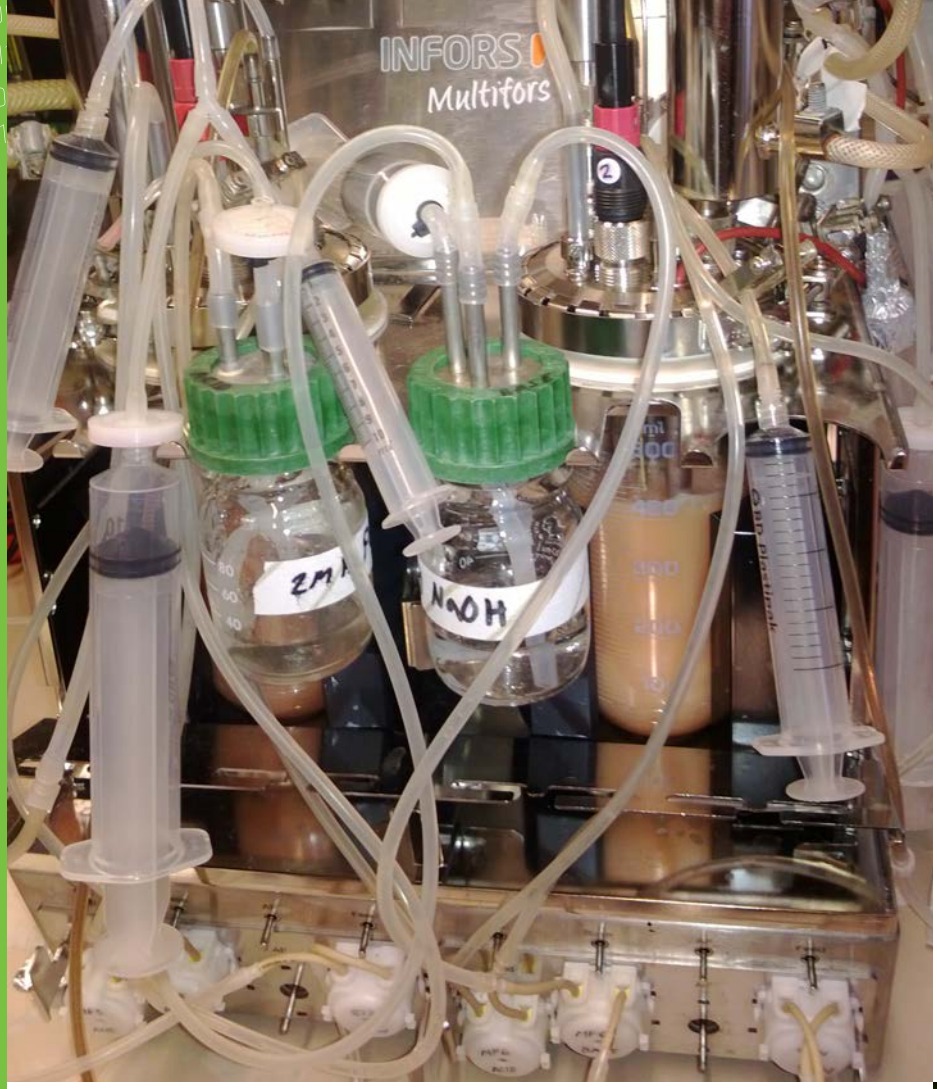

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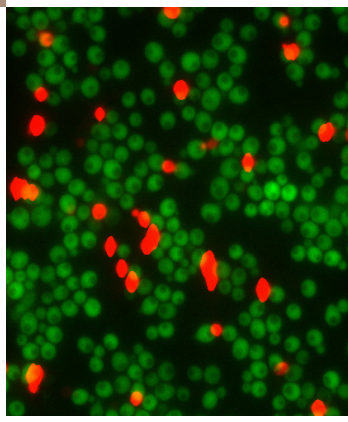
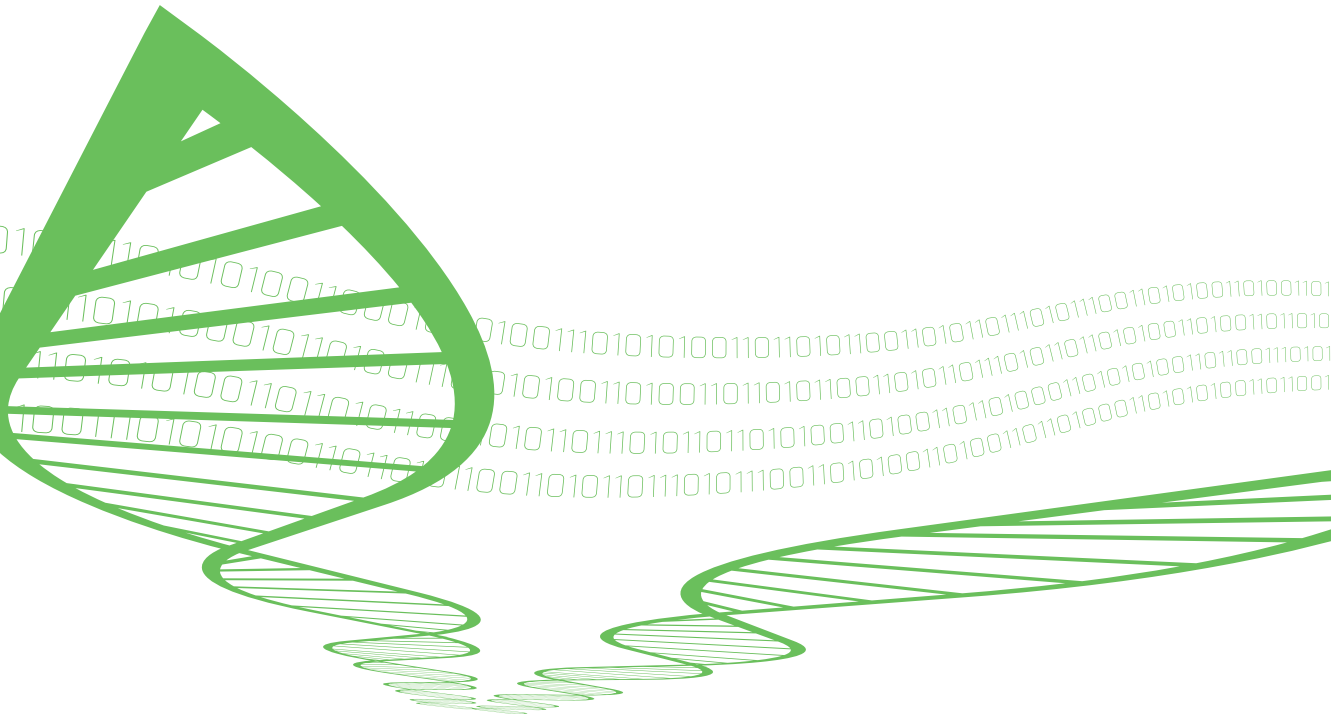


Production of D-xylonate and organic acid tolerance in yeast

Yvonne Nygård

VTT



VTT SCIENCE 58

Production of D-xylonate and organic acid tolerance in yeast

Yvonne Nygård

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Production of D-xylonate and organic acid tolerance in yeast

D-xylonat produktion och tolerans mot organiska syror i jäster. **Yvonne Nygård.** Espoo 2014. VTT Science 58. 99 p. + app. 84 p.

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 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.

Keywords yeast, D-xylonate, metabolic engineering, organic acids, stress responses, cytosolic pH, Pdr12, D-xylose

D-xylonat produktion och tolerans mot organiska syror i jäster

Production of D-xylonate and organic acid tolerance in yeast. **Yvonne Nygård.** Espoo 2014. VTT Science 58. 99 p. + app. 84 p.

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, 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.

Nyckelord yeast, D-xylonate, metabolic engineering, organic acids, stress responses, cytosolic pH, Pdr12, D-xylose

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.

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Academic dissertation

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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.

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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 substrates, 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 reproduction. 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).

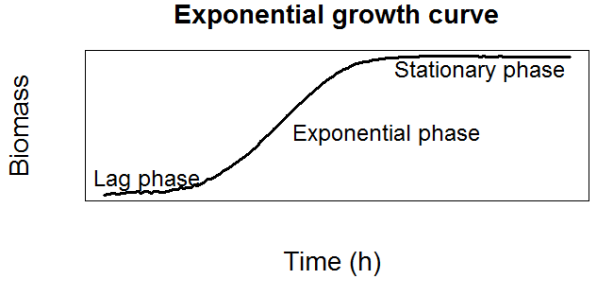

Figure 1. A typical exponential growth curve.

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 processes leading to the production of new cellular materials and compounds, and catabolic 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 dephosphorylation 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 molecules. (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.

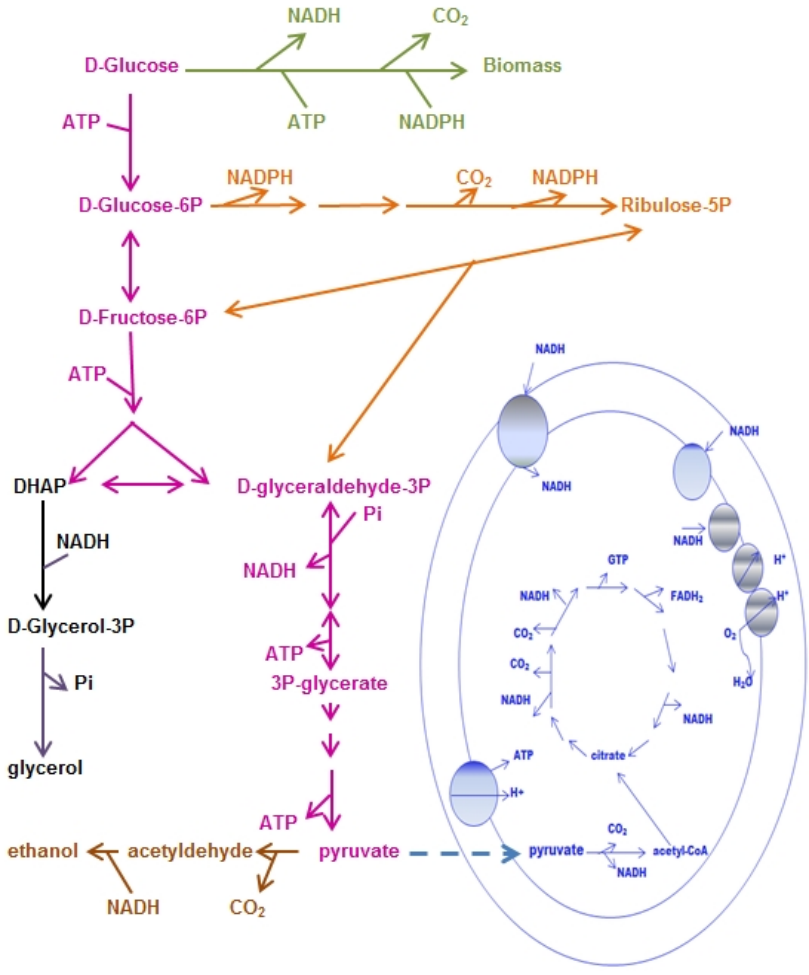

Figure 2. Central metabolism in *Saccharomyces cerevisiae*, showing NADH, NADPH, $FADH_2$ and ATP production and utilization. NADPH is produced by the oxidative part of the pentose phosphate pathway (PPP, in orange), while glycolysis (in pink) provides ATP and NADH. Ethanol is produced by pyruvate reduction (in brown) with NADH as a co-factor. Glycerol can be formed as a by-product to restore 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 dehydrogenases 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 ↔ 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 dehydrogenase 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 intracellular 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 solubility of a protein and as a result, the activity of an enzyme and interaction between 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 organelles 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).

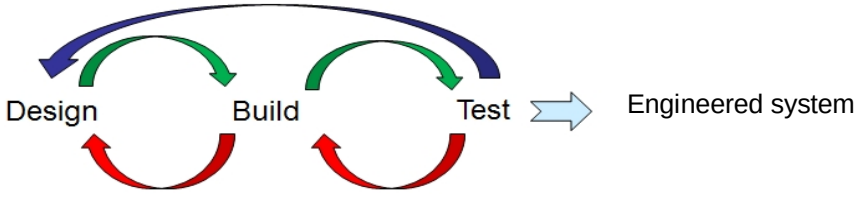

Figure 3. A scheme for synthetic biology as iterative circles of designing, building and testing. The figure is modified from a figure by Lynn Andrea Stein, in Interactive Programming in Java, available at http://www.cs101.org/ipij/design, cited 5.3.2014.

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.

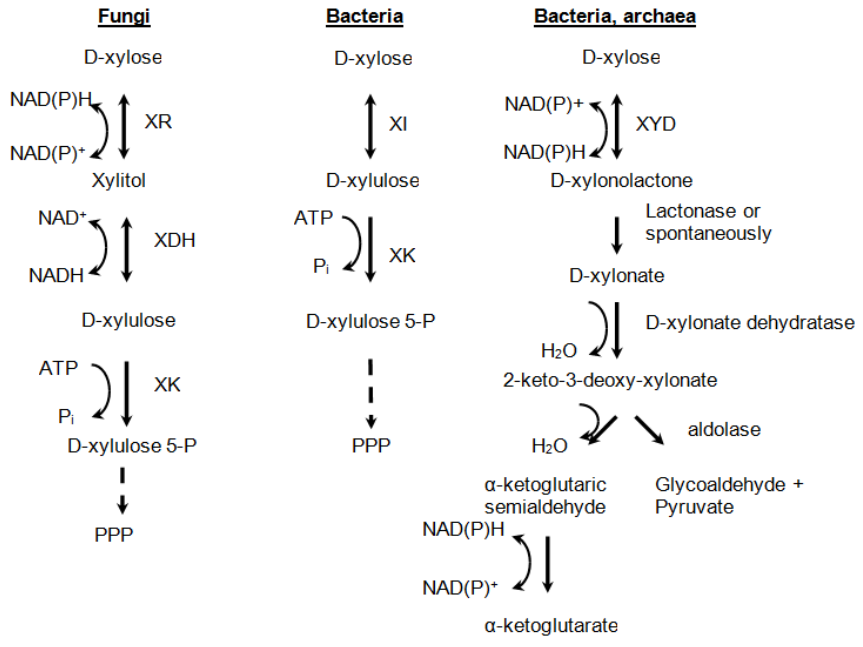

Figure 4. D-xylose metabolic pathways of fungi, bacteria or archaea. Figure is based on Figures shown by Hahn-Hägerdal et al. (2007) and Meijnen et al. (2009). XR = D-xylose reductase; XDH = xylitol dehydrogenase; XK = D-xylulokinase; XI = D-xylose isomerase; XYD = D-xylose dehydrogenase.

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 biofuels 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).

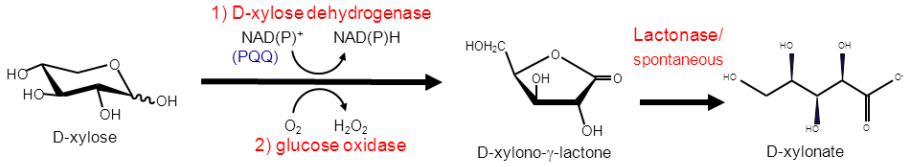

Figure 5. The reaction from D-xylose via D-xylono-γ-lactone to D-xylonate.

