁻¹ (pH 5.5) or 153 g l⁻¹ (pH 3) D-xylose in pulses at ~24 h intervals (indicated by dashed line). YP medium initially contained ~15 g l⁻¹ D-glucose and ~54 g D-xylose l⁻¹. D-Glucose, ~9 g l⁻¹ was also added at 28.4 h. Error bars represent ± SEM for duplicate cultures. D-xylonate and xylitol were measured by the hydroxamate method and/or by HPLC.

The initial D-xylonate production rate of *P. kudriavzevii* VTT-C-12903 (1.4 g l⁻¹ h⁻¹) was comparable to that of *Pseudomonas fragi* (1.4 g l⁻¹ h⁻¹, Buchert and Viikari (1988) and *E. cloacae* (1.6 g l⁻¹ h⁻¹, Ishizaki et al. 1973) producing similar concentrations of D-xylonate (150–200 g l⁻¹, at pH 6.5). However, for high D-xylonate production by *P. kudriavzevii*, the D-xylose had to be provided in pulses, whereas the bacteria produced high amounts of D-xylonate in batch culture. When more than 50 g D-xylose l⁻¹ was added to the cultures *P. kudriavzevii*, this resulted in a lower D-xylonate titre (data not shown). Due to technical limitations it was not possible to evaluate the production of D-xylonate in a continuous cultivation system.

P. kudriavzevii VTT-C-12903 efficiently produced D-xylonate at pH 3 (Fig. 17): up to 146 ± 5 g l⁻¹ from 153 ± 1 g D-xylose l⁻¹ at a rate of 1.2 ± 0.03 g l⁻¹ h⁻¹. The intracellular accumulation of D-xylonate was similar at pH 3 and 5, being initially very high (115–143 mg D-xylonate [g biomass]⁻¹, within 6–9 h of providing D-xylose to the culture), but subsequently decreasing to a concentration of 63 ± 2 mg [g biomass]⁻¹, until D-xylose was consumed.

A radical increase in cell death and decreased vitality was observed when *S. cerevisiae* produced D-xylonate at pH 3. Similarly, there was more cell death at pH 3.0 than at pH 5.5 for *P. kudriavzevii*. However, 47 ± 1% of the *P. kudriavzevii* cells remained metabolically active (30% viable) after 145 h at pH 3. At pH 3, only 10% of the *S. cerevisiae* B-67002 xylB cells remained viable after 67 h cultivation. After 146 h at pH 5.5, 61 ± 2% of the *P. kudriavzevii* cells remained metabolically active (52 ± 2% viable) in cultures provided with 171 g D-xylose l⁻¹. At 120 h, the vitality of *S. cerevisiae*

B-67002 xylB cells in similar conditions was only 23 ± 1%. The vitality of *P. kudriavzevii* cells producing D-xylonate was negatively correlated with extracellular D-xylonate concentration both at pH 5.5 and pH 3 (R^2 = 0.86 or 0.82, respectively).

Unexpectedly, the *in vitro* activity of xylB in *P. kudriavzevii* was much lower compared to the activity of xylB in *S. cerevisiae*. However *P. kudriavzevii* produced much higher amounts of D-xylonate than *S. cerevisiae*, demonstrating that high *in vitro* activity of an enzyme does not necessarily reflect high production.

The tolerance to low pH of yeast and more so of *P. kudriavzevii*, is poorly understood. Halm et al. (2004) demonstrated that the pH homeostasis of *P. kudriavzevii* in the presence of extracellular lactic acid at pH 2.5 was superior to that of *S. cerevisiae*. *P. kudriavzevii* has been engineered to produce 67–70 g lactic acid l^{-1} (Suominen et al. 2007). The intracellular pH of *S. cerevisiae* cells decreased in response to synthesising L-lactic acid (Valli et al. 2006) and D-xylonate (Zdraljevic et al. 2013). Therefore, superior pH maintenance might be one of the reasons for high D-xylonate production by *P. kudriavzevii*. However, efficient D-xylose utilization for generation of maintenance energy or a better capacity of D-xylonate export are other possible explanations for *P. kudriavzevii* being able to produce more D-xylonate than *S. cerevisiae*. The intracellular amounts of D-xylonate were smaller in *P. kudriavzevii* when compared to *S. cerevisiae* B-67002 xylB. Comparative genomic studies and transcriptome analyses of the D-xylonate producing strains would be needed to determine what factor(s) makes *P. kudriavzevii* such a good D-xylonate producer.

The results presented above show that *P. kudriavzevii* was able to produce over 10-fold more D-xylonate at pH 3.0 than any other reported strain producing D-xylonate, at rates which are almost as high as those at pH 5.5. The high stress tolerance (Kitagawa et al. 2010) and demonstrated capacity to ferment sugars in lignocellulosic hydrolysate (Kwon et al. 2011) make *P. kudriavzevii* an interesting candidate for D-xylonate production from biomass hydrolysate.

3.3 The role of Pdr12 in tolerance to weak organic acids in *S. cerevisiae*

The transport system for D-xylonate is unknown, and as D-xylonate accumulates inside the cells, a fair assumption is that there are no transporters in yeast that efficiently transport the acid out of the cells. In consequence, transport of D-xylonate would become a bottleneck in D-xylonate production, and accumulation of D-xylonate would lead to acidification of the cytosol and subsequent cell death.

However, as the cells are capable of excreting at least some D-xylonate there must be one or several transporters that transport D-xylonate out of the cell, although with a low affinity, specificity or efficiency. Therefore, the deletion and overexpression of *S. cerevisiae* transporters known to be involved in, or upregulated during, weak organic acid stress was tested. Unfortunately, none of the transporters we studied (Pdr12, Fps1, Tpo2, Tpo3 and Fun34) could significantly improve or decrease the production of D-xylonate. The deletion of a transporter that would

indeed be involved in transport of D-xylonate would be expected to decrease the production. The overexpression of these transporter proteins was achieved with multicopy plasmids that constitutively expressed the transporters. However, a too high expression of a transporter protein may lead to challenges such as proliferation of the endoplasmic reticulum (reviewed by Hyde et al. 2002) and therefore a more moderate upregulation or a controlled induction of the transporters should be tested. The results of this study cannot be considered conclusive.

Pdr12, an ABC transporter localized in the plasma membrane, which has been shown to be involved in resistance to several weak organic acids (Piper et al. 1998, Holyoak et al. 1999, Bauer et al. 2003, Ullah et al. 2012), was further studied, as its role in weak organic acid tolerance was yet poorly understood. Hence, yeast strains with altered expression of *PDR12* and/or *CMK1*, a gene encoding a protein kinase associated with post-transcriptional negative regulation of Pdr12, were exposed to seven different weak organic acids which are widely used as preservatives, present in lignocellulosic hydrolysates or attractive as industrial precursors: acetic, formic, glycolic, lactic, propionic, sorbic and levulinic acid. "The reasoning behind including the deletion of *CMK1* in this study was due to the reported benefit from this modification on acid tolerance. The Δpdr12Δcmk1 was originally thought to function as a control.

Acids exist in pH dependent equilibrium between the dissociated and undissociated forms. This means that when the undissociated acid pass through the cell wall and into the cells, the equilibrium changes. This leads to more undissociated acid that can pass the cell membrane and that has to be actively pumped out. The pKa and lipophilicity of an acid determines how the acid is able to enter and exit the cell at a certain pH. Therefore, the inhibitory concentration of an acid is highly dependent on the acid as well as the pH. For instance, although the pKa of sorbic and acetic acid is similar, ~4.8, the hydrophilic acetic acid is far less inhibitory to yeast compared to the highly lipophilic sorbic acid that dissolves into the cell membranes (Bracey et al. 1998). The concentrations of acids used in this study were chosen based on concentrations found to lead to the greatest differences between the mutant and the reference strain(s).

3.3.1 Deletion of *PDR12* leads to improved tolerance to acetic and formic acid

Deletion of *PDR12* was desirable for growth in acetic acid (as shown previously by Bauer et al. 2003) and this also applied to formic and glycolic acid (Fig. 18 a–b, V: data for glycolic acid not shown). The Δ*pdr12* and Δ*pdr12*Δ*cmk1* strains had shorter lag phases, before resuming growth in the presence of 100 mM acetic or 25 mM formic acid (Fig. 18a–b), and reached higher final biomass concentrations within the 70 h experiments, when compared to the reference strain. Although some earlier reports suggested that deletion of *PDR12* would increase sensitivity to acetic acid (Piper et al. 1998, Holyoak et al. 2000), this was later shown to be an artefact from the use of a Trp1 deficient strain (Bauer et al. 2003). Tryptophan auxotrophy increased tolerance to all the acids studied (data not shown).
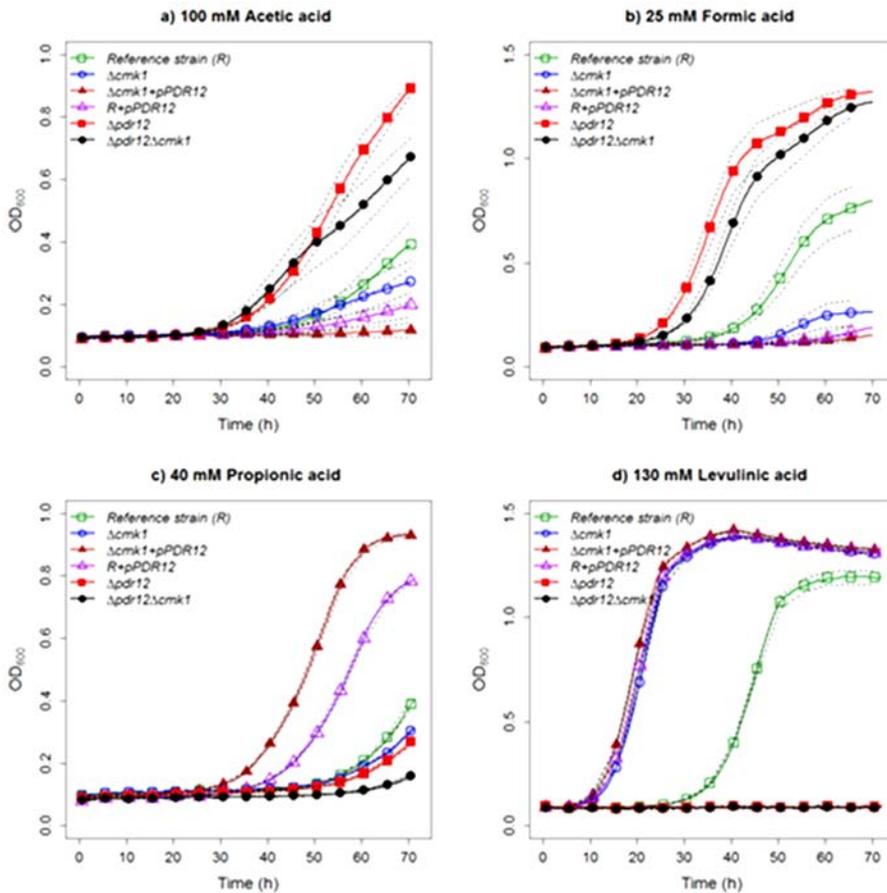
Figure 18. Measurement of biomass in bioscreen cultures of the reference strain (open squares), Δ*cmk1* (open circles), Δ*cmk1* + pPDR12 (solid triangles), R (reference) + pPDR12, (open triangles), Δ*pdr12* (solid squares) and Δ*pdr12*Δ*cmk1* (solid circles) strains in in SCD-leu medium containing 20 g D-glucose l⁻¹, in the presence of a) 100 mM acetic acid (pH 3.3), b) 25 mM formic acid (pH 3.1), c) 40 mM propionic acid (pH 3.9) and d) 130 mM levulinic acid (pH 3.1). Dashed lines represent SEM of 15 (a), 20 (b) or 5 (c, d) replicates.