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 ΔXYL2	H3763; H3632Δ*xyl2::KMX, xyd1*	*xyd1, Trichoderma reesei*	I
xyd1 ΔXYL1	H3765; H3632Δ*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, *leu*2-3/112 *ura3*-52 *trpI*-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_2S0_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 ^1H 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 autofluorescence 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 unbound 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.

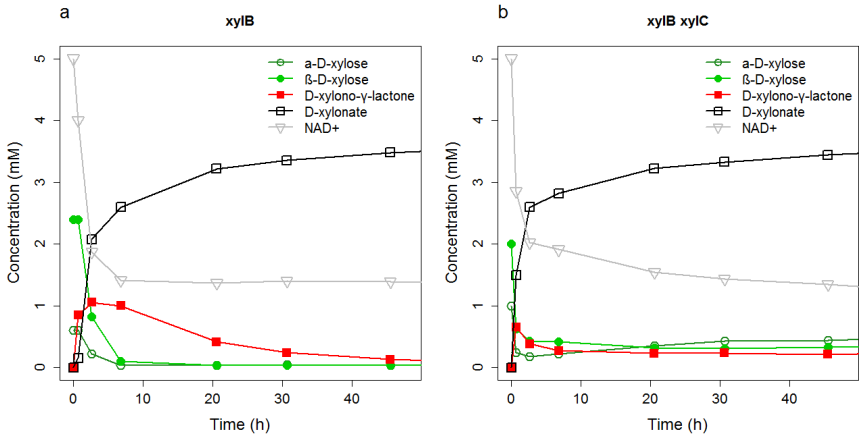

Figure 6. Conversion of D-xylose (green open (α-D-xylose) or closed (β-D-xylose) circles) to D-xylono-γ-lactone (red solid squares) by cell extracts of *S. cerevisiae* expressing *xylB* (a) or *xylB* and *xylC* (b) from *C. crescentus*, monitored by ^{1}H NMR spectroscopy. The production of D-xylono-γ-lactone 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}.

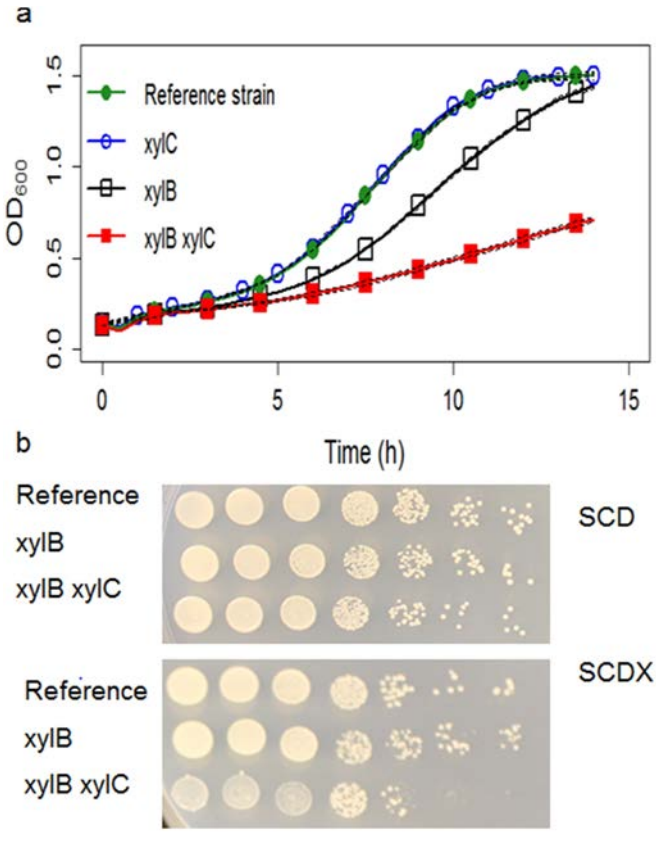

Figure 7. a) Growth curves of of *S. cerevisiae* expressing *xylB* (black open squeres), *xylB* and *xylC* (red solid squares) or *xylC* (blue open circles) from *C. crescentus* or without any genetic modification (reference strain, green solid circles), grown in SC medium containing 10 g D-glucose l^{-1} 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 consumption). 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).

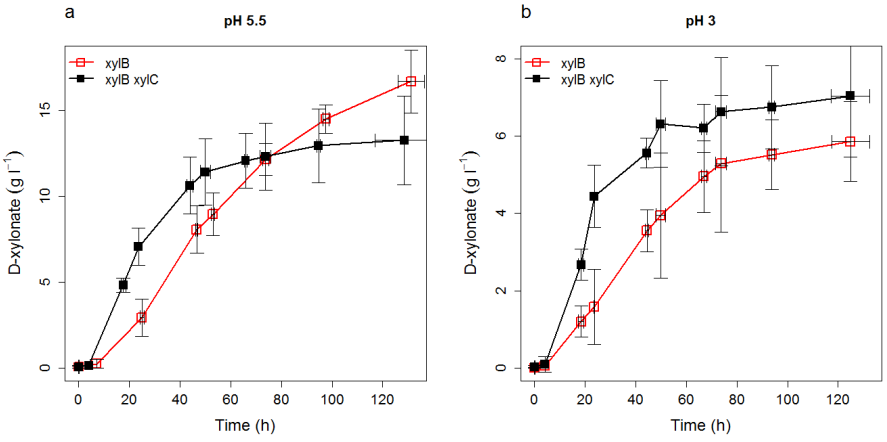

Figure 8. Production of D-xylonate at pH 5.5 (a) or at pH 3.0 (b) by *S. cerevisiae* expressing *xylB* (red open symbols) or *xylB* and *xylC* (black solid symbols) from *C. crescentus* in bioreactors, in SC medium with 9 ± 1 g D-glucose l^{-1} and 23 ± 1 g D-xylose l^{-1}, supplemented with 5 ± 1 g ethanol l^{-1} after ~50 h. Cells were maintained at 30 °C, at 500 rpm and 1.0 vvm aeration. Error bars show ± 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).

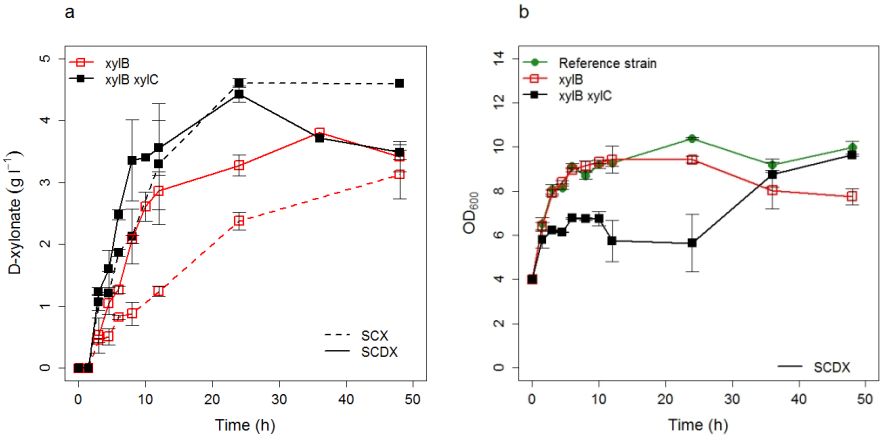

Figure 9. a) Production of D-xylonate by *S. cerevisiae* expressing *xylB* (red open symbols) or *xylB* and *xylC* (black solid symbols) from *C. crescentus* in SC medium and b) growth (as OD_{600}) of these strains and *S. cerevisiae* without gentic modifications (reference strain, green solid circles) 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.

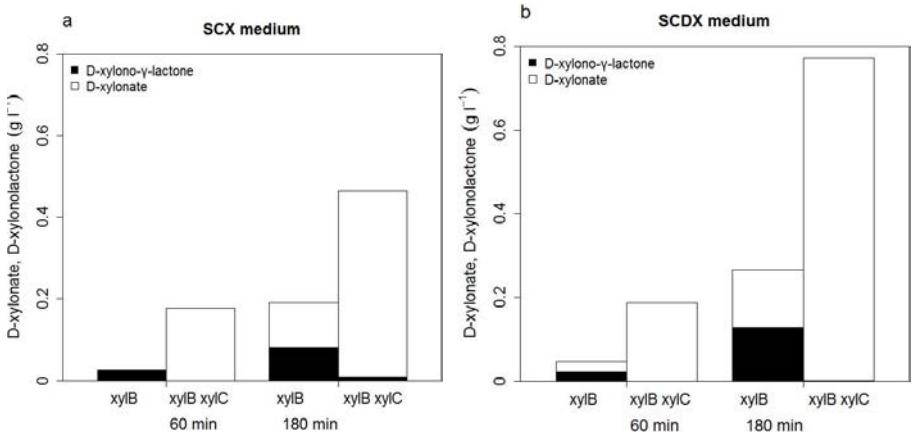

Figure 10. Concentrations of D-xylono-γ-lactone (black) and D-xylonate (white) in SC medium with a) 20 g D-xylose l^{-1} (SCX) or b) 10 g D-glucose l^{-1} and 20 g D-xylose l^{-1} (SCDX) in shake flasks by *S. cerevisiae* expressing *xylB* or *xylB* and *xylC* from *C. crescentus*. The 60 min samples 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).

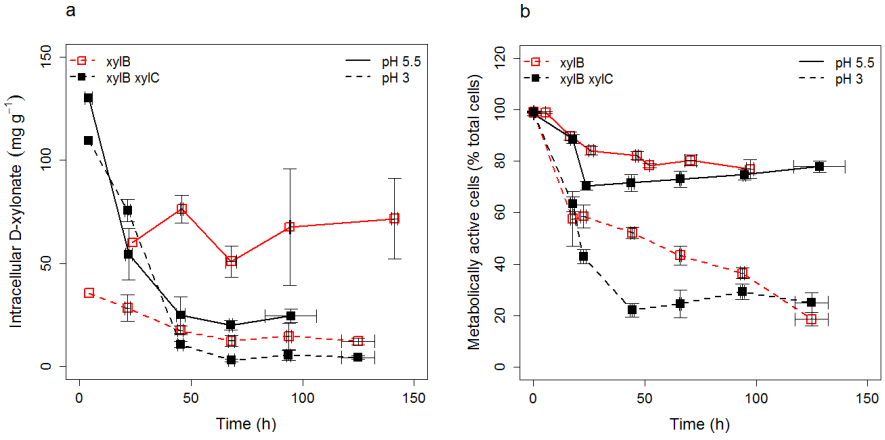

Figure 11. a) Intracellular D-xylonate (expressed as mg [g dry biomass]$^{-1}$) of and b) percentage of metabolically active cells (as determined by methylene blue staining) in populations of *S. cerevisiae* expressing *xylB* (red open squares) or *xylB* and *xylC* (black solid squares) from *C. crescentuS. c*ells 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.

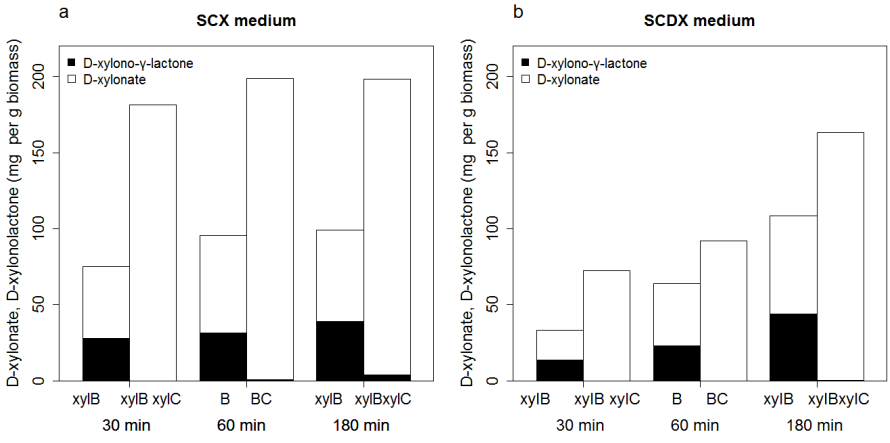

Figure 12. a) Intracellular concentrations of D-xylonate and D-xylono-γ-lactone (expressed as mg [g dry biomass]$^{-1}$, calculated from OD_{600} values, 1 OD l^{-1} estimated to correspond to 0.25 g l^{-1}) in *S. cerevisiae* expressing *xylB* or *xylB* and *xylC* (black solid squares) from *C. crescentus* (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).

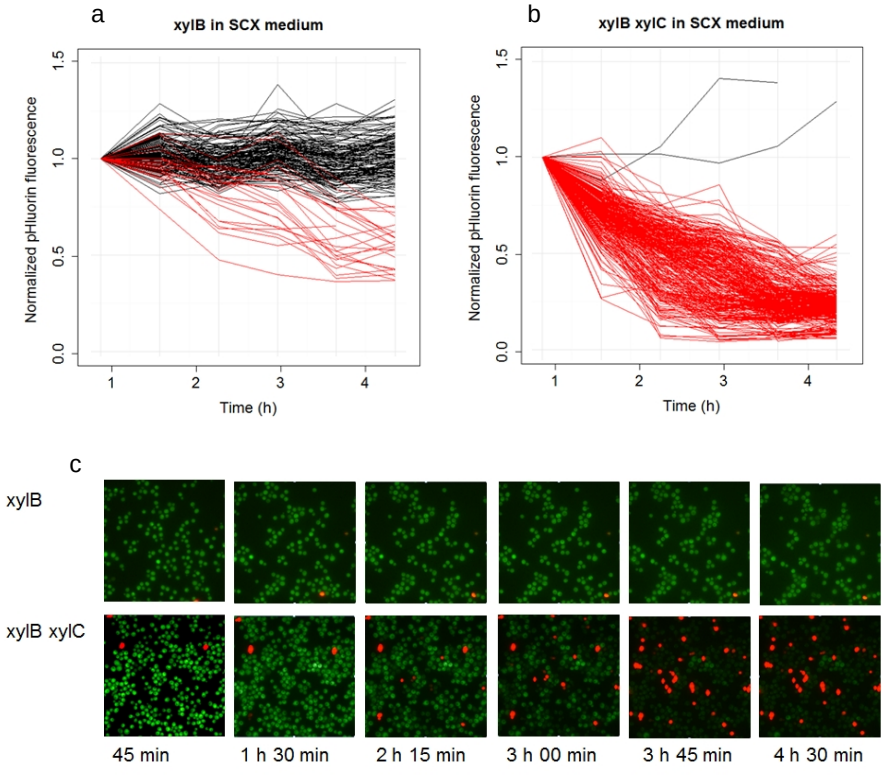

Figure 13. Fluorescent intensity, measured at 410 nm, of a) xylB and b) xylB xylC cells incubated in SC medium with 20 g D-xylose l^{-1} (SCX). Cells were attached to wells of glass bottom plates and imaged over time. Each line represents a single cell. Lines in red represent cells which lost pHluorin 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.