In contrast, deletion of *PDR12* had a negative effect on the tolerance to propionic, levulinic or sorbic acid (Fig. 18c–d, V: data for sorbic acid not shown). The Δ*pdr12* strains were unable to grow in the presence of 70 (data not shown) to 130 mM levulinic acid (Fig. 18d) or 45–50 mM propionic acid (data not shown). When exposed to 40 mM propionic acid, the difference in the lag phase between the reference and the Δ*pdr12* strains was 5 h (Fig. 18c).

71

3.3.2 Overexpression of *PDR12* leads to improved tolerance to sorbic, propionic and levulinic acid

Strains overexpressing *PDR12* ($\Delta cmk1$ + pPDR12, μ = 0.08 h^{-1}, and reference + pPDR12, μ = 0.09 h^{-1}) had higher specific growth rates ($p < 0.05$) than the reference strain (μ = 0.05 h^{-1}) and reached a higher final OD (> 0.5) compared to the reference strain (final OD < 0.4) when exposed to 20 mM sorbic acid (V: data not shown). The strains overexpressing *PDR12* also had shorter lag phases, compared to the reference strain, in the presence of 40 mM propionic acid or 130 mM levulinic acid (Fig. 18c–d).

In contrast, overexpression of *PDR12* made cells more sensitive to acetic and formic acids, and also to lactic and glycolic acids (Fig. 18a–b, V: data for lactic and glycolic acids not shown), both in the reference and Cmk1 deficient background. The specific growth rate of the overexpression strains was lower and the lag phase longer than that of the reference strain in the presence of these acids, and the length of the lag phases increased with increasing acid concentration (data not shown).

Cmk1 is a negative regulator of Pdr12 (Holyoak et al. 2000), thus it was expected that the deletion of *CMK1* would lead to a phenotype similar to that of *PDR12* overexpression. Indeed, deletion of *CMK1* improved growth in the presence of 130 mM levulinic acid and 30–35 mM propionic acid (Fig. 18d, data for propionic acid not shown), as shown by Holyoak et al. (2000). In 40 mM propionic acid however, the $\Delta cmk1$ strain grew slightly worse compared to the reference strain (Fig. 18c), demonstrating that the tolerance to weak organic acids is highly dependent on the concentration of acid. Overexpression of *PDR12* in the $\Delta cmk1$ strain increased tolerance to propionic acid (Fig. 18c) and as expected, deletion of *CMK1* led to decreased resistance to formic, acetic, glycolic and lactic acid, as was seen with strains overexpressing *PDR12* (V: data for glycolic and lactic acid not shown).

3.3.3 High concentrations of acetic or propionic acid leads to cell death

Acetic acid and propionic acid induced cell death (Fig. 19), as has been demonstrated previously (Bauer et al. 2003). However, deletion of *PDR12* did not reduce acetic acid-induced cell death, as suggested by Bauer et al. (2003), but did increase the rate of recovery of the small surviving population, on acetic but not on propionic acid (Fig. 19). When exposed to 100 mM acetic acid or 40 mM propionic acid, the viability of all the strains studied was reduced; less than 1% of the cells were viable within 24 h of incubation. The percentage of viable cells of the reference, $\Delta pdr12$ and $\Delta pdr12\Delta cmk1$ strains had increased within 40 h in the presence of 100 mM acetic acid (Fig. 19a). In contrast, and also in line with the length of lag phases, the Cmk1 deficient strains and the reference strain were more viable after 72 h in 40 mM propionic acid than either of the $\Delta pdr12$ strains or the strain overexpressing *PDR12* (Fig. 19b).
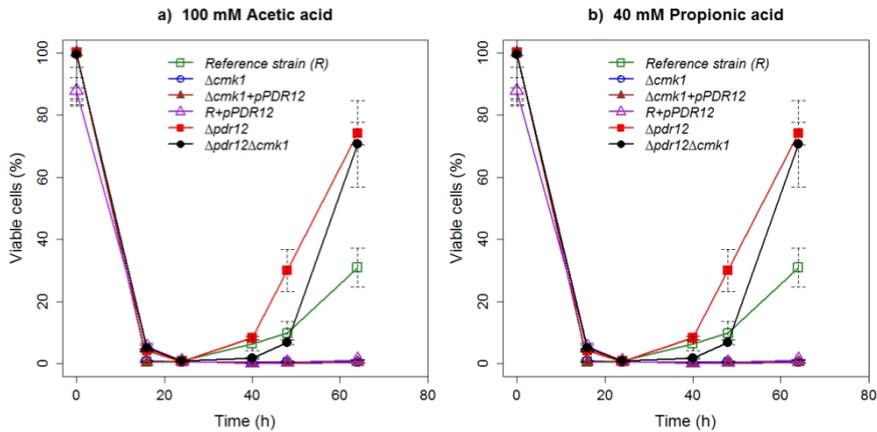
Figure 19. Percentage of viable cells in populations of reference (open squares), Δ*cmk1* (open circles), Δ*cmk1* + pPDR12 (solid triangles), R (reference) + pPDR12, (open triangles), Δ*pdr12* (solid squares) and Δ*pdr12*Δ*cmk1* (solid circles) cells grown in SCD-leu medium containing 20 g D-glucose l^{-1}, in the presence of a) 100 mM acetic acid (pH 3.3) or b) 40 mM propionic acid (pH 3.9), expressed as the percentage of colony forming units, CFU, relative to the total cell number determined with a Cellometer Auto T4 cell counter. Error bars show ± SEM for 2 biological replicates and reflect both the error in the CFU determination and in the estimation of the total cell number.

3.3.4 Adaptation to weak organic acids leads to increased acid tolerance

Adaptation is an important feature in organic acid tolerance; after adaptation, cells are more tolerant to subsequent acid stress. This adaptive response mechanism is poorly understood. Changes in cell wall composition (Fernandes et al. 2005, Simões et al. 2006, Ullah et al. 2013) and limitation of diffusional entry of the acid (Piper et al. 2001, Ullah et al. 2013), upregulation of the Pma1 plasma membrane ATPase as well as the vacuolar ATPase (reviewed by Mira et al. 2010) are reported acid adaptation mechanisms in yeast. Yeast can maintain the adaptation to weak acids as long as they are kept under acid stress, and retain this adaptation for numerous generations (reviewed by Dragosits and Mattanovich 2013). However, when the cells are allowed to grow without acid this adaptive phenotype is lost.

Pre-adaptation to sorbic acid stress has been shown to also render the culture more tolerant to other weak organic acids (Holyoak et al. 2000). Exposure of precultures to 0.45 mM sorbic acid improved growth of the reference and the Cmk1 deficient strain in the presence of propionic or levulinic acid (Table 5). After pregrowth in 0.45 mM sorbic acid these strains were as resistant to propionic acid or levulinic acid, as the strains overexpressing *PDR12* (Δ*cmk1* + pPDR12 and R (reference) + pPDR12), which had shown the highest tolerance when not pre-adapted (data not shown).

Table 5. Effect of adaptation in sorbic or acetic acid on tolerance to weak organic acidS. cells were pre-grown in SCD-leu medium supplemented with 0.45 mM sorbic acid (pH 6.0) or 50 mM acetic acid (pH 4.0) for 16 h, and then transferred to new medium supplemented with acid. FA = 25 mM formic acid, pH 3.1, AA = 100 mM acetic acid, pH 3.3, PA = 40 mM propionic acid, pH 3.9, LA = 140 mM levulinic acid, pH 3.0, SA = 20 mM sorbic acid, pH 6.0. The plus sign (+) indicates that pre-adaptation increased tolerance to the acid whereas the minus sign (-) indicates that tolerance was decreased. no indicates that tolerance was not affected. Experiment was performed with 5 biological replicates.

Strain	0.45 mM Sorbic acid adaptation					50 mM Acetic acid adaptation			
	FA	AA	PA	LA	SA	FA	AA	PA	SA
Reference	-	+	+	+	no	-	-	-	-
$\Delta cmk1$	-	+	+	+	+	-	-	-	-
$\Delta cmk1$ + p*PDR12*	-	+	+	+	-	-	-	-	-
Reference + p*PDR12*	-	+	+	no	-	-	-	-	-
$\Delta pdr12$	-	+	-	no	-	+	+	-	-
$\Delta pdr12\Delta cmk1$	+	+	-	no	-	+	+	-	-

The lag phase of all strains in medium supplemented with 100 mM acetic acid was shorter after pre-growth in the presence of 0.45 mM sorbic acid, and the $\Delta pdr12$ and the $\Delta pdr12\Delta cmk1$ cells were able to grow in the presence of 120 mM acetic acid after sorbic acid pre-treatment, the $\Delta pdr12\Delta cmk1$ strain having a shorter lag phase than the $\Delta pdr12$ strain (data not shown). The sorbic acid pre-treatment led to improved tolerance to 25 mM formic acid of the $\Delta pdr12\Delta cmk1$ strain, whereas this treatment had a negative effect on all the other strains (Table 5).

After adaptation to 50 mM acetic acid, the $\Delta pdr12\Delta cmk1$ was more tolerant to acetic and formic acids compared to the $\Delta pdr12$ strain, showing that Cmk1 plays a significant role in weak organic acid tolerance beyond its role in regulation of Pdr12.

When the strains were pre-grown in the presence of 50 mM acetic acid, only the Pdr12 deficient strains ($\Delta pdr12$ and $\Delta pdr12\Delta cmk1$) had improved growth (~25 h shorter lag phase) when subsequently exposed to 100 mM acetic acid (Table 5). The long lag phase after pre-growth in presence of acetic acid was likely due to lost viability; very few cells were viable after 8 h of incubation in medium supplemented with 100 mM acetic acid, when pre-cultures were grown in 50 mM acetic acid (data not shown). Similar to results without pre-growth in acetic acid, the $\Delta pdr12$ strains regained viability much faster than the Cmk1 deficient strain. In these conditions the reference and *PDR12* overexpressing strains contained essentially no viable cells during the interval studied (data not shown).