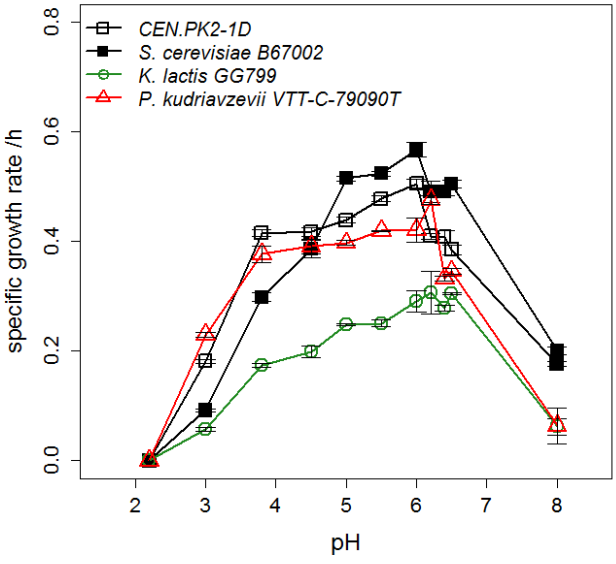

Figure 14. Comparison of specific growth rates of *S. cerevisiae* CEN.PK2-1D (open black squares), B-67002 (solid black squares), *K. lactis* GG799 (open green circles) and *P. kudriavzevii* VTT C-79090T (open red triangles) at pH 2–8 in defined medium, described by Verduyn et al. (1992), with 20 g D-glucose 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).

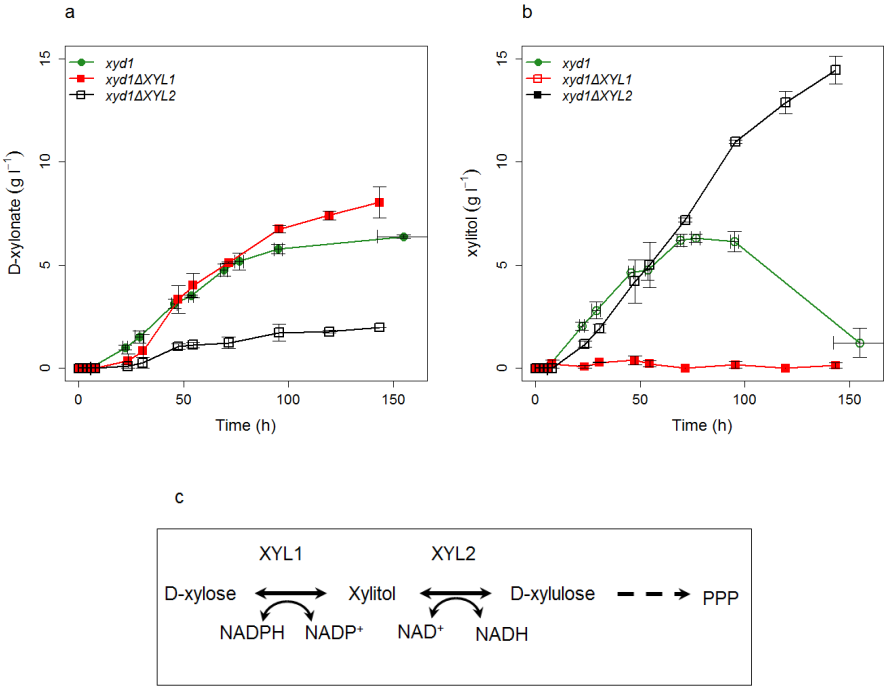

Figure 15. Extracellular accumulation of (a) D-xylonate and (b) xylitol by *K. lactis* xyd1 (solid red squares), xyd1Δ*XYL1* (solid green circles) and xyd1Δ*XYL2* (open black squares) on 10 g D-galactose l^{-1} and 20 g D-xylose l^{-1} in bioreactors, at pH 5.5, 30 °C. Cells were pre-grown on D-xylose. 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 (Δ*XYL1*) or 31 (Δ*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}.

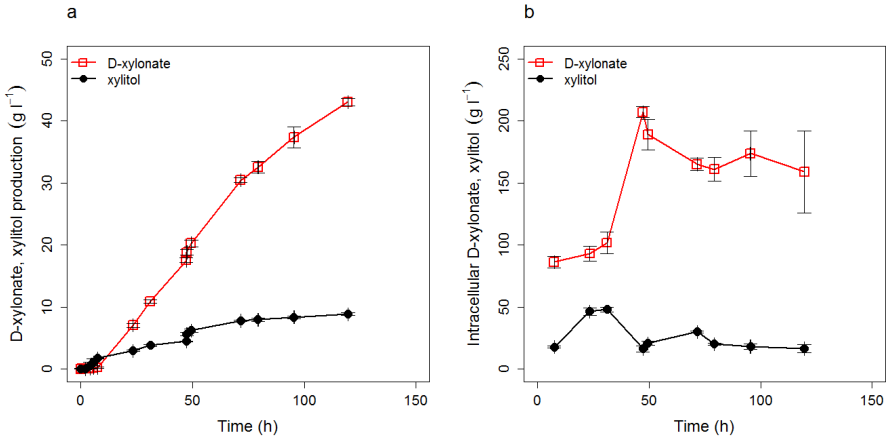

Figure 16. Extracellular (a) and intracellular accumulation (b) of D-xylonate (red open squares) and xylitol (black solid) by *S. cerevisiae* VTT B-67002 *xylB* at pH 5.5 in bioreactors, in YP medium from 49 D-xylose l^{-1}. The cultures initially contained 8 g D-glucose l^{-1} and 21 g D-xylose l^{-1} 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.

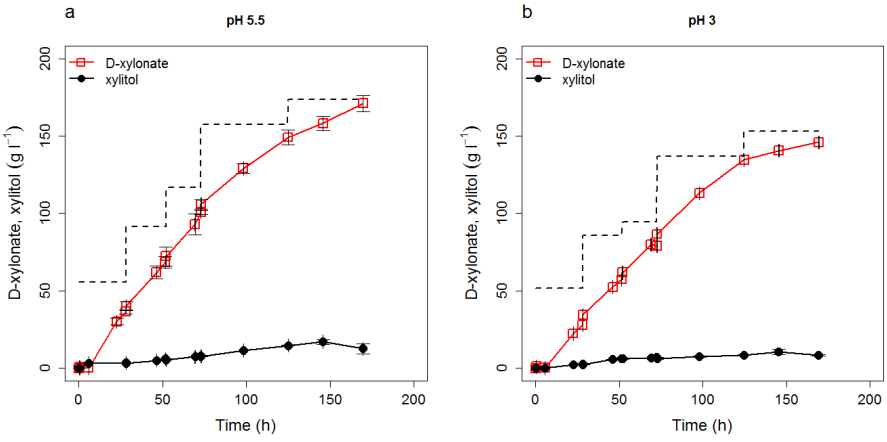

Figure 17. D-xylonate (red open squares) and xylitol (back solid circles) accumulation by *P. kudriavzevii* VTT-C-12903 at a) pH 5.5 b) pH 3 in bioreactor cultures provided with a total of 171 g l^{-1} (pH 5.5) or 153 g l^{-1} (pH 3) D-xylose in pulses at ~24 h intervals (indicated by dashed line). YP 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).

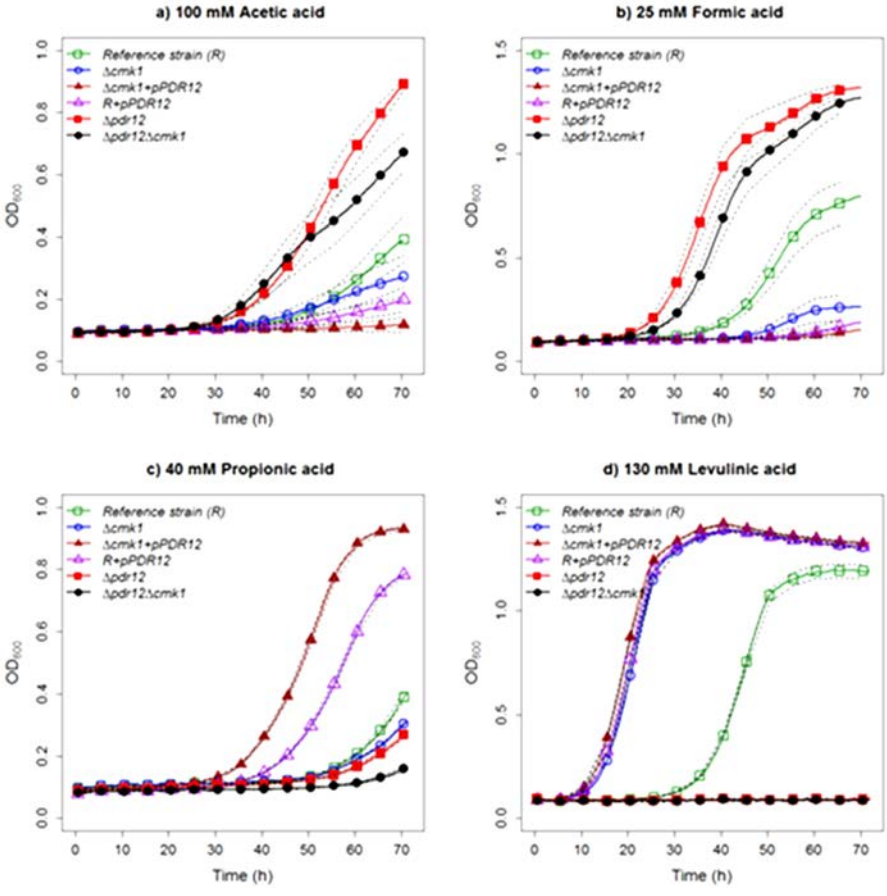

Figure 18. Measurement of biomass in bioscreen cultures of the reference strain (open squares), Δ*cmk1* (open circles), Δ*cmk1* + pPDR12 (solid triangles), R (reference) + pPDR12, (open triangles), Δ*pdr12* (solid squares) and Δ*pdr12*Δ*cmk1* (solid circles) strains in in SCD-leu medium containing 20 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).

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

Strains overexpressing *PDR12* (Δ*cmk1* + pPDR12, $\mu = 0.08\ h^{-1}$, and reference + pPDR12, $\mu = 0.09\ h^{-1}$) had higher specific growth rates ($p < 0.05$) than the reference strain ($\mu = 0.05\ h^{-1}$) 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 Δ*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 Δ*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, Δ*pdr12* and Δ*pdr12*Δ*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 Δ*pdr12* strains or the strain overexpressing *PDR12* (Fig. 19b).

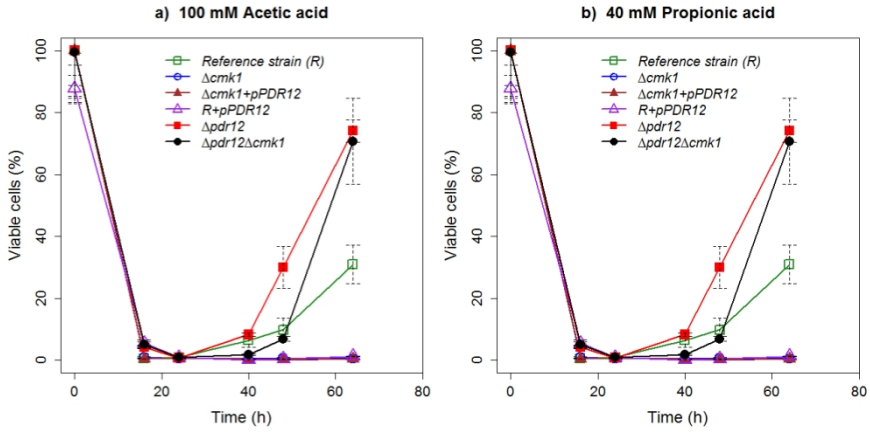

Figure 19. Percentage of viable cells in populations of reference (open squares), Δ*cmk1* (open circles), Δ*cmk1* + pPDR12 (solid triangles), R (reference) + pPDR12, (open triangles), Δ*pdr12* (solid squares) and Δ*pdr12*Δ*cmk1* (solid circles) cells grown in SCD-leu medium containing 20 g D-glucose 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 preadapted (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.

	0.45 mM Sorbic acid adaptation					**50 mM Acetic acid adaptation**			
Strain	FA	AA	PA	LA	SA	FA	AA	PA	SA
Reference	-	+	+	+	no	-	-	-	-
Δ*cmk1*	-	+	+	+	+	-	-	-	-
Δ*cmk1* + p*PDR12*	-	+	+	+	-	-	-	-	-
Reference + p*PDR12*	-	+	+	no	-	-	-	-	-
Δ*pdr12*	-	+	-	no	-	+	+	-	-
Δ*pdr12*Δ*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 Δ*pdr12* and the Δ*pdr12*Δ*cmk1* cells were able to grow in the presence of 120 mM acetic acid after sorbic acid pre-treatment, the Δ*pdr12*Δ*cmk1* strain having a shorter lag phase than the Δ*pdr12* strain (data not shown). The sorbic acid pre-treatment led to improved tolerance to 25 mM formic acid of the Δ*pdr12*Δ*cmk1* strain, whereas this treatment had a negative effect on all the other strains (Table 5).

After adaptation to 50 mM acetic acid, the Δ*pdr12*Δ*cmk1* was more tolerant to acetic and formic acids compared to the Δ*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 (Δ*pdr12* and Δ*pdr12*Δ*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 Δ*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.

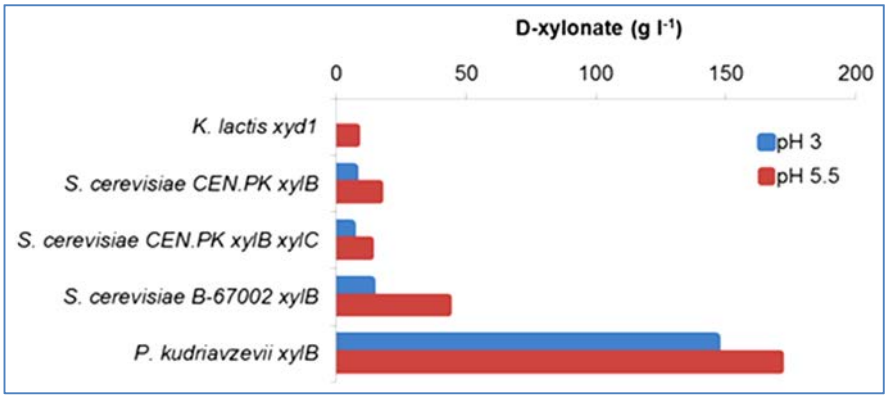

Figure 20. Production of D-xylonate at pH 3 (blue bars) and at pH 5.5 (red bars) using genetically modified yeast. Values presented are an overview of best results achieved in specific, strain-dependent setups.

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.

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Bioconversion of D-xylose to D-xylonate with *Kluyveromyces lactis*

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ABSTRACT

D-Xylonate was produced from D-xylose using *Kluyveromyces lactis* strains which expressed the gene for $NADP^+$-dependent D-xylose dehydrogenase from *Trichoderma reesei* (*xyd1*). Up to 19 ± 2 g D-xylonate l^{-1} was produced when *K. lactis* expressing *xyd1* was grown on 10.5 g D-galactose l^{-1} and 40 g D-xylose l^{-1}. Intracellular accumulation of D-xylonate (up to ~70 mg [g biomass]$^{-1}$) was observed.

D-Xylose was metabolised to D-xylonate, xylitol and biomass. Oxygen could be reduced to 6 mmol O_2 l^{-1} h^{-1} without loss in titre or production rate, but metabolism of D-xylose and xylitol were more efficient when 12 mmol O_2 l^{-1} h^{-1} were provided.

D-Xylose uptake was not affected by deletion of either the D-xylose reductase (*XYL1*) or a putative xylitol dehydrogenase encoding gene (*XYL2*) in *xyd1* expressing strains. *K. lactis xyd1ΔXYL1* did not produce extracellular xylitol and produced more D-xylonate than the *xyd1* strain containing the endogenous *XYL1*. *K. lactis xyd1ΔXYL2* produced high concentrations of xylitol and significantly less D-xylonate than the *xyd1* strain with the endogenous *XYL2*.

1. Introduction

With the current wide interest in using plant biomass derived products to replace fossil fuel derived materials, the ability of micro-organisms to convert the pentose sugar D-xylose, abundant in lignocellulosic biomass, into D-xylonic acid is worth noting. D-Xylonic acid is an organic acid similar to D-gluconic acid, which is widely used in the food, chemical, construction and pharmaceutical industries (Singh and Kumar, 2007). D-Xylonate can be produced by bacteria such as *Pseudomonas* and *Gluconobacter* species, which have periplasmic, membrane bound, PQQ-dependent, as well as cytoplasmic, dehydrogenases (Buchert et al., 1986, 1988; Buchert, 1990). D-Xylose is converted to D-xylono-γ-lactone by D-xylose dehydrogenase and the D-xylono-γ-lactone is subsequently hydrolysed either spontaneously or enzymatically by a lactonase to yield D-xylonate (Buchert and Viikari, 1988). Several bacteria are able to convert D-xylose to D-xylonate with high yields and production rates. However, commercial production of D-xylonate has not been established. *Pseudomonas* and *Gluconobacter* species produce a wide range of oxidising enzymes, with various degrees of specificity, resulting not only in the conversion of D-xylose to D-xylonate, but also in conversion of other sugars, which would be present in lignocellulosic hydrolysates, into other acids, such as D-gluconate and L-arabinoate.

Fungal species have high potential for production of organic acids (Porro et al., 1999; Magnuson and Lasure, 2004; Ilmén et al., 2007). Several fungi have the added benefits of being well known industrial production organisms, with generally regarded as safe (GRAS) status. Media requirements are generally simple and the availability of various sequenced genomes has further improved the molecular biology tools available. We recently described D-xylonate production with the yeast *Saccharomyces cerevisiae* using the D-xylose dehydrogenase (XYD) from *T. reesei* (Toivari et al., 2010), but production rates (25–36 mg D-xylonate l^{-1} h^{-1}) and yields (~0.4 g D-xylonate [g D-xylose consumed]$^{-1}$) were low.

S. cerevisiae does not naturally utilise D-xylose as a carbon source and it does not have specific uptake for D-xylose. D-Xylose is transported by hexose transporters, which have much lower K_m values for D-xylose compared to D-glucose (Kötter and Ciriacy, 1993; Hamacher et al., 2002), so that D-xylose transport is inhibited in the presence of excess D-glucose (Saloheimo et al., 2007) and alternative carbon needs to be provided for biomass and energy generation during D-xylonate production in batch culture (Toivari et al., 2010). In contrast, *Kluyveromyces lactis* can naturally use D-xylose as a carbon source and may have more efficient uptake of D-xylose than *S. cerevisiae*. Since D-xylose can be metabolised, it will also serve as a carbon, redox balance and energy source for *K. lactis*. Further, the *T. reesei* XYD requires $NADP^+$ as a co-factor (Berghäll et al., 2007) and *K. lactis* has several routes, in addition to the pentose phosphate pathway, for regeneration of intracellular $NADP^+$, such as a NAD(P)H-accepting external dehydrogenase (Tarrío et al., 2006), $NAD(P)^+$-accepting glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Verho et al., 2002) and glutathione reductase (*KlGLR1*, González-Siso et al., 2009) and would not require redox engineering to regenerate $NADP^+$ (Verho et al., 2002, 2003; Martínez et al., 2008). Although redox balancing was not able to improve D-xylonate

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production by *S. cerevisiae* (Toivari et al., 2010), these inherent routes for $NADP^+$ regeneration may still be beneficial in *K. lactis*. None-the-less, the activity of the native pathway for D-xylose conversion may need to be reduced in order to redirect D-xylose to D-xylonate rather than to biomass and/or xylitol. This can be achieved by deleting either the D-xylose reductase (XR) or the xylitol dehydrogenase (XDH) encoding gene of the native pathway or by controlling oxygen provision.

K. lactis is used in the industrial production of proteins. Although it is not particularly tolerant to hydrolysate inhibitors (M. Wiebe, unpublished results), with a sequenced genome and well defined tools for genetic modification it can serve as a model for the more hydrolysate tolerant *Kluyveromyces marxianus*, shown to have potential for ethanol production from biomass hydrolysates (Levine, 2008; Zhang et al., 2010), but for which fewer genetic tools are available.

Here we present the results of expressing the *T. reesei xyd1* gene, encoding $NADP^+$-dependent D-xylose dehydrogenase, in an industrial *K. lactis* strain as a model for D-xylonate production in a D-xylose utilising yeast. To reduce the conversion of D-xylose to biomass, either the XR encoding gene or the potential XDH encoding gene was deleted from the *xyd1* expressing strain. Biomass production was also controlled by the concentration of oxygen provided. The best results were obtained with the *xyd1* expressing strain provided with 40 g D-xylose l^{-1} and 10.5 g D-galactose l^{-1}, agitated at 500 rpm with 1 volume air per volume culture per minute. Under these conditions 19 ± 2 g D-xylonate l^{-1} were produced at a rate of 153 mg D-xylonate l^{-1} h^{-1}. These results provide a basis for further improvements to the oxidative bioconversion of D-xylose.

2. Materials and methods

2.1. Strains and strain construction

K. lactis H3632 (equivalent to GG799, New England Biolab, MA) was transformed to produce D-xylonate. *S. cerevisiae* strain H3488 (equivalent to FY834 (Winston et al., 1995)) was used for recombination cloning.

The *xyd1* gene, encoding the *T. reesei* D-xylose dehydrogenase, was digested from vector B2871, described in Berghäll et al. (2007), with *Bgl*II. The fragment was made blunt ended and ligated into the pKLAC1 (Protein expression kit, New England Biolab, MA) vector digested with *Hind*III and *Bgl*II and made blunt ended, generating plasmid B3260. Plasmid B3260 was digested with *Sac*II and introduced to *K. lactis* H3632 as instructed by the manufacturer. *xyd1* was placed under control of the modified, D-galactose-inducible *LAC4* promoter from *K. lactis* in the pKLAC1 vector. Transformants were selected by growth on acetamide and integration of *xyd1* was verified by PCR. The resulting strain was named *K. lactis xyd1* (H3677).

The D-xylose reductase (XR) encoding gene of *K. lactis* (*XYL1* described by Billard et al. (1995)) was deleted by amplifying an upstream fragment (440 bp non-coding sequence+40 bp coding sequence) with primers KlXYL1p_1YE1 (GATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTCTCACATAACAATTATTG) and KlXYL1p_2KMXp (GGTTGTCGACCTGCAGCGTACGAAGCTTCAGCTGGCGGCCCGCCATTGTTTAAAGTAAC) and a downstream fragment (290 bp of coding sequence+250 bp of non-coding sequence) with primers KlXYL1t_KMXt (GAAGTTATTAGGTGATATCAGATCCACTAGTGGCCTATGCAACAAGAAAGCTCTTAAC) and KlXYL1t_YE2 (TGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTATCAATTGGTTTGAAC) using chromosomal DNA of strain H3632 as template. The primers contained an additional 40 bp sequence that was homologous to sequence in either the YEplac195 vector (Gietz and Sugino, 1988) or to the dominant kanamycin resistance (kan^r) marker from plasmid pUG6 (Güldener et al., 1996). The amplified fragments were introduced together with the kan^r marker from pUG6, digested with *Not*I, and vector YEplac195, digested with *Bam*HI and *Pst*I, into *S. cerevisiae* strain H3488. The fragments were recombined *in vivo* to form a yeast expression vector with the kan^r gene flanked by upstream and downstream regions of the *K. lactis XYL1* gene basically as described in Colot et al. (2006). The resulting plasmid B3469 was digested with *Sma*I and *Sph*I to release the deletion cassette, which was subsequently transformed into *K. lactis* strain H3632. Transformants were screened for reduced growth on D-xylose medium and verified by PCR. The D-xylose reductase deficient strain was named H3765.

For deletion of the putative xylitol dehydrogenase (XDH) encoding gene, KLLA0D05511g (subsequently referred to as *XYL2*), an upstream fragment (574 bp non-coding sequence) was amplified with primers KlXDHp1KMXt (GAAGTTATTAGGTGATATCAGATCCACTAGTGGCCTATGCATTTGTACAAGGCCGTG) and KlXDHp2YE2 (TGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTAACCGATAGATGCAAG) and a downstream fragment (118 bp of coding sequence+449 bp of non-coding sequence) with primers KlXDHt1YE1 (GATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTTGACGGAGACGATGTG) and KlXDHt2KMXp (GGTTGTCGACCTGCAGCGTACGAAGCTTCAGCTGGCGGCCCAACGTTAAGAAAATG) using chromosomal DNA of strain H3632 as template. The disruption cassette was constructed as described above for deletion of *XYL1*, resulting in construct B3468. The XDH deficient strain H3763 was created as described above for H3765.

2.2. Media and culture conditions

Yeast peptone (YP) medium, containing yeast extract (10 g l^{-1}) and bacto peptone (20 g l^{-1}), with D-glucose (20 g l^{-1}) as a carbon source, G418 (200 mg l^{-1}), and solidified by the addition of 15 g agar l^{-1} was used to select strains in which the *XYL1* and *XYL2* genes had been deleted. All strains were stored on agar-solidified YP medium containing 20 g D-xylose l^{-1} as sole carbon source. Previous experiments demonstrated that a strong history of D-xylose utilisation was needed for *K. lactis* strains to have efficient D-xylose utilisation. Pre-growth on D-xylose increased the D-xylose consumption rate of the control strain H3632 by 77% and of *xyd1*-expressing strains by almost 12-fold (unpublished data). The deletion strains grew weakly on D-xylose, but sufficient to maintain the strains and generate inoculum with induced D-xylose uptake.

For small scale cultures, yeast were grown in either 20 or 50 ml modified yeast synthetic complete (YSC, Sherman et al., 1983) medium containing 20 g D-xylose l^{-1} in 100 or 250 ml Erlenmeyer flasks, respectively, with 250 rpm shaking at 30 °C. For larger scale cultures, yeast were grown in 500 ml yeast nitrogen base (YNB, Becton, Dickinson and Company, USA) medium containing 20 or 40 g D-xylose l^{-1} with 10 g D-galactose l^{-1} to induce the *LAC4* promoter, in Multifors bioreactors (max. working volume 500 ml, Infors HT, Switzerland) with 2 marine impellors (4 blades) at pH 5.5, 30 °C, 0.3–1.4 volume air [volume culture]$^{-1}$ min^{-1} (vvm) and 150–700 rpm agitation, to provide estimated oxygen transfer rates (OTRs) between 3 and 22 mmol l^{-1} h^{-1} (Table 1). Cultures provided with 40 g D-xylose l^{-1} were given an additional 0.5 g D-galactose l^{-1} after 53 h to sustain expression of *xyd1*. The pH was maintained constant by addition of 2 M NaOH or 1 M H_3PO_4. Clerol antifoaming agent (Cognis, France, 0.08–0.10 µl l^{-1}) was added to prevent foam accumulation. Gas concentration (CO_2, $^{13}CO_2$, O_2, N_2 and Ar) was analysed continuously in an Omnistar quadrupole mass spectrometer (Balzers AG, Liechtenstein), calibrated with 3% CO_2 in Ar.

2.3. Measurements of biomass

Biomass was measured as optical density at 600 nm (OD_{600}) or as dry mass of the cells. For dry mass measurement, samples were

Table 1
Agitation and aeration, with approximate k_La, oxygen transfer rates and oxygen uptake rates used in the cultivation of *K. lactis*.

Agitation (rpm)	Aeration ($l\ min^{-1}$)	k_La (s^{-1})	Oxygen transfer rate ($mmol\ l^{-1}\ h^{-1}$)	Maximum oxygen uptake rate ($mmol\ l^{-1}\ h^{-1}$)
150	0.14–0.15	0.003	3	2.5
300	0.3	0.007	6	5
500	0.5	0.014	12	9
700	0.6–0.7	0.024	22	13

collected in 2 ml pre-dried, pre-weighed microcentrifuge tubes, washed twice with equal volume distilled water and dried at 100 °C. One OD_{600} unit was equal to 0.22 $g\ l^{-1}$ cell dry mass.

2.4. Chemical analyses

Culture supernatant was stored at −20 °C for substrate and product analyses. To determine the intracellular concentrations of D-xylose, D-xylonate and xylitol, cells from 10 ml of culture were collected by centrifugation. Pellets were washed with 1.8 ml deionised water to remove extracellular compounds and frozen at −20 °C to disrupt membranes. The frozen pellets were freeze-dried using a Christ Alpha 2-4 lyophiliser (Biotech international, Belgium) to remove the interstitial liquid enabling the concentrations to be measured in a known volume. Intracellular D-xylose, D-xylonate and xylitol were extracted from the lyophilised pellets (~60 mg biomass ml^{-1}) in 5 mM H_2SO_4. The resuspended pellets were incubated for approximately 1 h. Samples could be refrozen at this stage if desired. Thawed samples were centrifuged and the supernatant collected for analyses. Cells could also be disrupted with glass beads, resulting in release of the same amounts of D-xylose, D-xylonate and xylitol as slow freezing with mild acid extraction. Results are presented as mg substrate or product per g dry biomass.

Intracellular and extracellular substrates and products (D-xylonic acid, ethanol, glycerol, pyruvate and acetate, D-glucose and D-xylose) were analysed by HPLC on a Fast Acid Analysis Column linked to an Aminex HPX-87H column (BioRad Labs, USA) with 2.5 mM H_2SO_4 as eluent and a flow rate of 0.5 $ml\ min^{-1}$. The column was maintained at 55 °C. Peaks were detected using a Waters 410 differential refractometer and a Waters 2487 dual wavelength UV (210 nm) detector. D-Xylose could not be accurately determined using this HPLC method when D-xylonic acid was present in the sample.