4. Conclusions and future prospects

The production of D-xylonate was substantially increased to a maximum titre of 171 g l^{-1}. D-xylonate production was successfully demonstrated in *Saccharomyces cerevisiae*, *Kluyveromyces lactis* and *Pichia kudriavzevii* yeast and the consequences of D-xylonate production on the physiology of *S. cerevisiae* was studied in detail. By combining single cell studies of pHluorin expressing, D-xylonate producing cells with sensitive and non-invasive ^{1}H NMR spectroscopic measurements of intra- and extracellular D-xylonate and D-xylono-γ-lactone, we have identified opening of the lactone as a critical step in D-xylonate production in yeast, with important consequences for cellular physiology.

The stress response to weak organic acids was highly dependent on the properties of the acids and the role of Pdr12 in resistance to weak organic acids was found to be highly dependent on the acid. The presence of high concentrations of acetic and propionic acids led to lost viability.

4.1 Improving D-xylonate production in yeast

D-xylonate production titres and yields were significantly improved (Fig. 20) via a combination of strategies. Various production hosts were used, including D-xylose-utilising yeast (*K. lactis* and *P. kudriavzevii*) and yeast incapable of metabolizing D-xylose (*S. cerevisiae*), as well as robust industrial and easily manipulated laboratory strains (*S. cerevisiae* CEN.PK). Production of D-xylonate was achieved by expressing several different D-xylose dehydrogenase encoding genes (*xyd1* from *Trichoderma reesei*, *xylB* from *Caulobacter crescentus*, SU2DD from *Sus domesticus*) and endogenous aldose reductase encoding genes were deleted in order to reduce xylitol formation. The highest D-xylonate production titres were obtained by the expression of the *xylB* D-xylose dehydrogenase encoding gene which originated from *C. crescentus*.

However, the greatest improvement was achieved by host selection. In fact, *P. kudriavzevii* was the best production organism, capable of producing D-xylonate as well, or better than, natural D-xylonate producers, such as *G. oxydans*, and it had the benefit of also producing D-xylonate very efficiently at low pH. *P. kudriavzevii was* capable of producing 171 or 146 g D-xylonate l^{-1} (Fig. 20), at a rate of 1.4 or 1.2 g l^{-1} h^{-1}, at pH 5.5 or pH 3, respectively.
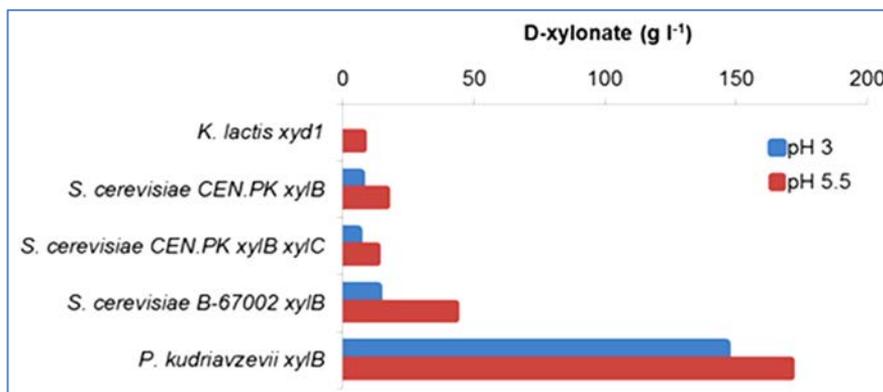
Figure 20. Production of D-xylonate at pH 3 (blue bars) and at pH 5.5 (red bars) using genetically modified yeast. Values presented are an overview of best results achieved in specific, strain-dependent setups.

4.2 D-xylonate production leads to accumulation of D-xylonate, decreased vitality and cell death

Large amounts of D-xylonate and D-xylono-γ-lactone accumulated inside the cells during production of D-xylonate. However, D-xylonate was also produced and exported from the cells from the very beginning of cultivation. D-xylose was taken up and converted to D-xylonate even in presence of up to 10 g D-glucose l^{-1}.

D-xylonate is produced from D-xylose via the D-xylono-γ-lactone that can be hydrolysed to D-xylonate spontaneously or with the aid of a lactonase. Both D-xylonate and D-xylono-γ-lactone were exported from the *S. cerevisiae* cells although neither very efficiently. There was no apparent preference for export of either compound.

The specific growth rate of D-xylonate producing *S. cerevisiae* cells in medium with D-xylose and D-glucose was decreased compared to non-producing cells because of loss in viability. This was more pronounced in cells co-expressing the *xylC* D-xylonolactone lactonase encoding gene with the *xylB* D-xylose dehydrogenase encoding gene.

Co-expression of *xylB* and *xylC* lead to an increased the D-xylonate production rate compared to expression of only *xylB*. Accumulation of D-xylonate, or more precisely, the anions and/or protons released by its dissociation, was very harmful for the cells. The accumulation of D-xylonate and protons was correlated to loss of fluorescence of the pH indicator pHluorin, indicating that the cytosol had become acidified. This loss of fluorescence occurred faster when the cells were co-expressing the lactonase encoding gene *xylC* with *xylB*, than in cells expressing *xylB* alone. Thus the acidification of the cytosol occurred more rapidly in the xylB xylC cells. The acidification of the cytosol was shown to be correlated to decreased vitality of the D-xylonate producing cells. The rate of loss of pHluorin fluorescence and loss in vitality was highly dependent on the pH of the production

medium. This likely reflects the added stress of low extracellular pH and maintenance of intracellular pH as well as stress caused by the intracellular accumulation of D-xylonate. Further work is needed to understand how yeast regulate intracellular pH in order to improve this mechanism. An improved capacity for adjusting intracellular pH in yeast would be most beneficial for organic acid production and tolerance.

In addition to improving export of D-xylonate, the production could potentially by improved by adjusting the stress of D-xylonate production to a more tolerable level. This could be achieved by a synthetic control circuit that senses early stress of D-xylonate production and transiently arrests xylB expression allowing the cells to recover before D-xylonate synthesis is re-initialized. A genetic circuit for controlling D-xylonate production at the single cell level could improve the viability of the production organisms.

4.3   Pdr12 and weak organic acid stress tolerance

The effect of *PDR12* deletion or overexpression on tolerance/sensitivity to the short-chain, hydrophilic acids, formic, acetic, lactic and glycolic acids, was to a large extent, opposite to that of the more hydrophobic sorbic, propionic and levulinic acids. Deletion of Pdr12 led to improved tolerance to formic and acetic acids, a feature that makes these strains interesting for use in biorefining of lignocellulosic hydrolysates. Acetic and formic acids are the most common acids in most lignocellulosic hydrolysates and are typically present at concentrations in the range investigated here. Overexpression of *PDR12* improved tolerance to sorbic, propionic and levulinic acids.

The presence of high concentrations of propionic or acetic acid led to cell death – most of the cells died within 24 h of incubation. The rate of recovery from propionic or acetic acid stress, of the small surviving population, corresponded to the length of the lag phase, in the presence of these acids.

4.4 Future prospects

Biotechnological production of D-xylonic acid with yeast clearly has the potential of becoming an industrially applicable process. Production titres and yields achieved with *P. kudriavzevii* from D-xylose were remarkable, but production of D-xylonate from lignocellulosic hydrolysates still needs to be addressed. In order for biotechnological production processes to become economically feasible, biorefinery approaches, in which lignocellulosic hydrolysates or other side- or waste streams are used as raw materials, need to be employed. As lignocellulosic hydrolysates contain compounds which are inhibitory to yeast such as organic acids, the finding of the current study that deletion of *PDR12* from *S. cerevisiae* improves tolerance to formic and acetic acid is potentially commercially very valuable. In fact, *S. cerevisiae* is a widely used production host and the toxicity of hydrolysates and especially acids present in these is a severe obstacle for efficient conversion of lignocellulosic biomass sugars.

In order to establish an economically feasible production of D-xylonate cell viability needs to be improved. Production at low pH is desirable, but at low pH even the robust *P. kudriavzevii* shows significantly decreased viability. Increased viability may be achieved by genetic engineering or process optimization. Metabolic modelling of D-xylonate production in yeast could potentially suggest genetic modifications for improving the fitness of the cells and also provide leads for optimization of the production conditions. Different process conditions such as D-xylose or co-substrate feeding strategy, oxygenation rate and/or product recovery during the production process should be evaluated. A continuous culture system with a constant feed of co-substrate may increase the vitality of the cells and lead to higher D-xylonate production. The optimization of the production process should eventually be done with biomass hydrolysate as substrate.

Scientifically, it would be most interesting to study what makes *P. kudriavzevii* such a good D-xylonate producer. A transcriptomic comparison of D-xylonate producing *P. kudriavzevii* and *S. cerevisiae* strains would potentially lead to better understanding of pH homeostasis and acid adaptation. Also, it would be very interesting to study D-xylonate production in *P. kudriavzevii* in detail, using the methods developed in this study. The combination of single cell studies with sensitive and non-invasive ^{1}H NMR spectroscopy measurements provides generally applicable tools for determining cause and effect relationships during heterologous production of compounds that are inhibitory to the production host. Biotechnological production processes are becoming more important in the production of chemicals and thus profound understanding of cellular physiology of the production organisms is essential.

This thesis has provided various means to study, understand and potentially also improve production of D-xylonic and other acids. Several acids have been identified as biochemicals with high potential (Werpy et al. 2004, de Jong 2011, Nita et al. 2013) and biotechnological production of a few acids, including lactic and itaconic acids, which are applied in the polymer industry has already been established. Decrease in intracellular pH has been observed in lactic acid producing *S. cerevisiae* (Valli et al. 2006) and the membrane transporters Jen1 and Ady2 were shown to modulate lactic acid production by *S. cerevisiae* (Pacheco et al. 2012). Acid tolerance and transport are likely important in microbial production of any acid. Pdr12 has been shown to have a diverse role in resistance to a number of acids, including lactic acid, and its role in the production of acids other than D-xylonate should be evaluated.

The field of industrial biotechnology has moved rapidly in recent years because international political desire has led to legislation and economic incentives to promote use of alternative raw materials. There has also been great progress in molecular biology. Biotechnical processes hold large potential for improving resource and energy efficiency and providing the means to reduce greenhouse gas emissions. Production of value-added by-products in combination with substitutes for fossil-fuel based compounds will be cornerstones in the future bioeconomy and synthetic biology is expected to make revolutionary improvements in biotechnological capabilities.