Extracellular D-xylonic acid concentrations were also measured using the hydroxamate method described by Lien (1959). Samples were diluted in 1.3 M HCl and heated at 100 °C to convert D-xylonic acid to D-xylono-γ-lactone before adding 50 μl of the diluted sample to 100 μl hydroxylamine reagent (2 M hydroxylamine HCl in 2 M NaOH). HCl (65 μl, 3.2 M) was added, followed by addition of 50 μl $FeCl_3$ (100 $g\ l^{-1}$ in 0.1 M HCl). Absorbance was measured immediately at 550 nm and D-xylono-γ-lactone concentration was determined by comparison with a standard curve. This assay was sensitive to <0.1 g D-xylono-γ-lactone l^{-1} and correlated well with HPLC measurements of D-xylonic acid, since D-xylonate was the only reacting compound present in the supernatant. The hydroxamate method was not used for intracellular D-xylono-γ-lactone measurement, since it was not necessarily the only lactone present in the cell extract.

2.5. Enzyme activity

D-Xylose dehydrogenase activity was measured, according to Berghäll et al. (2007) from crude cell extracts. The specific D-xylose dehydrogenase activity was calculated as enzyme activity in nanokatal per mg protein. The protein concentration was determined using the Bio-Rad protein kit, based on the assay developed by Bradford (1976). Assays were performed at 30 °C using a Konelab Arena photometric analyzer (Thermo Electron Oy, Finland). The sample volume from which cells were harvested for the enzyme assays was 7–50 ml, depending on the OD_{600} and expected activity.

3. Results

3.1. Production of D-xylonate from D-xylose in K. lactis expressing the T. reesei D-xylose dehydrogenase encoding gene xyd1

The *xyd1* expressing strain H3677 was grown with 20 or 40 g D-xylose $l^{-1}$and 10 g D-galactose l^{-1} which induced the *LAC4* promoter as well as providing for biomass generation and maintenance of the energy and redox state of the cells. With 20 g D-xylose l^{-1}, 6.3 ± 0.1 g D-xylonate l^{-1} was produced at an initial rate of 80 ± 1 mg D-xylonate $l^{-1}\ h^{-1}$, whereas no D-xylonate was produced by the control strain, H3632 (Fig. 1). D-Galactose was consumed during the first 10–15 h, after which D-xylose consumption started.

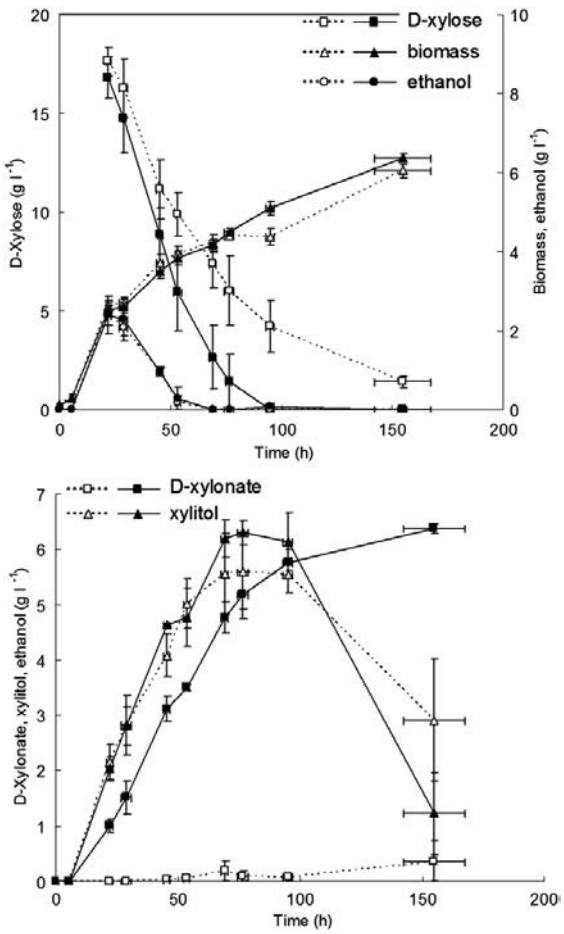

Fig. 1. Biomass, D-xylose, D-xylonate, xylitol and ethanol concentrations of *K. lactis* H3677 (*xyd1*, solid symbols) and H3632 (parent, open symbols) during growth on 10 g D-galactose l^{-1} and 20 g D-xylose l^{-1} at pH 5.5, 30 °C, OTR ~ 12 mmol $O_2\ l^{-1}\ h^{-1}$. Error bars show ± SEM for duplicate cultures.

A maximum of 2.9 ± 0.2 g ethanol l^{-1} was produced from D-galactose and was consumed simultaneously with D-xylose. The D-xylose consumption rate of *xyd1* expressing H3677 (0.27 ± 0.02 g D-xylose l^{-1} h^{-1}) was significantly ($p < 0.05$) higher than that of the control strain, H3632 (0.20 ± 0.02 g D-xylose l^{-1} h^{-1}). Both strains produced 6.1 ± 0.1 g biomass l^{-1}, but H3677 produced more ($p < 0.05$) xylitol (6.4 ± 0.1 g l^{-1}) than the parent strain (5.8 ± 0.3 g xylitol l^{-1}). Biomass was produced primarily during D-galactose and ethanol consumption, but at least 1.8 g biomass l^{-1} was produced by the control and 2.2 g biomass l^{-1} by H3677 when D-xylose and xylitol were the only carbon-sources being consumed. The yield of D-xylonate and xylitol on D-xylose and of biomass on total carbohydrate consumed are given in Table 2, along with production and consumption rates.

All D-xylose was consumed within 75 h when only 20 g D-xylose l^{-1} was provided. When the D-xylose concentration was increased to 40 g l^{-1} and an additional 0.5 g D-galactose l^{-1} was added at 53 h to induce the *LAC4* promoter, 18.9 ± 2.3 g D-xylonate l^{-1} was produced at an initial rate of 158 ± 11 mg D-xylonate l^{-1} h^{-1}. Biomass (6.2 ± 0.2 g l^{-1}), xylitol (6.2 ± 0.2 g l^{-1}) and ethanol (2.5 ± 0.4 g l^{-1}) concentrations did not differ significantly ($p > 0.05$) from that produced with 20 g D-xylose l^{-1}. Yields and rates of D-xylonate production are presented in Table 2.

H3677 had approximately 2 nkat XYD activity [mg protein]$^{-1}$ after 24 h cultivation in D-galactose and D-xylose containing medium. From 48 to 140 h XYD activity was constant at 1.5 ± 0.0 nkat [mg protein]$^{-1}$. The control strain H3632 showed no XYD activity.

3.2. Effect of aeration on D-xylonate production by K. lactis

K. lactis H3677 was grown in bioreactors with 20 g D-xylose l^{-1} at various OTRs, controlled by changing the agitation and aeration rates (Table 1). Oxygen uptake rate (OUR) was highest at the end of the exponential growth phase on D-galactose and remained below the estimated potential OTR throughout the cultivations in all conditions (Table 1).

Similar ($p > 0.05$) concentrations of D-xylonate were produced at OTRs of 6, 12 and 22 mmol O_2 l^{-1} h^{-1}, but initial D-xylonate production rates were higher ($p < 0.05$) with oxygen provided at 6 mmol O_2 l^{-1} h^{-1} (77 ± 2 mg D-xylonate l^{-1} h^{-1}) and 12 mmol O_2 l^{-1} h^{-1} (80 ± 1 mg D-xylonate l^{-1} h^{-1}) than at 22 mmol O_2 l^{-1} h^{-1} (64 mg D-xylonate l^{-1} h^{-1}, Fig. 2). In contrast, significantly less ($p < 0.05$) D-xylonate was produced at a lower volumetric rate when oxygen provision was reduced to 3 mmol O_2 l^{-1} h^{-1} (Fig. 2). However, initial specific production rates, ~33 mg [g biomass]$^{-1}$ h^{-1}, were similar in all conditions. As expected, less biomass and more ethanol were produced at lower OTRs (Fig. 2). Little or no apparent xylitol consumption occurred at OTRs of 3 or 6 mmol O_2 l^{-1} h^{-1}, whereas xylitol was consumed at 12 mmol O_2 l^{-1} h^{-1} after D-xylose had been consumed from the supernatant. D-Xylose metabolism at 22 mmol O_2 l^{-1} h^{-1} appeared inefficient, with less biomass, ethanol and xylitol being produced than at 12 mmol O_2 l^{-1} h^{-1} (Fig. 2). Although xylitol was produced at 22 mmol O_2 l^{-1} h^{-1}, it was not completely consumed (data not shown).

3.3. Altering the D-xylose flux in D-xylose reductase and xylitol dehydrogenase-deficient K. lactis strains

Deletion of *XYL1*, encoding XR, disrupted xylitol production in *K. lactis* H3765, while deletion of the putative *XYL2* gene, encoding XDH, disrupted metabolism of xylitol in *K. lactis* H3763. Deletion of *XYL1* resulted in no accumulation of xylitol (< 0.4 g xylitol l^{-1}) in the culture supernatant and production of 22% more D-xylonate (7.7 ± 0.4 g D-xylonate l^{-1}) than H3677 containing the endogenous *XYL1* (6.3 ± 0.1 g D-xylonate l^{-1}; Fig. 3). *K. lactis xyd1ΔXYL1* (127 ± 2 mg D-xylonate l^{-1} h^{-1}) had a significantly higher ($p < 0.05$) initial D-xylonate production rate than H3677 (80 mg D-xylonate l^{-1} h^{-1}, Table 2). Deletion of the putative *XYL2* resulted in accumulation 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 H3677 containing the endogenous *XYL2* (Fig. 3). The initial xylitol production rate was not affected (Table 2). The D-xylose consumption rate of H3677 (*xyd1*), H3765 (*xyd1* Δ*XYL1*) and H3763 (*xyd1* Δ*XYL2*) did not differ significantly ($p > 0.05$, Table 2).

Neither the XDH encoding gene nor the corresponding protein from *K. lactis* has been characterised, but the potential protein encoded by open reading frame KLLA0D05511g in the genome of *K. lactis* NRRL Y-1140 shows 53% identity and 68% sequence similarity with the *Scheffersomyces stipitis* (formerly *Pichia stipitis*) XDH (BLASTP 2.2.6; http://www.genolevures.org/blast.html). Thus this open reading frame is a potential XDH encoding gene. Since deletion of the potential *XYL2* gene resulted in significantly ($p < 0.05$) more xylitol being produced, with little apparent xylitol consumption, and a higher yield of xylitol on D-xylose than in H3677 or the parent H3632, we conclude that KLLA0D05511g encodes an XDH. However, *K. lactis xyd1ΔXYL2* grew on rich medium with D-xylose as the sole carbon source to produce inocula, and in D-xylonate production cultures approximately 3.4 g biomass l^{-1} was produced when D-xylose and xylitol were

Table 2
D-Xylose, D-xylonate, and xylitol consumption or production rates and yield of biomass, D-xylonate and xylitol (at maximum concentration) on total carbohydrate or D-xylose for *K. lactis* H3632, H3677, H3765 and H3763 grown in batch cultures with 20 (40 if indicated) g D-xylose l^{-1} and 10 g D-galactose l^{-1}, pH 5.5, 30 °C, and ~12 mmol O_2 l^{-1} h^{-1} OTR. Values are means ± SEM (n=2 for rates and 2–6 for yield). Values in the same column with the same superscript (a, b, c) did not differ significantly ($p < 0.05$, Fisher's multiple range test), while * indicates values which differ from that of H3677 growing in the presence of 20 g D-xylose l^{-1} (Student's t-test).

Strain (*K. lactis*)	Rate			Yield		
	D-xylose consumption rate (g l^{-1} h^{-1})	D-xylonate production rate (mg l^{-1} h^{-1})	Xylitol production rate (mg l^{-1} h^{-1})	Biomass (g [g carbohydrate consumed] $^{-1}$)	D-xylonate (g [g D-xylose consumed]$^{-1}$)	Xylitol (g [g D-xylose consumed]$^{-1}$)
H3632	0.20 ± 0.02*	0	104 ± 6	0.26 ± 0.01	0	0.36 ± 0.02*
H3677 (*xyd1*) (20 g l^{-1})	0.27 ± 0.02^a	80 ± 1^a	119 ± 1^a	0.27 ± 0.01^a	0.29 ± 0.01^a	0.30 ± 0.02^a
H3677 (*xyd1*) (40 g l^{-1})	0.37 ± 0.01*	158 ± 11*	104 ± 4	0.17 ± 0.01*	0.60 ± 0.03*	0.23 ± 0.02*
H3765 (*xyd1* Δ*XYL1*)	0.29 ± 0.03^a	127 ± 2^b	18 ± 5^b	0.22 ± 0.02^a	0.40 ± 0.01^b	0.02 ± 0.01^b
H3763 (*xyd1* Δ*XYL2*)	0.29 ± 0.00^a	31 ± 5^c	135 ± 5^a	0.25 ± 0.01^a	0.09 ± 0.01^c	0.59 ± 0.03^c

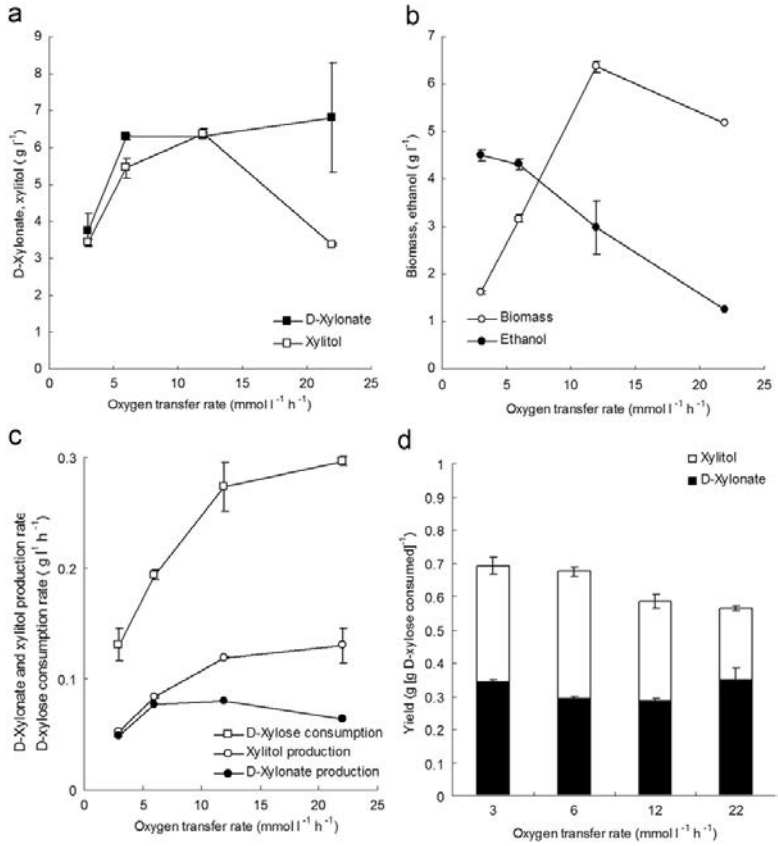

Fig. 2. The effect oxygen provision (estimated OTR, Table 1) on the production of (a) D-xylonate, xylitol, and (b) ethanol and biomass by *K. lactis* H3677, (c) on the production or consumption rates and (d) on the approximate yield of D-xylonate and xylitol on D-xylose. *K. lactis* H3677 was grown in batch culture with 10 g D-galactose l^{-1} and 20 g D-xylose l^{-1}, pH 5.5, 30 °C. Error bars show ± SEM for duplicate cultures.

the only available carbon sources, indicating that *XYL2* is not the only gene encoding XDH present in *K. lactis*. Other putative xylitol dehydrogenase encoding sequences in the *K. lactis* genome have lower sequence similarity with *S. stipitis* XDH (28% or 27% identity and 45% or 46% sequence similarity for KLLA0B00451g and KLLA0D19929g, respectively).