Acknowledgements

References

Abbott D.A., Zelle R.M., Pronk J.T., van Maris A.J.A. 2009. Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: current status and challenges. FEMS Yeast Res. 9: 1–14.

Akita O., Nishimori C., Shimamoto T., Fujii T., Iefuji H. 2000. Transport of pyruvate in *Saccharomyces cerevisiae* and cloning of the gene encoded pyruvate permease. Biosci. Biotechnol. Biochem. 64: 980–984.

Albers E., Larsson C. 2009. A comparison of stress tolerance in YPD and industrial lignocellulose-based medium among industrial and laboratory yeast strains. J. Ind. Microbiol. Biotechnol. 36: 1085–1091.

Allen C., Buttner S., Aragon A.D., Thomas J.A., Meirelles O., Jaetao J.E., Benn D., Ruby S.W., Veenhuis M., Madeo F., Werner-Washburne M. 2006. Isolation of quiescent and nonquiescent cells from yeast stationary-phase cultures. J. Cell. Biol. 174: 89–100.

Almeida J.R.M., Modig T., Petersson A., Hähn-Hägerdal B., Lidén G., Gorwa-Grauslund M.-F. 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae.* J. Chem. Technol. Biotechnol. 82: 340–349.

Ambesi A., Miranda M., Petrov V.V., Slayman C.W. 2000. Biogenesis and function of the yeast plasma-membrane H$^+$-ATPase. J. Exp. Biol. 203: 155–60.

Ask M., Bettiga M., Mapelli V., Olsson L. 2013. The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing *Saccharomyces cerevisiae*. Biotechnol. Biofuels 6: 22.

Bagar T., Altenbach K., Read N.D., Benčina M. 2009. Live-Cell Imaging and measurement of intracellular pH in filamentous fungi using a genetically encoded ratiometric probe. Eukaryot. Cell 8: 703–712.

Bakker B.M., Overkamp K.M., van Maris A.J., Kötter P., Luttik M.A., van Dijken J.P., Pronk J.T. 2001. Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 25: 15–37.

Bauer B.E., Rossington D., Mollapour M., Mamnun Y., Kuchler K., Piper P.W. 2003. Weak organic acid stress inhibits aromatic amino acid uptake by yeast, causing a strong influence of amino acid auxotrophies on the phenotypes of membrane transporter mutants. Eur. J. Biochem. 270: 3189–3195.

Berg J., Hung Y.P., Yellen G. 2009. A genetically encoded fluorescent reporter of ATP:ADP ratio. Nat. Methods 6: 161–166.

Berghäll S., Hilditch S., Penttilä M., Richard P. 2007. Identification in the mould *Hypocrea jecorina* of a gene encoding an NADP($^+$): D-xylose dehydrogenase. FEMS Microbiol. Lett. 277: 249–253.

Bermejo C., Ewald J.C., Lanquar V., Jones A.M., Frommer W.B. 2011. *In vivo* biochemistry: quantifying ion and metabolite levels in individual cells or cultures of yeast. Biochem. J. 438: 1–10.

Bertilsson M., Andersson J., Lidén G. 2008. Modeling simultaneous glucose and xylose uptake in *Saccharomyces cerevisiae* from kinetics and gene expression of sugar transporters. Bioproc. Biosyst. Eng. 31: 369–377.

Billard P., Ménart S., Fleer R., Bolotin-Fukuhara M. 1995. Isolation and characterization of the gene encoding xylose reductase from *Kluyveromyces lactis*. Gene 162: 93–97.

Boles E., Hollenberg C.P. 1997. The molecular genetics of hexose transport in yeasts. FEMS Microbiol. Rev. 21: 85–111.

Bracey D., Holyoak C.D., Coote P.J. 1998. Comparison of the inhibitory effect of sorbic acid and amphotericin B on *Saccharomyces cerevisiae*: is growth inhibition dependent on reduced intracellular pH? J. Appl. Microbiol. 85: 1056–1066.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Brohée S., Barriot R., Moreau Y., André B. 2010. YTPdb: A wiki database of yeast membrane transporters. Biochim. Biophys. Acta. 1798 :1908–1912.

Bruinenberg P.M., Van Dijken J.P., Scheffers W.A. 1983. A theoretical analysis of NADPH production and consumption in yeasts. J. Gen. Microbiol. 129: 953–964.

Buchert J. 1990. Biotechnical oxidation of D-xylose and hemicellulose hydrolyzates by *Gluconobacter oxydans*. PhD thesis. Publication 70, VTT Technical Research Centre of Finland. 53 p. + app. 46 p.

Buchert J., Puls J., Poutanen K. 1988. Comparison of *Pseudomonas fragi* and *Gluconobacter oxydans* for production of xylonic acid from hemicellulose hydrolyzates. Appl. Microbiol. Biotechnol. 28: 367–372.

Buchert J., Viikari L. 1988. The role of xylonolactone in xylonic acid production by *Pseudomonas fragi.* Appl. Microbiol. Biotechnol. 27: 333–336.

Cai Z., Zhang B., Li Y. 2012. Engineering *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: Reflections and perspectives. Biotechnol. J. 7: 34–46.

Cao Y., Xian M., Zou H., Zhang H. 2013. Metabolic Engineering of *Escherichia coli* for the production of xylonate. PLoS ONE 8: e67305.

Casal M., Cardoso H., Leao C. 1996. Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae.* Microbiology 142: 1385–1390.

Casal M., Paiva S., Andrade R.P., Gancedo C., Leão C. 1999. The lactate-proton symport of *Saccharomyces cerevisiae* is encoded by *JEN1.* J. Bacteriol. 181: 2620–2623.

Chalfie M., Tu Y., Euskirchen G., Ward W.W., Prasher D.C. 1994. Green fluorescent protein as a marker for gene expression. Science 263: 802–805.

Chan G.F., Gan H.M., Ling H.L., Rashid N.A.A. 2012. Genome sequence of *Pichia kudriavzevii* M12, a potential producer of bioethanol and phytase. Eukaryot. Cell 11: 1300–1301.

Chun B.W., Dair B., Macuch P.J., Wiebe D., Porteneuve C., Jeknavorian A. 2006. The development of cement and concrete additive: based on xylonic acid derived via bioconversion of xylose. Appl. Biochem. Biotechnol. 129–132: 645–658.

Colman-Lerner A., Gordon A., Serra E., Chin T., Resnekov O., Endy D., Gustavo Pesce C., Brent R. 2005. Regulated cell-to-cell variation in a cell-fate decision system. Nature 437: 699–706.

Cui Z., Hirata D., Tsuchiya E., Osada H., Miyakawa T. 1996. The multidrug resistance-associated protein (MRP) subfamily (Yrs1/Yor1) of *Saccharomyces cerevisiae* is important for the tolerance to a broad range of organic anions. J. Biol. Chem. 271: 14712–14716.

Dahms S.A.1974. 3-Deoxy-D-pentulosonic acid aldolase and its role in a new pathway of D-xylose degradation. Biochem. Biophys. Res. Commun. 4: 1433–1439.

de Jong E. 2011. Bio-based chemicals – Value added products from biorefineries. Report prepared for IEA Bioenergy – Task 42 Biorefinery.

de Koning W., van Dam K. 1992. A method for the determination of changes of glycolytic metabolites in yeast on a subsecond time scale using extraction at neutral pH. Anal. Biochem. 204: 118–123.

Dhaliwal S.S., Oberoi H.S., Sandhu S.K., Nanda D., Kumar D., Uppal S.K. 2011. Enhanced ethanol production from sugarcane juice by galactose adaptation of a newly isolated thermotolerant strain of *Pichia kudriavzevii*. Bioresour. Technol. 102: 5968–5975.

Diakov T.T., Kane P.M. 2010. Regulation of vacuolar proton-translocating ATPase activity and assembly by extracellular pH. J. Biol. Chem. 285: 23771–23778.

Dragosits M., Mattanovich D. 2013. Adaptive laboratory evolution-principles and applications for biotechnology. Microb. Cell Fact. 12: 64.

Dunlop A. 1948. Furfural formation and behavior. Ind. Eng. Chem. 40: 204–209.

Edwards M.C., Doran-Peterson J. 2012. Pectin-rich biomass as feedstock for fuel ethanol production. Appl. Microbiol. Biotechnol. 95: 565–575.

Engler C., Gruetzner R., Kandzia R., Marillonnet S. 2009. Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. PLoS One 4: e5553.

Fernandes A.R., Mira N.P., Vargas R.C., Canelhas I., Sá-Correia I. 2005. *Saccharomyces cerevisiae* adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes. Biochem. Biophys. Res. Commun. 337: 95–103.

Finogenova T., Morgunov I., Kamzolova S., Chernyavskaya O. 2005. Organic acid production by the yeast *Yarrowia lipolytica*: a review of prospects. Appl. Biochem. Microbiol. 41: 418–425.

Fonseca G.G., Heinzle E., Wittmann C., Gombert A.K. 2008. The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl. Microbiol. Biotechnol. 79: 339–354.

Förster J., Famili I., Fu P., Palsson B.Ø, Nielsen J. 2003. Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. Genome Res. 13: 244–253.

Gancedo C., Serrano R. 1989. Energy yielding metabolism. In: Rose A.H., Harrison J.S. (eds.). The Yeasts. Academic Press, London, UK. Pp. 205–259.

Gárdonyi M., Jeppsson M., Lidén G., Gorwa-Grauslund M.-F., Hahn-Hägerdal B. 2003. Control of xylose consumption by xylose transport in recombinant *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 82: 818–824.

Giaever G., Chu A.M., Ni L., Connelly C., Riles L., Veronneau S., Dow S., Lucau-Danila A., Anderson K., Andre B., Arkin A.P., Astromoff A., El-Bakkoury M., Bangham R., Benito R., Brachat S., Campanaro S., Curtiss M., Davis K., Deutschbauer A., Entian K.D., Flaherty P., Foury F., Garfinkel D.J., Gerstein M., Gotte D., Guldener U., Hegemann J.H., Hempel S., Herman Z., Jaramillo D.F., Kelly D.E., Kelly S.L., Kotter P., LaBonte D., Lamb D.C., Lan N., Liang H., Liao H., Liu L., Luo C., Lussier M., Mao R., Menard P., Ooi S.L., Revuelta J.L., Roberts C.J., Rose M., Ross-Macdonald P., Scherens B., Schimmack G., Shafer B., Shoemaker D.D., Sookhai-Mahadeo S., Storms R.K., Strathern J.N., Valle G., Voet M., Volckaert G., Wang C.Y., Ward T.R., Wilhelmy J., Winzeler E.A., Yang Y., Yen G., Youngman E., Yu K., Bussey H., Boeke J.D., Snyder M., Philippsen P., Davis R.W., Johnston M. 2002. Functional profiling of the Saccharomyces *cerevisiae* genome. Nature 418: 387–391.

Gibson D.G., Glass J.I., Lartigue C., Noskov V.N., Chuang R., Algire M.A., Benders G.A., Montague M.G., Ma L., Moodie M.M. 2010. Creation of a bacterial cell controlled by a chemically synthesized genome. Science 329: 52–56.

Gibson D.G., Young L., Chuang R., Venter J.C., Hutchison C.A., Smith H.O. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods. 6: 343–345.

Gietz D., St. Jean A., Woods R.A., Schiestl R.H. 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. 20: 1425.

Goffeau A., Slayman C.W. 1981. The proton-translocating ATPase of the fungal plasma membrane. Biochim. Biophys. 639: 197–223.

Goffeau A., Barrell B.G., Bussey H., Davis R.W., Dujon B., Feldmann H., Galibert F., Hoheisel J.D., Jacq C., Johnston M., Louis E.J., Mewes H.W., Murakami Y.,

Philippsen P., Tettelin H., Oliver S.G. 1996. Life with 6000 genes. Science 274: 546, 563–567.

González-Siso M.I., García-Leiro A., Tarrío N., Cerdán M.E. 2009. Sugar metabolism, redox balance and oxidative stress response in the respiratory yeast *Kluyveromyces lactis*. Microb. Cell Fact. 8: 46.

Graham L.A., Flannery A.R., Stevens T.H. 2003. Structure and assembly of the yeast V-ATPase. J. Bioenerg. Biomembr. 35: 301–312.

Gregori C., Schüller C., Frohner I.E., Ammerer G., Kuchler K. 2008. Weak organic acids trigger conformational changes of the yeast transcription factor War1 in vivo to elicit stress adaptation. J. Biol. Chem. 283: 25752–25764.

Guadalupe Medina V.G. 2013. Novel strategies for engineering redox metabolism in Saccharomyces cerevisiae. PhD thesis, Delft University of Technology, The Netherlands.

Hahn-Hägerdal B., Karhumaa K., Jeppsson M., Gorwa-Grauslund M.-F. 2007. Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. Adv. Biochem. Eng. Biotechnol. 108: 147–77.

Halm M., Hornbaek T., Arneborg N., Sefa-Dedeh S., Jespersen L. 2004. Lactic acid tolerance determined by measurement of intracellular pH of single cells of *Candida krusei* and *Saccharomyces cerevisiae* isolated from fermented maize dough. Int. J. Food Microbiol. 94: 97–103.

Harhangi H.R., Akhmanova A.S., Emmens R., van der Drift C., de Laat W. T.A.M., van Dijken J.P., Jetten M.S.M., Pronk J.T., Op den Camp H.J.M. 2003. Xylose metabolism in the anaerobic fungus *Piromyces* sp. strain E2 follows the bacterial pathway. Arch. Microbiol. 180:134-141.

Hatzixanthis K., Mollapour M., Seymour I., Bauer B.E., Krapf G., Schüller C., Kuchler K., Piper P.W. 2003. Moderately lipophilic carboxylate compounds are the selective inducers of the *Saccharomyces cerevisiae* Pdr12p ATP-binding cassette transporter. Yeast 20: 575-585.

Hayashi M., Fukuzawa T., Sorimachi H., Maeda T. 2005. Constitutive activation of the pH-responsive Rim101 pathway in yeast mutants defective in late steps of the MVB/ESCRT pathway. Mol. Cell. Biol. 25: 9478–9490.

Hedbacker K., Carlson M. 2008. SNF1/AMPK pathways in yeast. Front. Biosci. 1: 2408–20.

Heggart H., Margaritis A., Stewart R., Pilkington H., Sobczak J., Russell I. 2000. Measurement of brewing yeast viability and vitality: a review of methods, Mast. Brew. Ass. Amer. Tech. Quart. 37: 409–430.

Heipieper H.J., Weber F.J., Sikkema J., Keweloh H., de Bont J.A. 1994. Mechanisms of resistance of whole cells to toxic organic solvents. Trends Biotechnol. 12: 409–415.

Henne W.M., Buchkovich N.J., Emr S.D. 2011. The ESCRT pathway. Dev. Cell 21: 77–91.

Ho N.W., Chen Z., Brainard A.P. 1998. Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. Appl. Environ. Microbiol. 64: 1852–1859.

Holyoak C.D., Bracey D., Piper P.W., Kuchler K., Coote P.J. 1999. The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter Pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. J. Bacteriol. 181: 4644.

Holyoak C.D., Thompson S., Ortiz Calderon C., Hatzixanthis K., Bauer B., Kuchler K., Piper P.W., Coote P.J. 2000. Loss of Cmk1 Ca^{2+}-calmodulin-dependent protein kinase in yeast results in constitutive weak organic acid resistance, associated with a post-transcriptional activation of the Pdr12 ATP-binding cassette transporter. Mol. Microbiol. 37: 595–605.

Hung Y., Albeck J., Tantama M., Yellen G. 2011. Imaging cytosolic $NADH-NAD^+$ redox state with a genetically encoded fluorescent biosensor. Cell Metab. 14: 545–554.

Hyde M., Block-Alper L., Felix J., Webster P., Meyer D.I. 2002. Induction of secretory pathway components in yeast is associated with increased stability of their mRNA. J. Cell Biol. 156: 993–1001.

Ilmén M., Koivuranta K., Ruohonen L., Rajgarhia V., Suominen P., Penttilä M. 2013. Production of L-lactic acid by the yeast *Candida sonorensis* expressing heterologous bacterial and fungal lactate dehydrogenases. Microb. Cell Fact. 12: 53.

Ilmén M., Koivuranta K., Ruohonen L., Suominen P., Penttilä M. 2007. Efficient production of L-lactic acid from xylose by *Pichia stipitis*. Appl. Environ. Microbiol. 73: 117–123.

Imamura H., Huynh Nhat K.P., Togawa H., Saito K., Iino R., Kato-Yamada Y., Nagai T., Noji H. 2009. Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators. PNAS 106: 15651–15656.

Ishizaki H., Ihara T., Yoshitake J., Shimamura M., Imai T. 1973. D-xylonic acid production by *Enterobacter cloacae*. Nippon Nogei Kagaku Kaishi 47: 755–761.

Isom D.G., Sridharan V., Baker R., Clement S.T., Smalley D.M., Dohlman H.G. 2013. Protons as second messenger regulators of G protein signaling. Mol. Cell 51: 531–538.

Isono N., Hayakawa H., Usami A., Mishima T., Hisamatsu M. 2012. A comparative study of ethanol production by *Issatchenkia orientalis* strains under stress conditions. J. Biosci. Bioeng. 113: 76–78.

Jeffries T.W., Jin Y. 2004. Metabolic engineering for improved fermentation of pentoses by yeasts. Appl. Microbiol. Biotechnol. 63: 495–509.

Jeffries T.W., Shi N.Q. 1999. Genetic engineering for improved xylose fermentation by yeasts. Adv. Biochem. Eng. Biotechnol. 65:117-61.

Johnsen U., Schönheit P. 2004. Novel xylose dehydrogenase in the halophilic archaeon *Haloarcula marismortui*. J. Bacteriol. 186: 6198–6207.

Johnsen U., Dambeck M., Zaiss H., Fuhrer T., Soppa J., Sauer U., Schönheit P. 2009. D-xylose degradation pathway in the halophilic archaeon *Haloferax volcanii*. J. Biol. Chem. 284: 27290–27303.

Kanauchi M., Bamforth C. 2003. Use of xylose dehydrogenase from *Trichoderma viride* in an enzymic method for the measurement of pentosan in barley. J. I. Brewing. 109: 203–207.

Kane P.M. 1995. Disassembly and reassembly of the yeast vacuolar H^+-ATPase *in vivo*. J. Biol. Chem. 270: 17025–17032.

Kane P.M. 2006. The where, when, and how of organelle acidification by the yeast vacuolar H-ATPase. Microbiol. Mol. Biol. Rev. 70: 177–191.

Karhumaa K., Hahn-Hägerdal B., Gorwa-Grauslund M.-F. 2005. Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. Yeast 22: 359–368.

Karpenshif Y., Bernstein K.A. 2012. From yeast to mammals: recent advances in genetic control of homologous recombination. DNA Repair 11: 781–788.

Keasling J.D. 2012. Synthetic biology and the development of tools for metabolic engineering. Metab. Eng. 14: 189–195.

Kiesling H., Lindberg B., McKay J. 1962. Some products of the metabolism of D-xylose by *Pullularia pullulans*. Acta. Chem. Scand. 16: 1858–1862.

Keil R.L., Roeder G.S. 1984. *Cis*-acting, recombination-stimulating activity in a fragment of the ribosomal DNA of *S. cerevisiae*. Cell 39: 377–386.

Kitagawa T., Tokuhiro K., Sugiyama H., Kohda K., Isono N., Hisamatsu M., Takahashi H., Imaeda T. 2010. Construction of a β-glucosidase expression system using the multistress-tolerant yeast *Issatchenkia orientalis*. Appl. Microbiol. Biotechnol. 87: 1841–1853.

Koivistoinen O.M., Kuivanen J., Barth D., Turkia H., Pitkänen J., Penttilä M., Richard P. 2013. Glycolic acid production in the engineered yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Microb. Cell Fact. 12: 82.

Konings W., Lolkema J., Bolhuis H., Van Veen H., Poolman B., Driessen A. 1997. The role of transport processes in survival of lactic acid bacteria, Energy transduction and multidrug resistance. A. Van Leeuw. 71: 117–128.

Kötter P., Amore R., Hollenberg C.P., Ciriacy M. 1990. Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene, *XYL2*, and construction of a xylose-utilizing Saccharomyces cerevisiae transformant. Curr. Genet. 18: 493–500.

Kötter P., Ciriacy M. 1993. Xylose fermentation by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 38: 776–783.

Krahulec S., Petschacher B., Wallner M., Longus K., Klimacek M., Nidetzky B. 2010. Fermentation of mixed glucose-xylose substrates by engineered strains of *Saccharomyces cerevisiae*: role of the coenzyme specificity of xylose reductase, and effect of glucose on xylose utilization. Microb. Cell Fact. 9: 16.

Kren A., Mamnun Y.M., Bauer B.E., Schüller C., Wolfger H., Hatzixanthis K., Mollapour M., Gregori C., Piper P., Kuchler K. 2003. War1p, a novel transcription factor controlling weak acid stress response in yeast. Mol. Cell. Biol. 23: 1775–1785.

Kuyper M., Harhangi H.R., Stave A.K., Winkler A.A., Jetten M.S.M., de Laat W.T., Ridder J.J.J., Op den Camp H.J.M., Dijken J.P., Pronk J.T. 2003. High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? FEMS Yeast Res. 4: 69–78.