Similarly, H3765 (*xyd1* Δ*XYL1*) grew on D-xylose as sole carbon source and produced 3 ± 1 g biomass l^{-1} from D-xylose in the D-xylonate production cultures, indicating the presence of other D-xylose or aldose reductase(s) in the genome. The *K. lactis* XR shows 60% identity and 77% sequence similarity with the *S. stipitis* XR, but in addition, there are at least 5 hypothetical aldose reductases with app. 30% identity and 50% sequence similarity to the *S. stipitis* XR in the *K. lactis* genome. Deletion of *XYL1* or *XYL2* did not affect the yield of biomass on total carbohydrate consumed (Table 2).

Both *K. lactis xyd1* Δ*XYL1* and *K. lactis xyd1* Δ*XYL2* had similar levels of XYD activity as strain H3677.

3.4. Intracellular accumulation of D-xylonate, xylitol and D-xylose

D-Xylonate accumulated intracellularly in *K. lactis* H3677, expressing *xyd1*, when grown in the presence of D-xylose (Fig. 4). At 31 h, when only 0–3 g l^{-1} extracellular D-xylonate was detected, the intracellular concentration in *K. lactis xyd1* was approximately 71 mg [g biomass]$^{-1}$. This represents an intracellular concentration of 35–47 g D-xylonate l^{-1}, depending on the volume of cytoplasm per g dry biomass, which was not measured in this study but has been reported to be between 1.5 and 2 ml per g dry cell mass for *S. cerevisiae* (de Koning and van Dam, 1992; Gancedo and Serrano, 1989), and may vary depending on the stage of growth (Lagunas and Moreno, 1985; Austriaco, 1996) and differences in the volume of organelles, in particular the cell wall and the vacuole. Intracellular D-xylonate concentration then generally decreased as extracellular D-xylonate concentration increased, with a strong negative correlation (r=0.98). When either *XYL1* or *XYL2* were deleted, intracellular D-xylonate did not accumulate above 44 mg [g biomass]$^{-1}$, and remained approximately constant at 26 (Δ*XYL1*) or 31 (Δ*XYL2*) mg D-xylonate [g biomass]$^{-1}$ between 31 and 143 h. No (< 3 mg [g biomass]$^{-1}$ background) D-xylonate was detected in the cell extracts of the parent strain H3632.

Xylitol also accumulated intracellularly in *K. lactis*. Concentrations over 135 mg xylitol [g biomass]$^{-1}$ (65–90 g xylitol l^{-1}) were observed in both H3677 and the parent H3632 when cultivated in 20 g D-xylose l^{-1} with 12 mmol O_2 l^{-1} h^{-1}. High intracellular concentrations were maintained in H3677 and H3632 even when extracellular xylitol was being consumed. In *K. lactis xyd1* Δ*XYL1* ~19 mg intracellular xylitol [g biomass]$^{-1}$ was observed, whereas in *K. lactis xyd1* Δ*XYL2*, xylitol initially accumulated to ~100 mg [g biomass]$^{-1}$, similar to H3677 and H3632.

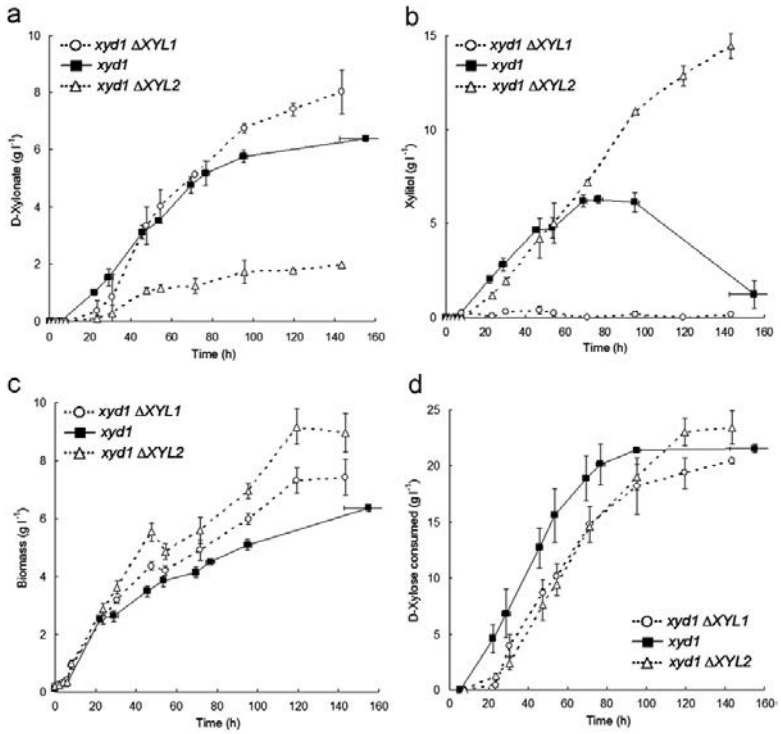

Fig. 3. Concentrations of (a) D-xylonate, (b) xylitol, (c) biomass, and (d) D-xylose consumed during cultivation of *K. lactis* H3677 (*xyd1*, solid squares) and H3765 (*xyd1*Δ*XYL1*, open circles) and H3763 (*xyd1*Δ*XYL2*, open triangles) on 10 g D-galactose l^{-1} and 20 g D-xylose l^{-1} at pH 5.5, 30 °C, OTR~12 mmol O_2 l^{-1} h^{-1}. Error bars show ± SEM for duplicate cultures.

After 72 h the intracellular xylitol concentration in *K. lactis xyd1* Δ*XYL2* decreased to a concentration of only ~8 mg [g biomass]$^{-1}$ at the end of the cultivation.

Along with D-xylonate and xylitol, D-xylose was detected in the intracellular extracts and was positively correlated to extracellular concentrations. After 31 h cultivation, approximately 70 mg D-xylose [g biomass]$^{-1}$ (35–47 g D-xylose l^{-1}) was present intracellularly in H3632 and H3677, when the extracellular D-xylose concentration was ~16 g l^{-1}. Less D-xylose accumulated intracellularly in the Δ*XYL1* (max. 44 mg D-xylose [g biomass]$^{-1}$) and Δ*XYL2* (max. 12 mg D-xylose [g biomass]$^{-1}$) strains than in H3677, but as in H3677, intracellular D-xylose was positively correlated with the extracellular concentration.

4. Discussion

Expression of *T. reesei xyd1* in *K. lactis* resulted in a 5-fold increase in titre, 4-fold increase in production rate and a 50% increase in the yield of D-xylonate from D-xylose, compared to expression of the same gene in *S. cerevisiae* (Toivari et al., 2010). Activity levels of the XYD in *K. lactis* and *S. cerevisiae* were similar. *K. lactis* H3677 produced 19 ± 2 g l^{-1} D-xylonate from D-xylose at a maximum rate of 158 ± 11 mg l^{-1} h^{-1} and yield of ~0.60 ± 0.03 g D-xylonate [g D-xylose consumed]$^{-1}$ when grown on 10 g D-galactose l^{-1} and 40 g D-xylose l^{-1}, with re-induction of the *LAC4* promoter with 0.5 g D-galactose l^{-1} after 53 h. D-Galactose was provided to *K. lactis* instead of D-glucose or ethanol (cf. Toivari et al., 2010), in order to induce the *LAC4* promoter, but also provided carbon for biomass and as an energy source.

Various factors may contribute to the higher D-xylonate production by *K. lactis* than *S. cerevisiae*, such as more efficient uptake of D-xylose, better tolerance to intracellular acid accumulation and/or better D-xylonate export capacity. In contrast to *S. cerevisiae*, *K. lactis* is able to use D-xylose as a carbon source and produced biomass as well as D-xylonate and xylitol when grown on D-xylose. The uptake of D-xylose has not been studied in *K. lactis*. *K. lactis* has several uniporters for sugar transport (Palma et al., 2009), and also a putative active sugar transporter belonging to the putative D-xylose proton symporter family (Palma et al., 2007). Although evolved strains of *S. cerevisiae* have been shown to take up D-xylose at rates of 0.2–1.1 g D-xylose l^{-1} h^{-1}, 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). D-Xylonate-producing strains of *S. cerevisiae* consumed D-xylose at 0.06 g D-xylose l^{-1} h^{-1} (Toivari et al., 2010), which was three to six times slower than the rate of D-xylose consumption observed for *K. lactis* strains (0.2–0.4 g D-xylose l^{-1} h^{-1}). Further, since D-xylose could be used as both carbon and energy source by *K. lactis*, it would not be necessary to add a co-substrate during the cultivation to sustain D-xylonate production, as was necessary with *S. cerevisiae* (Toivari et al., 2010).

Production of D-xylonate from D-xylose is an oxidative reaction and production occurs under aerobic conditions (Buchert et al., 1986; Hardy et al., 1993; Toivari et al., 2010). Oxygen is also essential for the growth of *K. lactis*. However, since D-xylose provides carbon for both D-xylonate and biomass production, it would be desirable to limit biomass production by limiting oxygen provision, while providing sufficient oxygen for D-xylose oxidation. In oxygen limited conditions (e.g. < 12 mmol O_2 l^{-1} h^{-1}), biomass production decreased as expected. However, D-xylonate production

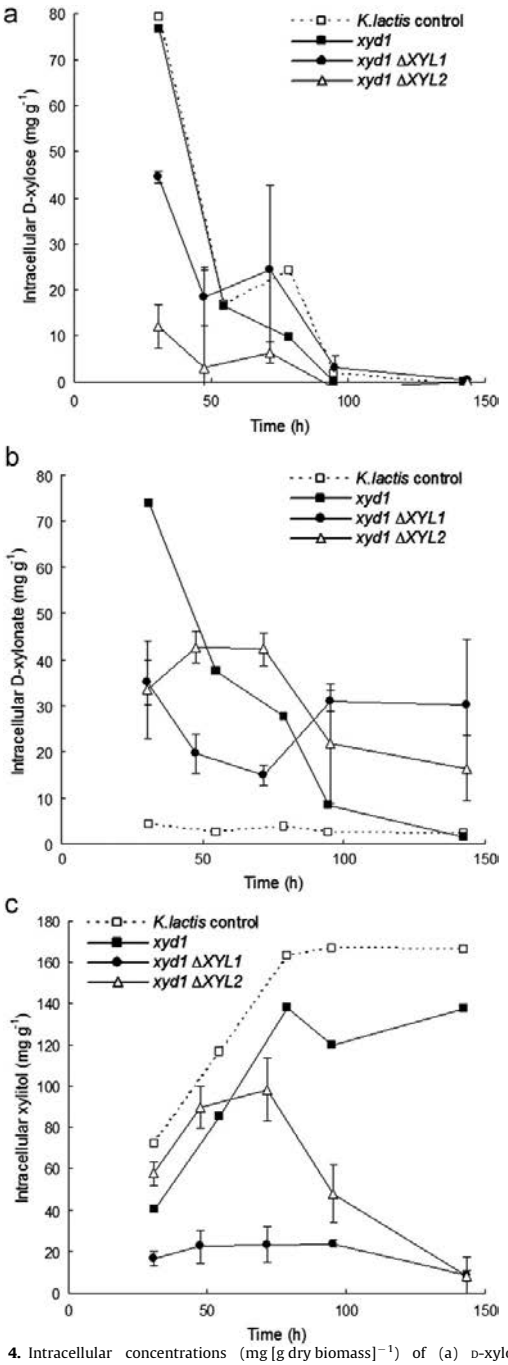

Fig. 4. Intracellular concentrations (mg [g dry biomass]$^{-1}$) of (a) D-xylose, (b) D-xylonate and (c) xylitol during cultivation of *K. lactis* H3677 (*xyd1*, solid squares) and H3765 (*xyd1ΔXYL1*, solid circles), H3763 (*xyd1ΔXYL2*, open triangles) and H3632 (control, open squares) on 10 g D-galactose l^{-1} and 20 g D-xylose l^{-1} at pH 5.5, 30 °C, OTR~12 mmol O_2 l^{-1} h^{-1}. Error bars show ± SEM for duplicate cultures. Extracellular concentrations are shown in Figs. 1 and 3.

was not affected until the oxygen supply was reduced to 3 mmol O_2 l^{-1} h^{-1}. Thus, completely aerobic conditions were not necessary and *K. lactis* could produce D-xylonate with OTRs as low as 6 mmol O_2 l^{-1} h^{-1}. None-the-less, D-xylose uptake rate was significantly ($p < 0.05$) slower with 6 mmol O_2 l^{-1} h^{-1} (0.19 g D-xylose l^{-1} h^{-1}) than with 12 mmol O_2 l^{-1} h^{-1} (0.27 g D-xylose l^{-1} h^{-1}) and D-xylose consumption may be incomplete, so 12 mmol O_2 l^{-1} h^{-1} was used as the standard condition for strain comparisons.

Deletion of the XR encoding gene improved the D-xylonate titre, yield and production rate in comparison with the strain with the intact XR encoding gene and completely eliminated extracellular xylitol production. In *S. cerevisiae* expressing *xyd1*, deletion of the aldose reductase encoding gene *GRE3* did not improve the titre or rate of D-xylonate production (Toivari et al., 2010). This may indicate that xylitol production was not as important in *K. lactis* as in *S. cerevisiae* in regenerating $NADP^+$ for XYD.

Although extracellular xylitol was low or undetected, the *xyd1ΔXYL1* derivative (H3765) of the industrial *K. lactis* strain GG799 was able to grow and produce biomass with D-xylose as the sole carbon source. Intracellular xylitol (~19 mg [g biomass]$^{-1}$) was also detected in this strain. Thus, although Billard et al. (1995) described *XYL1* as the gene encoding XR in the genome of *K. lactis* MW270-7B, it appears that H3632 (GG799) has alternative aldose reductases which enable growth on D-xylose. Indeed, there are at least 5 hypothetical aldose reductases with approx. 30% identity and 50% sequence similarity to the *S. stipitis* XR in the *K. lactis* genome.

The *K. lactis* XDH has not been characterised. We suggest that the open reading frame KLLA0D05511g of *K. lactis* strain NRRL Y-1140, which has 53% identity to the *S. stipitis* XDH encoded by *XYL2*, codes for xylitol dehydrogenase, since deletion of the corresponding gene in strain H3632 diminished the ability to use D-xylose and xylitol as carbon sources. However, the *xyd1ΔXYL2* strain (H3763) was still able to grow on D-xylose as the sole carbon source, suggesting that there are other enzymes present which perform the same reaction. There are other putative xylitol dehydrogenase encoding sequences in the *K. lactis* genome which have lower sequence similarity with *S. stipitis* XDH than that encoded by KLLA0D05511g.

Deletion of the XDH encoding gene resulted in significantly lower D-xylonate and increased xylitol production compared to H3677. Extracellular xylitol accumulated, as with H3632 and H3677 which contain the intact XDH encoding gene, during the first ca. 70 h of growth, but unlike strains with an intact endogenous D-xylose utilisation pathway, there was no apparent xylitol consumption. Intracellular xylitol concentrations decreased after 79 h, indicating the activity of an alternative xylitol dehydrogenase, increased export of xylitol or increased cell lysis.

D-Xylonate (0–70 mg [g biomass]$^{-1}$) and xylitol (10–150 mg [g biomass]$^{-1}$) both accumulated within the cytoplasm. The mechanism of D-xylonate excretion across the cell membrane is not known and no eukaryotic D-xylonate transporters have been described. Organic acids, in the charged dissociated form at intracellular pH, may be transported by specific ABC-type transporters or by facilitated or passive diffusion across the membrane (Nicolaou et al., 2010), but are generally thought to not freely diffuse through the membrane as the free acid is thought to do (Casal et al., 2008; Abbott et al., 2009). Maintenance of a high extracellular pH is thus useful in reducing the re-import of acid to the cell, but would contribute less to the initial export of the acid. Intracellular D-xylonate concentration was generally negatively correlated to the extracellular concentration, indicating either that the cells became more efficient at exporting D-xylonate with time (e.g. by induction of specific transporters) or that cell lysis contributed to its release. Intracellular xylitol concentrations did not decrease simultaneously.

Concentrations of intracellular xylitol similar to those observed here have been observed in the D-xylose utilising yeast

Pachysolen tannophilus (Xu and Taylor, 1993) and in D-xylose utilising *S. cerevisiae* (Krallish et al., 1997). The benefit of maintaining high intracellular xylitol concentrations is not clear. Although high concentrations of polyol may be a response to stress, such as high intracellular D-xylonate concentrations, this was not the case in *K. lactis*, since the parent H3632 and the D-xylonate producing strain H3677 had similar intracellular xylitol concentrations. Low intracellular xylitol concentrations in both Δ*XYL1* and Δ*XYL2* strains after 70 h cultivation may indicate poor xylitol import. Xylitol production and excretion has not been studied in *K. lactis*, but transporters for xylitol have been found in both fungi and bacteria (Suzuki et al., 2002; van de Vondervoort et al., 2006).

The high concentration of intracellular D-xylose ($\sim$70 mg [g biomass]$^{-1}$, i.e. >35 g l^{-1}) supports the hypothesis that *K. lactis* has an active D-xylose transporter. D-Xylose did not accumulate in *P. tannophilus* (Xu and Taylor, 1993). Intracellular D-xylose concentrations around 15 g l^{-1} have been observed in *S. cerevisiae* and *Debaryomyces hansenii*, while higher concentrations ($\sim$30 g l^{-1}) were observed in hybrid (*S. cerevisiae* with *D. hansenii*) yeast (Loray et al., 1997; Gárdonyi et al., 2003). In the case of *S. cerevisiae* and *D. hansenii* intracellular D-xylose concentration was also highly correlated with the extracellular concentration, as observed here for *K. lactis*. The high intracellular concentrations suggest that transport does not limit D-xylose metabolism in *K. lactis*.

In this study we have significantly increased the production of D-xylonate over that achieved earlier with *S. cerevisiae*, demonstrating the benefit of using a strain with good D-xylose uptake. Using a more robust D-xylose utilising yeast, such as *K. marxianus* and introducing xylanases for a consolidated process, as already demonstrated for ethanol production in other yeast (Katahira et al., 2004; den Haan et al., 2007; Voronovsky et al., 2009), would further improve the process. In *K. lactis*, the deletion of *XYL1* was beneficial for D-xylonate production. However, the accumulation of D-xylonate in the cytoplasm may indicate difficulties with export.

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PUBLICATION II

Metabolic engineering of *Saccharomyces cerevisiae* for bioconversion of D-xylose to D-xylonate

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Metabolic engineering of *Saccharomyces cerevisiae* for bioconversion of D-xylose to D-xylonate

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ABSTRACT

An NAD^+-dependent D-xylose dehydrogenase, XylB, from *Caulobacter crescentus* was expressed in *Saccharomyces cerevisiae*, resulting in production of 17 ± 2 g D-xylonate l^{-1} at 0.23 g l^{-1} h^{-1} from 23 g D-xylose l^{-1} (with glucose and ethanol as co-substrates). D-Xylonate titre and production rate were increased and xylitol production decreased, compared to strains expressing genes encoding *T. reesei* or pig liver $NADP^+$-dependent D-xylose dehydrogenases. D-Xylonate accumulated intracellularly to ~70 mg g^{-1}; xylitol to ~18 mg g^{-1}. The aldose reductase encoding gene *GRE3* was deleted to reduce xylitol production. Cells expressing D-xylonolactone lactonase *xylC* from *C. crescentus* with *xylB* initially produced more extracellular D-xylonate than cells lacking *xylC* at both pH 5.5 and pH 3, and sustained higher production at pH 3. Cell vitality and viability decreased during D-xylonate production at pH 3.0. An industrial *S. cerevisiae* strain expressing *xylB* efficiently produced 43 g D-xylonate l^{-1} from 49 g D-xylose l^{-1}.

1. Introduction

D-Xylose is an abundant pentose sugar present in lignocellulosic plant material, which is currently considered primarily as a potential feed-stock for ethanol or xylitol production (Akinterinwa and Cirino, 2009; Nair and Zhao, 2010; Skorupa Parachin et al., 2011). However, its oxidation product D-xylonic acid or its conjugate base D-xylonate has potential applications as chelator, dispersant, clarifying agent, antibiotic, health enhancer, polyamide or hydrogel modifier or 1,2,4-butanetriol precursor (Millner et al., 1994; Chun et al., 2006; Markham, 1991; Tomoda et al., 2004; Pujos, 2006; Niu et al., 2003; Zamora et al., 2000). D-Xylonate could also serve as a non-food derived replacement of D-gluconic acid.

Microbial production of D-xylonate with bacteria e.g., *Pseudomonas sp.* or *Gluconobacter oxydans* has been well described (Buchert et al., 1986, 1988; Buchert, 1990). High D-xylonate yields and relatively high production rates from D-xylose are obtainable with bacteria, but when birch wood hydrolyzates were used as substrate, the conversion of D-xylose to D-xylonate decreased (Buchert et al., 1989, 1990). *Gluconobacter* species have periplasmic, membrane bound PQQ-dependent and intracellular $NAD(P)^+$-dependent dehydrogenases which oxidise D-xylose to D-xylonate. These enzymes are responsible for the oxidation of a variety of sugars and sugar alcohols, and the lack of specificity results in a mixture of acids when complex substrates such as lignocellulosic hydrolysate are provided (Buchert, 1991; Rauch et al., 2010; Hölscher et al., 2009). Resently, an *Escherichia coli* strain was engineered to produce D-xylonate from D-xylose (Liu et al., in press). However, for an industrial production process, an inhibitor tolerant organism such as the yeast *Saccharomyces cerevisiae* expressing a D-xylose specific D-xylose dehydrogenase would be advantageous. We recently described D-xylonate production with *S. cerevisiae* (Toivari et al., 2010) and *Kluyveromyces lactis* (Nygård et al., 2011) using D-xylose preferring D-xylose dehydrogenase from *T. reesei* (Berghäll et al., 2007). The activity of the *T. reesei* D-xylose dehydrogenase was relatively low in both hosts, even though the encoding gene was expressed in multiple copies. In addition, this enzyme is $NADP^+$-dependent, and reoxidation of the NADPH formed is not necessarily efficient. A D-xylose dehydrogenase with high activity, using NAD^+ as a cofactor, may provide improved D-xylonate production.

A NAD^+-dependent D-xylose dehydrogenase has been described in the oxidative catabolic D-xylose pathway (Dahms, 1974; Weimberg, 1961) of the fresh water bacterium *Caulobacter*

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crescentus (Stephens et al., 2007). In addition, NAD^+-dependent D-xylose dehydrogenase activity has been observed in *Trichoderma viridea* (Kanauchi and Bamforth, 2003) and an NAD^+-dependent D-xylose dehydrogenase was purified from *Arthrobacter sp.* (Yamanaka et al., 1977), but no gene has been annotated to these activities. Other enzymes, e.g., from mammalian tissues (dimeric dihydrodiol dehydrogenase e.g., from pig liver) (Zepeda et al., 1990; Aoki et al., 2001), Archaea *Haloarcula marismortui* (Johnsen and Schönheit, 2004), *Haloferax volcanii* (Johnsen et al., 2009), and *Pichia querquum* (Suzuki and Onishi, 1973), with D-xylose dehydrogenase activity use $NADP^+$ as cofactor. Overall, relatively little is known about the substrate specificities and kinetic properties of D-xylose dehydrogenases.

D-Xylose dehydrogenases convert D-xylose to D-xylonolactone, which is subsequently hydrolyzed either spontaneously or by a lactonase enzyme to yield D-xylonate. Only a few lactonases have been described and these have not been characterised. Spontaneous hydrolysis is relatively slow, and accumulation of D-xylonolactone inhibits growth of both *Pseudomonas fragi* (Buchert and Viikari, 1988) and recombinant *Pseudomonas putida* S12 (Meijnen et al., 2009), indicating an important role for the lactonase. In non-engineered bacteria, D-xylonolactone hydrolyzing lactonase enhances D-xylonate production, but its role in yeast has not been defined.

Here we describe the production of D-xylonate by *S. cerevisiae* using the NAD^+-dependent D-xylose dehydrogenase XylB from *C. crescentus*, and compare it with D-xylonate production using the *T. reesei* Xyd1 and another $NADP^+$-dependent enzyme, *SUS2DD* from pig liver. The recombinant XylB enzyme was purified to determine its substrate specificity and kinetic properties when expressed in yeast. This D-xylose dehydrogenase was also expressed in a strain deficient in the aldose reductase Gre3p for reduced xylitol production and in an industrial *S. cerevisiae* strain for high D-xylonate production. A strain expressing the D-xylonolactone lactonase encoding gene *xylC* of *C. crescentus* together with the D-xylose dehydrogenase encoding gene *xylB* was studied at pH 3 and pH 5. Physiological effects, such as intracellular pH, cell vitality and viability, of D-xylonolactone and D-xylonate production in *S. cerevisiae* were also assessed.

2. Materials and Methods

2.1. Strains and strain construction

Saccharomyces cerevisiae CEN.PK 113-17A (H2802; MATα, *ura3-52 HIS3 leu2-3/112 TRP1 MAL2-8^c SUC2*; Entian and Kötter, 1998) and B67002 (VTT Culture Collection) were used as parental strains. *S. cerevisiae* strain FY834 (Winston et al., 1995) was used for recombination cloning. The strains used in the study are listed in Table 1.

The D-xylose dehydrogenase encoding genes from *C. crescentus* (*xylB*, Stephens et al., 2007, CC_0821, Gene ID: 941308, NCBI) and pig liver (*SUS2DD*, Gene ID: 397337, NCBI) were obtained as synthetic genes, codon optimized for *S. cerevisiae* (Gene Art, Germany). The genes were ligated into the *Bgl*II site between the *PGK1* promoter and terminator of B1181 (derived from the multicopy plasmid YEplac195+*PGK1*PT containing *URA3*; Toivari et al., 2010), generating plasmids B3441 and B3443, respectively. Plasmids were introduced to *Saccharomyces cerevisiae* CEN.PK 113-17A strain H2802 to generate strains H3698 and H3700, respectively (Table 1). A control strain was created by introducing plasmid B1181 to *S. cerevisiae* CEN.PK 113-17A (H2802) to generate strain H3680 (Table 1). Plasmid B3441 was also introduced into the Gre3p-deficient strain H3613 (Toivari et al., 2010), resulting in strain H3722.

The D-xylonolactone lactonase encoding gene *xylC* ((Stephens et al., 2007) CC_0820, Gene ID: 941305, NCBI) was obtained as a synthetic gene, codon optimized for *S. cerevisiae* (Gene Art, Germany). The *xylC* gene was cloned using recombination. The gene was amplified with oligonucleotides 5′-TGCTTAAATCTA-TAACTACAAAAAACACATACAGGAATTCACAATGACTGCTCAAGTTAC-3′ and 5′-CTTATTCAGTTAGCTAGCTGAGCTCGACTCTAGAGGATCCC-AGATCTTTAAACCAATC-3′, and introduced into strain FY834 together with an *Eco*RI and *Bam*HI linearised plasmid B2158, modified from pYX242 (R&D systems, UK) as previously described for B2159 (Toivari et al., 2010). The resulting plasmid was named B3574. The lactonase containing plasmid was introduced into strain H2802 along with the *xylB* gene (on B3441) to create strain H3938 (Table 1).

The *xylB* expression cassette *pPGK-xylB-tPGK* was released as a *Hind*III-fragment from plasmid B3441 and the ends were made blunt with T4-polymerase. The fragment was ligated into bacterial plasmids pMLV23 and pMLV39, which had been cut with *Bam*HI and the ends modified to blunt ends. Plasmids pMLV23 and pMLV39 contain *loxP- S. cerevisiae MEL5* (*α-galactosidase*)*-loxP* and *loxP- pTEF*(*A. gossypii*)*- kan^r–tTEF*(*A. gossypii*) *-loxP* marker cassettes, respectively, with 60 bp flanking regions for targeting to the *GRE3* locus in *S. cerevisiae*. The *GRE3* flanking regions were from nucleotide −250 to −193 and from nucleotide 981 to +1040, where numbers are relative to nucleotide A in the *GRE3* ATG start codon. The *Bam*HI cloning site was included in one of the *GRE3* flanking sequences. The resulting plasmids, containing *xylB*, were named pMLV81B and pMLV82C.

Table 1
Strains of *S. cerevisiae* used or referred to in this study. Plasmids are described in the text.

Strain	H-number	Genotype or parent strain+plasmid	Dehydrogenase gene, source of gene
CEN.PK113-17A	H2802	MATα, *ura3-52 HIS3 leu2-3/112 TRP1 MAL2-8^c SUC2*	
Control	H3680	H2802+B1181	
xylB	H3698	H2802+B3441	*xylB, C. crescentus*
SUS2DD	H3700	H2802+B3443	*SUS2DD*, pig liver
xyd1[a]	H3725	H2802+B2871	*xyd1, T. reesei*
Gre3p deficient[a]	H3613	MATα, *ura3-52 HIS3 leu2-3/112 TRP1 MAL2-8^c SUC2 gre3Δ::kanMX*	
Gre3p deficient	H3720	H3613+B1181	
Gre3p deficient		H3613+B3441	*xylB, C. crescentus*
xylB	H3722		
xylB xylC	H3938	H2802+B3441+B3574	*xylB, C. crescentus*
pHluorin control	H3909	H3720+pMV118	
pHluorin *xylB*	H3910	H3722+pMV118	*xylB, C. crescentus*
N-Strep-tag *xylB*	H3779	H2802+B3694	*xylB, C. crescentus*
B67002 control		Isolated from spent sulfite liquor	
B67002 *xylB*	H3935	Two copies of *xylB*	*xylB, C. crescentus*

[a] Described in Toivari et al. (2010).