Kwon Y., Ma A., Li Q., Wang F., Zhuang G., Liu C. 2011. Effect of lignocellulosic inhibitory compounds on growth and ethanol fermentation of newly-isolated thermotolerant *Issatchenkia orientalis*. Bioresour. Technol. 102: 8099–8104.

Lachke A. 2002. Biofuel from D-xylose – The second most abundant sugar. Resonance 7: 50–58.

Lawlis V.B., Dennis M.S., Chen E.Y., Smith D.H., Henner D.J. 1984. Cloning and sequencing of the xylose isomerase and xylulose kinase genes of *Escherichia coli.* Appl. Environ. Microbiol. 47:15-21.

Li W., Mitchell A.P. 1997. Proteolytic activation of Rim1p, a positive regulator of yeast sporulation and invasive growth. Genetics 145: 63–73.

Lien O.G. 1959. Determination of gluconolactone, galactonolactone, and their free acids by hydroxamate method. Anal. Chem. 31: 1363–1366.

Lin C., Lovén J., Rahl P., Paranal R., Burge C., Bradner J., Lee T., Young R. 2012. Transcriptional amplification in tumor cells with elevated c-Myc. Cell 151: 56–67.

Liu E., Hu Y. 2010. Construction of a xylose-fermenting *Saccharomyces cerevisiae* strain by combined approaches of genetic engineering, chemical mutagenesis and evolutionary adaptation. Biochem. Eng. J. 48: 204–210.

Liu H., Valdehuesa K.N.G., Nisola G.M., Ramos K.R.M., Chung W. 2012. High yield production of D-xylonic acid from D-xylose using engineered *Escherichia coli.* Bioresour. Technol. 115: 244–248.

Lourenço A., Ascenso J., Sá-Correia I. 2011. Metabolic insights into the yeast response to propionic acid based on high resolution 1H NMR spectroscopy. Metabolomics 7: 457–468.

Ludovico P., Sousa M.J., Silva M.T., Leao C., Corte-Real M. 2001. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. Microbiology 147: 2409–2415.

Ma H., Kunes S., Schatz P.J., Botstein D. 1987. Plasmid construction by homologous recombination in yeast. Gene 58: 201–216.

Maeda T. 2012. The signaling mechanism of ambient pH sensing and adaptation in yeast and fungi. FEBS J. 279: 1407–1413.

Magnuson J.K., Lasure L.L. 2004. Organic acid production by filamentous fungi. In: Tkacz J.S., Lange L. (eds) Advances in fungal biotechnology for industry, agriculture and medicine. Kluwer Academic/Plenum Publishers, USA. Pp. 307–340.

Maresová L., Hodková B., Urbánková E., Chaloupka R., Sychrová H. 2010. New applications of pHluorin measuring intracellular pH of prototrophic yeasts and determining changes in the buffering capacity of strains with affected potassium homeostasis. Yeast 27: 317–325.

Margaritis A., Bajpai P. 1982. Direct fermentation of D-xylose to ethanol by *Kluyveromyces marxianus* strains. Appl. Environ. Microbiol. 44: 1039–1041.

Martínez K., de Anda R., Hernández G., Escalante A., Gosset G., Ramírez O.T., Bolívar F.G. 2008. Coutilization of glucose and glycerol enhances the production of aromatic compounds in an *Escherichia coli* strain lacking the phosphoenolpyruvate: carbohydrate phosphotransferase system. Microb. Cell Fact. 7: 1.

Martínez-Muñoz G.A., Kane P. 2008. Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast. J. Biol. Chem. 283: 20309–20319.

Matthews T.M., Webb C. 1991. Culture systems. In: Tuite M., Oliver S. (eds). *Saccharomyces* biotechnology handbooks (Volume 4). Springer, USA. Pp. 249–282.

Meijnen J., de Winde J.H., Ruijssenaars H.J. 2009. Establishment of oxidative D-xylose metabolism in *Pseudomonas putida* S12. Appl. Environ. Microbiol. 75: 2784–2791.

Micolonghi C., Corsi E., Conte R., Bianchi M.M. 2007. Heterologous products from the yeast *Kluyveromyces lactis*: exploitation of KlPDC1, a single-gene based system. Formatex Research Centre, Extremadura, Spain. pp 271–282.

Miesenböck G., De Angelis D.A., Rothman J.E. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 394: 192–195.

Miller C., Fosmer A., Rush B., McMullin T., Beacom D., Suominen P. 2011. Industrial Production of Lactic Acid. In: Murray Moo-Young (ed). Comprehensive Biotechnology (Second Edition). Academic Press, Burlington, USA. Pp. 179–188.

Mills T.Y., Sandoval N.R., Gill R.T. 2009. Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*. Biotechnol. Biofuels. 2: 26.

Mira N.P., Lourenço A.B., Fernandes A.R., Becker J.D., Sá-Correia I. 2009. The RIM101 pathway has a role in *Saccharomyces cerevisiae* adaptive response and resistance to propionic acid and other weak acids. FEMS Yeast Res. 9: 202–216.

Mira N.P., Teixeira M.C. Sá-Correia I. 2010. Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: A genome-wide view. OMICS J. Integr. Biol. 14: 525-540.

Mollapour M., Piper P.W. 2007. Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. Mol. Cell. Biol. 27: 6446.

Murray A.W., Szostak J.W. 1983. Construction of artificial chromosomes in yeast. Nature 305: 189–193.

Nelson H., Nelson N. 1990. Disruption of genes encoding subunits of yeast vacuolar H^+-ATPase causes conditional lethality. PNAS 87: 3503–3507.

Nita V., Benini L., Ciupagea C., Kavalov B., Pelletier N. 2013. Bio-economy and sustainability: a potential contribution to the Bio-economy Observatory. Report EUR 25743 EN. European Commission, JRC Scientific and policy reports.

Niu W., Molefe M.N., Frost J.W. 2003. Microbial synthesis of the energetic material precursor 1,2,4-Butanetriol. J. Am. Chem. Soc. 125: 12998-12999.

Olsson L., Hahn-Hägerdal B. 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb. Technol. 18: 312–331.

Orij R., Urbanus M.L., Vizeacoumar F.J., Giaever G., Boone C., Nislow C., Brul S., Smits G.J. 2012. Genome-wide analysis of intracellular pH reveals quan-

titative control of cell division rate by pH(c) in *Saccharomyces cerevisiae*. Genome Biol. 13: R80.

Orij R., Postmus J., Ter Beek A., Brul S., Smits G.J. 2009. In vivo measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in *Saccharomyces cerevisiae* reveals a relation between intracellular pH and growth. Microbiology 155: 268–278.

Pacheco A., Talaia G., Sá-Pessoa J., Bessa D., Gonçalves M.J., Moreira R., Paiva S., Casal M., Queirós O. 2012. Lactic acid production in *Saccharomyces cerevisiae* is modulated by expression of the monocarboxylate transporters Jen1 and Ady2. FEMS Yeast Res. 12: 375–381.

Painting K., Kirsop B. 1990. A quick method for estimating the percentage of viable cells in a yeast population, using methylene blue staining. World J. Microb. Biot. 6: 346–347.

Paiva S., Devaux F., Barbosa S., Jacq C., Casal M. 2004. Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*. Yeast 21: 201–210.

Palma M., Goffeau A., Spencer-Martins I., Baret P.V. 2007. A phylogenetic analysis of the sugar porters in hemiascomycetous yeasts. J. Mol. Microbiol. Biotechnol. 12: 241–248.

Palmqvist E., Hahn-Hägerdal B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour. Technol. 74: 25–33.

Papanikolaou S., Aggelis G. 2011. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. Eur. J. Lipid Sci. Tech. 113: 1031–1051.

Partow S. 2012. Novel synthetic biology tools for metabolic engineering of *Saccharomyces cerevisiae.* PhD thesis. Chalmers University of Technology.

Peñalva M.A., Tilburn J., Bignell E., Arst Jr. H.N. 2008. Ambient pH gene regulation in fungi: making connections. Trends Microbiol. 16: 291–300.

Perlin D.S., Harris S., Seto-Young D., Haber J. 1989. Defective H$^+$-ATPase of hygromycin B-resistant *pma1* mutants from *Saccharomyces cerevisiae*. J. Biol. Chem. 264: 21857–21864.

Pezzotti F., Therisod M. 2006. Enzymatic synthesis of aldonic acids. Carbohydr. Res. 341: 2290–2292.

Piper P.W. 1999. Yeast superoxide dismutase mutants reveal a pro-oxidant action of weak organic acid food preservatives. Free Radical Biol. Med. 27: 1219–1227.

Piper P., Mahé Y., Thompson S., Pandjaitan R., Holyoak C., Egner R., Mühlbauer M., Coote P., Kuchler K. 1998. The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J. 17: 4257–4265.

Piper P., Calderon C.O., Hatzixanthis K., Mollapour M. 2001. Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiology 147: 2635–2642.

Pitkänen J.P., Aristidou A., Salusjärvi L., Ruohonen L., Penttilä M. 2003. Metabolic flux analysis of xylose metabolism in recombinant *Saccharomyces cerevisiae* using continuous culture. Metab. Eng. 5: 16–31.

Pitkänen J.P., Rintala E., Aristidou A., Ruohonen L., Penttilä M. 2005. Xylose chemostat isolates of *Saccharomyces cerevisiae* show altered metabolite and enzyme levels compared with xylose, glucose, and ethanol metabolism of the original strain. Appl. Microbiol. Biotechnol. 67: 827–837.

Pollak N., Dolle C., Ziegler M. 2007. The power to reduce: pyridine nucleotides-small molecules with a multitude of functions. Biochem. J. 402: 205–218.

Porro D., Bianchi M.M., Brambilla L., Menghini R., Bolzani D., Carrera V., Lievense J., Liu C.L., Ranzi B.M., Frontali L., Alberghina L. 1999. Replacement of a metabolic pathway for large-scale production of lactic acid from engineered yeasts. Appl. Environ. Microbiol. 65: 4211–4215.

Prasher D.C., Eckenrode V.K., Ward W.W., Prendergast F.G., Cormier M.J. 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111: 229–233.

Preston R., Murphy R., Jones E. 1989. Assay of vacuolar pH in yeast and identification of acidification-defective mutants. PNAS 86: 7027–7031.

Puopolo K., Forgac M. 1990. Functional reassembly of the coated vesicle proton pump. J. Biol. Chem. 265: 14836–14841.

Rahikainen J. 2013. Cellulase-lignin interactions in the enzymatic hydrolysis of lignocellulose. PhD thesis. VTT Science 41 VTT Technical Research

Centre of Finland. 91 p. app. 44 p. http://www.vtt.fi/inf/pdf/science/2013/S41.pdf

Ramachandran S., Fontanille P., Pandey A., Larroche C. 20

06. Gluconic acid: properties, applications and microbial production. Food Technol Biotech 44: 185–195.

Ray B. 1996. Fundamental food microbiology. CRC Press, Boca Raton,USA.

Sachs K., Perez O., Pe'er D., Lauffenburger D.A., Nolan G.P. 2005. Causal protein-signaling networks derived from multiparameter single-cell data. Science 308: 523–529.

Saloheimo A., Rauta J., Stasyk O., Sibirny A., Penttilä M., Ruohonen L. 2007. Xylose transport studies with xylose-utilizing *Saccharomyces cerevisiae* strains expressing heterologous and homologous permeases. Appl. Microbiol. Biotechnol. 74: 1041–1052.

Sauer B. 1987. Functional expression of the cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. 7: 2087–2096.

Sauer M., Porro D., Mattanovich D., Branduardi P. 2008. Microbial production of organic acids: expanding the markets. Trends Biotech. 26: 100–108.

Sauer M., Porro D., Mattanovich D., Branduardi P. 2010. 16 years research on lactic acid production with yeast–ready for the market? Biotechnol. Genet. Eng. 27: 229–256.

Schlake T., Bode J. 1994. Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. Biochemistry 33: 12746–12751.

Schüller C., Mamnun Y.M., Mollapour M., Krapf G., Schuster M., Bauer B.E., Piper P.W., Kuchler K. 2004. Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in *Saccharomyces cerevisiae.* Mol. Biol. Cell 15: 706-720.

Semchyshyn H.M., Abrat O.B., Miedzobrodzki J., Inoue Y., Lushchak V.I. 2011. Acetate but not propionate induces oxidative stress in bakers' yeast *Saccharomyces cerevisiae*. Redox Rep. 16: 15–23.

Serrano R., Kielland-Brandt M., Fink G.R. 1986. Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+ + K^+), K^+- and Ca^{2+}-ATPases. Nature 319: 689–693.

Shah P., Zhu X., Chen C., Hu Y., Li C. 2013. Lab-on-chip device for single cell trapping and analysis. Biomed. Microdevices 1–7.

Sherman F., Fink G., Hicks J.B. 1983. Methods in yeast genetics. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, USA.

Shimomura O., Johnson F.H., Saiga Y. 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. J. Cell. Comp. Physiol. 59: 223–239.

Shinohara A., Ogawa H., Ogawa T. 1992. Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. Cell 69: 457–470.

Shoemaker D.D., Lashkari D.A., Morris D., Mittmann M., Davis R.W. 1996. Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar–coding strategy. Nat. Genet. 14: 450–456.

Simões T., Mira N.P., Fernandes A.R., Sá-Correia I. 2006. The *SPI1* gene, encoding a glycosylphosphatidylinositol-anchored cell wall protein, plays a prominent role in the development of yeast resistance to lipophilic weak-acid food preservatives. Appl. Environ. Microbiol. 72: 7168.

Singh V. 2014. Recent advancements in synthetic biology: Current status and challenges. Gene 535: 1–11.

Skory C. 2003. Lactic acid production by *Saccharomyces cerevisiae* expressing a *Rhizopus oryzae* lactate dehydrogenase gene. J. Ind. Microbiol. Biotech. 30: 22–27.

Sonderegger M., Schumperli M., Sauer U. 2004. Metabolic engineering of a phosphoketolase pathway for pentose catabolism in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 70: 2892–2897.

Spencer J., de Spencer A.R., Laluce C. 2002. Non-conventional yeasts. Appl. Microbiol. Biotechnol. 58: 147–156.

Stephanopoulos G. 2012. Synthetic biology and metabolic engineeringring. ACS Synth. Biol.1: 514–525.

Stephens C., Christen B., Fuchs T., Sundaram V., Watanabe K., Jenal U. 2006. Genetic analysis of a novel pathway for D-xylose metabolism in *Caulobacter crescentus*. J. Bacteriol. 189: 2181–2185.

Subtil T., Boles E. 2012. Competition between pentoses and glucose during uptake and catabolism in recombinant Saccharomyces cerevisiae. Biotechnol. Biofuels 5: 14.

Suominen P., Aristidou A., Penttilä M., Ilmén M., Ruohonen L., Koivuranta K., Robert-Perez K. 2007. Genetically modified yeast of the species *Issatchenkia orientalis* and closely related species and fermentation processes using same. US Patent 2009/0226989 A1.

Suzuki T., Onishi H. 1973. Oxidation and reduction of D-xylose by cell-free extract of *Pichia quercuum*. Appl. Microbiol. 25: 850–852.

Tarrío N., Cerdán M.E., González Siso M.I. 2006. Characterization of the second external alternative dehydrogenase from mitochondria of the respiratory yeast *Kluyveromyces lactis*. Biochim. Biophys. Acta 1757: 1476–1484.

Toivari M.H., Aristidou A., Ruohonen L., Penttilä M. 2001. Conversion of Xylose to Ethanol by Recombinant *Saccharomyces cerevisiae*: Importance of Xylulokinase (XKS1) and Oxygen Availability. Metab. Eng. 3: 236-249.

Toivari M.H., Ruohonen L., Richard P., Penttilä M., Wiebe M.G. 2010. *Saccharomyces cerevisiae* engineered to produce D-xylonate. Appl. Microbiol. Biotechnol. 88: 751–760.

Toivola A., Yarrow D., Van Den Bosch E., Van Dijken J.P., Scheffers W. A. 1984. Alcoholic fermentation of D-xylose by yeasts. Appl. Environ. Microbiol. 47: 1221–1223.

Ulbricht R.J., Northup S.J., Thomas J.A. 1984. A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions. Fund. Appl.Toxicol. 4: 843–853.

Ullah A., Chandrasekaran G., Brul S., Smits G.J. 2013. Yeast adaptation to weak acids prevents futile energy expenditure. Front. Microbiol. 4:142.

Ullah A., Orij R., Brul S., Smits G.J. 2012. Quantitative analysis of the modes of growth inhibition by weak organic acids in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 78: 8377–8387.

Valkonen M., Mojzita D., Penttilä M., Benčina M. 2013. Non-invasive high-throughput single-cell analysis of the intracellular pH of yeast by ratiometric flow cytometry. Appl. Environ. Microbiol. 79: 7179–87.

Valli M., Sauer M., Branduardi P., Borth N., Porro D., Mattanovich D. 2006. Improvement of lactic acid production in *Saccharomyces cerevisiae* by cell sorting for high intracellular pH. Appl. Environ. Microbiol. 72: 5492–5499.

van Beilen J.W., Brul S. 2013. Compartment-specific pH monitoring in *Bacillus subtilis* using fluorescent sensor proteins: a tool to analyze the antibacterial effect of weak organic acids. Front. Microbiol 4:157.

van Dijken J.P., Scheffers W.A. 1986. Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol. Lett. 32: 199–224.

van Dijken J.P., Weusthuis R.A., Pronk J.T. 1993. Kinetics of growth and sugar consumption in yeasts. A. Van Leeuw. 63: 343–352.

van Maris A.J.A., Konings W.N., van Dijken J.P., Pronk J.T. 2004. Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes. Metab. Eng. 6: 245–255.

Van Vleet J., Jeffries T. 2009. Yeast metabolic engineering for hemicellulosic ethanol production. Curr. Opin. Biotechnol. 20: 300–306.

Weimberg R. 1961. Pentose Oxidation by *Pseudomonas fragi.* J.Biol.Chem. 3: 629–635.

Veine D.M., Arscott L.D., Williams C.H. 1998. Redox potentials for yeast, *Escherichia coli* and human glutathione reductase relative to the NAD /NADH redox couple: enzyme forms active in catalysis. Biochemistry 37: 15575–15582.

Verduyn C., Postma E., Scheffers W.A., Van Dijken J.P. 1992. Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8: 501–517.

Verho R., Londesborough J., Penttilä M., Richard P. 2003. Engineering redox cofactor regeneration for improved pentose fermentation in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 69: 5892–5897.

Verho R., Richard P., Jonson P.H., Sundqvist L., Londesborough J., Penttilä M. 2002. Identification of the first fungal NADP-GAPDH from *Kluyveromyces lactis*. Biochemistry 41: 13833–13838.

Vojinović V., von Stockar U. 2009. Influence of uncertainties in pH, pMg, activity coefficients, metabolite concentrations, and other factors on the analysis of the thermodynamic feasibility of metabolic pathways. Biotechnol. Bioeng. 103: 780–795.

Wahlström R. 2014. Enzymatic hydrolysis of cellulose in aqueous ionic liquids. PhD thesis. VTT Technical Research Centre of Finland, Espoo. VTT Science 52. 102 p. + app. 57 p. http://www.vtt.fi/inf/pdf/science/2014/S52.pdf.

Walfridsson M., Bao X., Anderlund M., Lilius G., Bulow L., Hahn-Hägerdal B. 1996. Ethanolic fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus xylA* gene, which expresses an active xylose (glucose) isomerase. Appl. Environ. Microbiol. 62: 4648–4651.

Ward W.W. 2005. 3.4 Physical characteristics of GFP. In: Chalfie M., Kain S.R (ed.). Methods of biochemical analysis, Green fluorescent protein: properties, applications and protocols (second edition). John Wiley and Sons, USA, pp. 41–51.

Warren L. 2008. Single-cell gene-expression analysis by quantitative RT-PCR. PhD thesis. California Institute of Technology.

Weigert C., Steffler F., Kurz T., Shellhammer T.H., Methner F. 2009. Application of a short intracellular pH method to flow cytometry for determining *Saccharomyces cerevisiae* vitality. Appl. Environ. Microbiol. 75: 5615–5620.

Werpy T., Petersen G., Aden A., Bozell J., Holladay J., White J., Manheim A. 2004. Top value added chemicals from biomass. Volume I: Results of screening for potential candidates from sugars and synthesis gas. US Department of Energy report.

Wohlrab H., Flowers N. 1982. pH gradient-dependent phosphate transport catalyzed by the purified mitochondrial phosphate transport protein. J. Biol. Chem. 257: 28–31.

Zamora, F., Bueno, M., Molina, I., Iribarren, J.I., Muñoz-Guerra, S., Galbis, J.A. 2000. Stereoregular Copolyamides Derived from d-Xylose and l-Arabinose. Macromolecules. 33: 2030-2038.

Zdraljevic S., Wagner D., Cheng K., Ruohonen L., Jäntti J., Penttilä M., Resnekov O., Pesce G.C. 2013. Single-cell measurement of enzyme levels as a predic-

tive tool for cellular fates during organic acid production. Appl. Environ. Microbiol. 79: 7569–82.

Zepeda S., Monasterio O., Ureta T. 1990. NADP+-dependent D-xylose dehydrogenase from pig liver. Purification and properties. Biochem J. 266: 637–644.

Zhao Y., Jin J., Hu Q., Zhou H., Yi J., Yu Z., Xu L., Wang X., Yang Y., Loscalzo J. 2011. Genetically encoded fluorescent sensors for intracellular NADH detection. Cell Metab. 14: 555–566.

Title	**Production of D-xylonate and organic acid tolerance in yeast**
Author	Yvonne Nygård
Abstract	Various organic acids have huge potential as industrial platform chemicals. Biotechnological routes of organic acid production are currently being sought, so that fossil resources and petrochemistry could be replaced with renewable resources. Microbial production of organic acids imposes stresses on the organism and understanding the physiology of micro-organisms which have been genetically engineered to produce an organic acid, can make valuable contributions to the development of production organisms for biorefineries.

Production of D-xylonate, an industrial platform chemical with high application potential, was successfully demonstrated in various yeast species. D-xylonate is produced from D-xylose via D-xylono-γ-lactone that can be hydrolysed to D-xylonate spontaneously or with the aid of a lactonase enzyme. Various ways to improve production of D-xylonate in the yeast *Saccharomyces cerevisiae*, *Kluyveromyces lactis* or *Pichia kudriavzevii* as production organisms were successfully applied. The best D-xylonate production was obtained by expression of the D-xylose dehydrogenase encoding gene *xylB* from *Caulobacter crescentus* and the highest D-xylonate titre was achieved with *P. kudriavzevii* that produced 171 and 146 g D-xylonate l^{-1}, at a rate of 1.4 or 1.2 g l^{-1} h^{-1}, at pH 5.5 and pH 3, respectively.

The consequences of D-xylonate production on the physiology of *S. cerevisiae* were studied in detail, both at population and single-cell level. D-xylonate and D-xylono-γ-lactone were produced and also exported from the cells from the very start of cultivation in D-xylose, even in the presence of D-glucose. There was no apparent preference for export of either compound. However, great amounts of D-xylono-γ-lactone and/or D-xylonate was accumulated inside the cells during the production.

The D-xylonolactone lactonase encoding gene *xylC* was co-expressed with the D-xylose dehydrogenase encoding gene *xylB* (both genes from *C. crescentus*). This lead to a significant increase in the D-xylonate production rate compared to cells expressing only *xylB* and showed that accumulation of D-xylonate and protons releases during hydrolysis, was harmful for the cells. The accumulation of D-xylonate led to lost vitality and acidification of the cytosol, as determined by loss of pHluorin (a pH dependent fluorescent protein) fluorescence. This loss of fluorescence was faster in cells co-expressing *xylC* with *xylB* compared to cells expressing xylB alone. The decrease in vitality and challenges in export of D-xylonate are major obstacles for D-xylonate production by *S. cerevisiae*. The excellent D-xylonate producer, *P. kudriavzevii* also accumulated large amounts of D-xylonate and suffered decreased vitality, especially when D-xylonate was produced at low pH.

The stress response to weak organic acids is highly dependent on the properties of the acids and the presence of high concentrations of weak organic acids may lead to lost viability. The role of Pdr12, a membrane transporter, in resistance to weak organic acids was studied and found to be highly dependent on the acid. Deletion of *PDR12* led to improved tolerance to formic and acetic acids, a feature that makes this modification interesting for micro-organisms used in biorefining of lignocellulosic hydrolysates that commonly contain these acids.

Biotechnological production of D-xylonic acid with yeast clearly has the potential of becoming an industrially applicable process. In order for biotechnological production processes to become economically feasible, biorefinery approaches in which lignocellulosic hydrolysates or other biomass side- or waste streams are used as raw materials need to be employed. This thesis provides new understanding on how production of an organic acid affects the production host and presents novel approaches for studying and increasing the production. |
ISBN, ISSN	
Date	May 2014
Language	English, Swedish abstract
Pages	99 p. + app 84 p.
Keywords	Yeast, D-xylonate, metabolic engineering, organic acids, stress responses, cytosolic pH, Pdr12, D-xylose
Publisher	

Namn	# D-xylonat produktion och tolerans mot organiska syror i jäster
Författare	Yvonne Nygård
Referat	Organiska syror har en enorm potential som industriella plattformskemikalier. En bioteknisk produktion av organiska syror kunde ersätta produktionen av motsvarande, oljebaserade kemikalier. En mikrobiell produktion av organiska syror kan utgöra ett miljövänligt, hållbart sätt att producera kemikalier för industrin. För detta behövs effektiva processer och mikroorganismer med kapacitet att producera stora mängder syror. Dessvärre är syrorna ofta okända för produktionsorganismen och därmed medför produktionen av syra stora påfrestningar, vilket leder till stress. Denna stress påverkar vitaliteten, livskraften och produktiviteten hos cellerna i en bioprocess. Genom att förstå fysiologin hos mikroorganismer som är genetiskt manipulerade för att producera en organisk syra, kan nya produktionsorganismer för bioraffinaderier utvecklas. I ett bioraffinaderi kan jord-och skogsbruksavfall omvandlas till användbara kemikalier. D-xylonat, en industriell prekursorkemikalie med stor potential, kan produceras med hjälp av olika jästsvampar. D-xylonat framställs från D-xylos via D-xylono-γ-lakton, som kan hydrolyseras till linjär D-xylonat, spontant eller med hjälp av ett laktonas enzym. I denna studie förbättrades produktionen av D-xylonat märkbart med hjälp av jästerna *Saccharomyces cerevisiae*, *Kluyveromyces lactis* eller *Pichia kudriavzevii* som produktionsorganismer. Den bästa produktionen av D-xylonat erhölls genom att uttrycka *xylB*, en gen från *Caulobacter crescentus* som kodar för ett D-xylos dehydrogenas enzym. Den största D-xylonatproduktionen uppnåddes med *P. kudriavzevii*, som var kapabel att producera 171 eller 146 g D- xylonat l^{-1}, med en hastighet av 1.4 eller 1.2 g l^{-1} h^{-1}, vid pH 5.5 respektive pH 3. Det är fördelaktigt att producera syra vid ett lågt pH-värde, eftersom det gör uppsamlandet av syran enklare och därmed processen mer ekonomiskt lönsam. Konsekvenserna av D-xylonatproduktionen på *S. cerevisiae* jästens fysiologi studerades i detalj, både på populations- och encellsnivå. Under produktionen samlades stora mängder av D-xylonat och D-xylono-γ-lakton inuti cellerna. Ändå producerades och exporterades D-xylonat från cellerna från början av produktionsprocessen, även i närvaro av D-glukos. Både D-xylonat och D-xylono-γ-lakton exporterades från *S. cerevisiae* cellerna och det fanns ingen uppenbar preferens för någondera molekylen. Genom att uttrycka genen som kodar för D-xylonolakton laktonas enzymet, *xylC*, tillsammans med genen som kodar för D-xylos dehydrogenas enzymet, *xylB*, fastställdes att ackumulering av linjärt D-xylonat och i hydrolysen frigjorda protoner, var skadligt för cellerna. D-xylonatproduktionen skedde märkbart snabbare i celler som uttryckte både *xylB* och *xylC* jämfört med celler som uttryckte endast *xylB*. Ackumuleringen av D-xylonat ledde till att fluorescensen från pHluorin, ett pH-känsligt fluorescerande protein, försvann. Detta antyder att cellens cytosol försurnade då cellen producerade D-xylonat. Fluorescensen från pHluorin proteinet försvann snabbare i de celler som uttryckte både *xylC* och *xylB*, jämfört med de celler som uttryckte endast *xylB*. Denna försurning av cytosolen visade sig korrelera med en minskad livskraft bland cellerna som producerade D-xylonat och graden av försurning och förminskningen i viabiliteten var starkt beroende av pH-värdet i produktionsunderlaget. En förminskad livskraft och utmaningar i exporten av D-xylonat utgör stora hinder för D-xylonatproduktion med *S. cerevisiae*. Även i *P. kudriavzevii* cellerna samlades det stora mängder av D-xylonat och livskraften hos dessa var minskad, speciellt då D-xylonatet producerades vid lågt pH. Stressreaktionerna gentemot svaga organiska syror är starkt beroende av egenskaperna hos syrorna och höga koncentrationer av svaga organiska syror leder till en förlorad livskraft. Vid studier av den roll transportproteinet Pdr12 har i resistensen mot svaga organiska syror, framkom att syrans egenskaper har stor inverkan på cellernas syratolerans. Mikroorganismer med en deleterad *PDR12* gen uppvisade en förbättrad tolerans mot myr- och ättiksyra, vilket kan utnyttjas vid bioraffineringen av lignocelluloshydrolysat, som oftast innehåller dessa syror. En bioteknisk produktion av D-xylonsyra med hjälp av jästceller har stor potential att bli en industriellt användbar process. För att biotekniska produktionsprocesser skall kunna bli ekonomiskt möjliga, måste man utveckla bioraffinaderier där lignocellulosahydrolysat eller andra sido- eller avfallsströmmar används som råvaror. Denna avhandling ger ny förståelse för hur produktionen av en organisk syra påverkar produktionsorganismen och presenterar nya metoder för att studera och öka produktionen.
ISBN, ISSN	ISBN 978-951-38-8147-4 (nid.) ISBN 978-951-38-8148-1 (URL: http://www.vtt.fi/publications/index.jsp) ISSN-L 2242-119X ISSN 2242-119X (print) ISSN 2242-1203 (online)
Datum	Maj 2014
Språk	Engelska, svenskt abstrakt
Sidor	99 s. + bil. 84 s.
Nyckelord	Yeast, D-xylonate, metabolic engineering, organic acids, stress responses, cytosolic pH, Pdr12, D-xylose
Utgivare	VTT PL 1000, 02044 VTT, Puh. 020 722 111

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Production of D-xylonate, an industrial platform chemical with high application potential, was successfully demonstrated in the yeast *Saccharomyces cerevisiae*, *Kluyveromyces lactis* and *Pichia kudriavzevii*. The best D-xylonate production was obtained by expression of the D-xylose dehydrogenase encoding gene *xylB* from *Caulobacter crescentus* and the highest D-xylonate titre was achieved with *P. kudriavzevii* that produced 171 and 146 g D-xylonate l^{-1}, at a rate of 1.4 or 1.2 g l^{-1} h^{-1}, at pH 5.5 and pH 3, respectively.

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Biotechnological production of D-xylonic acid with yeast clearly has the potential of becoming an industrially applicable process. In order for biotechnological production processes to become economically feasible, biorefinery approaches in which lignocellulosic hydrolysates or other biomass side- or waste streams are used as raw materials need to be employed. This thesis provides new understanding on how production of an organic acid affects the production host and presents novel approaches for studying and increasing the production.