The expression cassette for XylB, together with the *loxP-kan*^{r}-*loxP* marker, was released from pMLV82C with *Not*I, introduced into B67002 cells by transformation and G418 resistant yeast colonies were collected. A second copy of *xylB* was introduced into the yeast chromosome of the B67002/pMLV82C transformant with the XylB expression cassette and *loxP-MEL5-loxP* marker, released from pMLV81B with *Not*I. Blue coloured, *MEL5* (α-galactosidase) expressing, G418 resistant yeast colonies were collected from agar-solidified YP containing 2% w/v D-galactose, supplemented with 5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside (*X-α-Gal*, 40 μg/ml) and G418 (200 μg/ml). To remove the marker cassettes from the yeast chromosome, the double transformant was retransformed with plasmid pKlNatCre (Steensma and Ter Linde, 2001), expressing the Cre recombinase. Integration of the expression cassette *pPGK-xylB-tPGK* into two *GRE3* loci in the B67002 genome was verified with PCR. The strain generated was named H3935.

Plasmid pYES2-PACT1-pHluorin (Orij et al., 2009), containing the pH responsive pHluorin gene (Miesenböck et al., 1998), was a kind gift from Dr. Smits (University of Amsterdam, The Netherlands). The pHluorin gene was cloned into vector B2158 with the constitutive *TPI1* promoter and terminator, resulting in plasmid pMV118. pMV118 was introduced into the Gre3p-deficient strain H3722, which also contains the *C. crescentus* xylose dehydrogenase *xylB*, resulting in strain H3910 (Table 1) and into the Gre3p-deficient control strain H3720, resulting in strain H3909 (Table 1).

For protein characterisation work, the *C. crescentus xylB* was expressed as an N-terminally Strep-tagged construct with an additional tobacco etch virus (TEV) cleavage site downstream of the Strep-tag. The Strep-tag was added by amplifying the *xylB* gene from plasmid B3441 by PCR using oligonucleotides 5′-GGTTCAGATCTACAATGTGGTCTCATCCACAATTCGAGAAGGAGA-ATTTGTATTTCCAATCATCTGCAATTTACCC-3′ and 5′-GGTTCAGATC-TTCATCTCCAACCAGC-3′. *Bgl*II sites were introduced to the 5′ and 3′ ends, respectively. Amplified fragments were digested with *Bgl*II and ligated into the *Bgl*II site of vector B1181, resulting in plasmid B3694. Plasmid B3694 was introduced to H2802 to generate strain H3779 (Table 1).

2.2. Media and culture conditions

Yeast-Peptone (YP) medium contained 10 g yeast extract l^{-1} and 20 g bacto-peptone l^{-1}, with D-glucose and D-xylose concentrations as indicated in the text. YP medium with 20 g D-glucose l^{-1} (YPD) was sometimes solidified with addition of 15 g agar l^{-1}.

For small scale cultures, yeast were cultured in 20 or 50 ml YP or modified synthetic complete medium (YSC, (Sherman et al., 1983)) in 100 or 250 ml Erlenmeyer flasks, respectively, at 250 rpm, 30 °C. Concentrations of added carbon source (D-glucose, D-xylose or ethanol) are indicated in the text. For larger scale cultures, yeast were grown in 500 ml medium (YP, YSC-ura, or YSC-ura-leu) in Multifors bioreactors (max. working volume 500 ml, Infors HT, Switzerland) at pH 5.5 (unless otherwise indicated), 30 °C, 1 volume air [volume culture]$^{-1}$ min^{-1} (vvm) and 500 rpm agitation with two marine impellors, as previously described (Toivari et al., 2010) or in 2 l YP medium in B. Braun Biotech International (Sartorius AG, Germany) Biostat® CT (2.5 l working volume) bioreactors at pH 5.5, 30 °C, 500 rpm and 0.5 vvm aeration. The pH was maintained constant by addition of 2 M NaOH or 1 M H_2PO_4. Clerol antifoaming agent (Cognis, France, 0.08–0.10 μl l^{-1}) was added to prevent foam formation.

2.3. Measurement of biomass

Biomass was measured as optical density (OD) at 600 nm (OD_{600}) or as dry weight. For dry weight, samples were collected in 2 ml pre-dried, pre-weighed microcentrifuge tubes, washed twice with equal volume distilled water and dried at 100 °C.

2.4. Chemical analyses

For determination of intracellular D-xylonate, D-xylose and xylitol, cells were collected from 10 ml culture. Cell pellets were washed with 1.8 ml deionised H_2O or NaCl (9 g l^{-1}), followed with 1.8 ml deionised water and frozen at −20 °C to disrupt membranes. The frozen pellets were freeze-dried using a Christ Alpha 2–4 lyophiliser (Biotech international, Belgium), removing all excess moisture. Intracellular D-xylonate, D-xylose and xylitol were extracted from the lyophilized pellets (~60 mg biomass ml^{-1}) in 5 mM H_2SO_4, as described by Nygård et al. (2011). Cell debris was removed by centrifugation and the supernatant analysed by HPLC. Intracellular concentrations are given as mg per g dry biomass, but a conservative estimate of intracellular concentrations can be derived by assuming that 1 g dry cell weight corresponds to 2 ml cell volume (Gancedo and Serrano, 1989; de Koning and van Dam, 1992). This estimate is conservative since it does not take into account the volume of intracellular organelles, variation in cell wall thickness, or the contribution of dead cells to the dry biomass.

Extracellular and intracellular compounds (D-xylonic acid and/or D-xylonolactone, ethanol, glycerol, pyruvate and acetate, D-glucose and D-xylose) were analysed by HPLC using a Fast Acid Analysis Column (100 mm × 7.8 mm, BioRad Laboratories, Hercules, CA) linked to an Aminex HPX-87 H column (BioRad Labs, USA) with 2.5 mM H_2SO_4 as eluent and a flow rate of 0.5 ml min^{-1}. The column was maintained at 55 °C. Peaks were detected using a Waters 410 differential refractometer and a Waters 2487 dual wavelength UV (210 nm) detector. When D-xylonic acid was present, D-xylose concentrations were estimated by subtraction of the D-xylonic acid (detected by UV) from the combined D-xylose and D-xylonic acid/D-xylonolactone peak detected by RI.

Extracellular D-xylonic acid concentrations were also measured as the lactone using the hydroxymate method (Lien, 1959) as described by Toivari et al. (2010).

2.5. Determination of vitality and viability of D-xylonate producing cells

The number of metabolically active (vital) cells was determined microscopically by methylene blue (0.25 g l^{-1} in 0.04 M NaCitrate buffer pH 8.3 or in 0.1 M phosphate buffer pH 4.6) staining. Viability was determined by comparing the number of viable colony forming units (CFU) on YPD to the total cell number determined microscopically or to the number of viable CFU of a control strain.

2.6. Enzyme purification

The *S. cerevisiae* strain expressing the Strep-tagged XylB (Strep-TEV-XylB) was grown in YCS-ura for 16 h and the cells were harvested by centrifugation at 3000 rpm (1600 × *g*) for 10 min, +4 °C. The cells were washed with Milli-Q water and disrupted with acid-washed glass beads (Ø 425–600 μm, Sigma–Aldrich Corporation, USA) in 50 mM sodium phosphate buffer pH 8.0, containing protease inhibitor (cOmplete Mini EDTA-free, Roche). The cell lysate was centrifuged at 4000 × *g* for 30 min at +4 °C. Crude protein extract was loaded on an affinity column (Strep-Tactin Superflow Plus (1 ml, QIAGEN) and the protein purified using an ÄKTAPurifier FPLC system (GE Healthcare). The affinity column was equilibrated with 50 mM sodium phosphate, 300 mM NaCl, pH 8 and washed with 10 column volumes

after sample loading. The same buffer containing 2.5 mM of desthiobiotin (Sigma) was used to elute the protein from the column. Fractions of 800 μl were collected and analyzed by SDS-PAGE 4–20% gradient gel (Criterion SF, Bio-Rad Laboratories) and using an NADH absorbance-based activity assay (see below). Fractions containing active enzyme were pooled and concentrated to a total volume of 2.5 ml with a VIVASPIN 20 concentrator tube ((Sartorius Vivascience) with a molecular weight cutoff of 10 kDa. The resulting concentrated solution was desalted using a PD 10 desalting column (GE Healthcare, Sephadex™ G-25 M) to a final volume of 3.5 ml of 50 mM Tris-Cl buffer pH 8. All purification steps were carried out at +4 °C.

The protein concentration of the purified Strep-TEV-XylB enzyme was determined by measuring absorbance at 280 nm using a theoretical molar extinction coefficient, $\varepsilon = 43{,}680\ M^{-1}\ cm^{-1}$, which was calculated from the primary amino acid sequence.

2.7. Enzyme activity

D-xylose dehydrogenase activity from crude cell extracts was measured according to Berghäll et al. (2007) in 100 mM Tris/HCl, pH 8.1 in the presence of 2 mM $MgCl_2$ using either 1 mM NAD^+ or $NADP^+$ as cofactor and 100 mM D-xylose (L-arabinose, D-glucose, or D-ribose) as substrate. The measurements were performed at 30 °C using a Konelab Arena photometric analyzer (Thermo Electron Oy, Finland). Protein concentration from crude cell extracts was determined using a Bio-Rad protein kit, based on the assay developed by Bradford (1976).

The pH optimum of the purified Strep-tagged XylB dehydrogenase (Strep-TEV-XylB) was determined in the pH range from 2.4 to 10.1 using 10 mM D-xylose and 5 mM NAD^+ with McIlvaine (50 mM, pH 2.4–7.3), Tris-HCl (100 mM, pH 8.0–9.2) and Glycine (100 mM, pH 10.1) buffers. Substrate specificity of the purified Strep-TEV-XylB was measured in 50 mM bis-Tris propane (BTP) buffer, pH 9.0 using 1, 10 and 100 mM substrate, 5 mM NAD^+ and 19 nM enzyme. Pentose sugars D-xylose, L-arabinose, D-lyxose and D-ribose, and hexose sugars D-glucose, D-galactose and D-mannose were used as substrates. The kinetic constants (k_{cat} and K_m values) of the purified Strep-TEV-XylB on D-xylose, L-arabinose and NAD^+ were determined in 50 mM BTP buffer pH 9.0, 50 mM BTP buffer pH 7.0 and in vivo like (IVL) buffer pH 6.8, described by van Eunen et al. (2010). Ten different substrate or cofactor concentrations (0–10 mM, 0–800 mM and 0—5 mM, for D-xylose, L-arabinose and NAD^+, respectively), were used. D-Xylose dehydrogenase activity was measured at 25 °C as the formation of NADH, detected at 340 nm using a Varioskan kinetic plate reader (Thermo Electron Corporation, USA). The kinetic parameters were obtained by curve fitting analysis using GraphPad Prism software 5.03 (GraphPadPrism Software Inc., USA). The effect of $MgCl_2$ up to 10 mM on XylB dehydrogenase activity was also tested, but was found to be negligible and thus omitted.

2.8. NMR analyses

NMR analysis of culture supernatant and cell extracts for D-xylonolactone lactonase activity measurements were carried out at 22 °C on a 500 MHz Bruker Avance III. The D_2O needed for the spectrometer lock was confined in a coaxially located glass capillary, so the samples were in 100% H_2O environment. ^1H NMR spectra were recorded with a standard 1 s presaturation of the water signal.

The hydrolysis of chemically produced xylono-1,4-lactone was monitored in vitro by ^1H NMR spectroscopy. The D-xylono-1,4-lactone was obtained from pure Ca-D-xylonate by incubating it in 0.1 M HCl at 95 °C for 3 h. Approximately 65% of the D-xylonate was converted to the 1,4-lactone form under these conditions and a small amount of another product, putatively 1,5-lactone, was detected. The pH of the D-xylonic acid – lactone mixture was adjusted by adding 50 mM Na-phosphate D_2O buffers of different pH and the hydrolysis was monitored by ^1H NMR. NMR spectra were recorded at 22 °C on a 500 MHz Varian Inova NMR spectrometer with 1 s presaturation of the water signal. The pH of the solutions was measured after the analysis.

2.9. In situ pHluorin calibration and measurement

Ratiometric pHluorin was used to determine intracellular pH as described by Miesenböck et al. (1998). For calibration of the probe, yeast strains expressing pHluorin were grown in flasks in YSC-ura-leu medium with 50 g D-glucose l^{-1} to an OD_{600} of approximately 1.0. Calibration buffers contained 50 mM MES, 50 mM HEPES, 50 mM KCl, 50 mM NaCl, 200 mM ammonium acetate, 10 mM NaN_3, 10 μM nigericin (Sigma) and the pH was adjusted with NaOH or HCl (Brett et al., 2005). For imaging, the cells were grown in flasks in YSC-ura-leu medium (pH5.5) with 10 g D-glucose l^{-1}, 20 g D-xylose l^{-1}, and 10 g $CaCO_3$ l^{-1} to buffer the cultures.

A Leica TCS SP5 laser scanning microscope mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems, Germany) with an HCX plan apo 63 × (NA 1.4) oil immersion objectives was used for imaging. For pH probe recording, a method described by Bagar et al. (2009) was adopted. For sequential excitation, a 50 mW 405 nm diode laser and the 476 nm line of a 25 mW argon laser were used. Laser powers 6% for the diode laser and 12% for the argon laser were employed. Successive images excited at 405 and 476 nm were captured within 1.2 s of each other.

Images were analysed using ImageJ (Rasband, 1997–2011; Abramoff et al., 2004) and ratio calculations and conversion to pH values were determined as in Bagar et al. (2009)

3. Results

3.1. D-xylose dehydrogenases and their activity in recombinant S. cerevisiae strains

Crude cell extract containing XylB had activity of 45.4 ± 3.0 nkat [mg protein]$^{-1}$ with D -xylose as substrate and was strictly NAD^+-dependent. The cell extract containing *SUS2DD* had activity of 1.32 ± 0.11 nkat [mg protein]$^{-1}$ and required $NADP^+$ as a cofactor. No D-xylose dehydrogenase activity was detectable in crude cell extracts from the control strain (H3680), lacking a D-xylose dehydrogenase gene.

The XylB was purified from *S. cerevisiae* using an *N*-terminal Strep-tag, and its kinetic properties and pH optimum determined (Table 2). XylB was most active at pH 9 but remained active over a wide pH range from pH 7 to 10, and showed 30% activity at pH 5 (data not shown). The purified enzyme had activity only with the pentose sugars D-xylose, L-arabinose and D-lyxose (measured at pH 9); D-xylose was clearly the best substrate and D-lyxose the worst (data not shown). There was no activity detected with D-ribose, D-glucose, D-galactose or D-mannose. The measured kinetic constants (K_m, k_{cat} and k_{cat}/K_m) for D-xylose and L-arabinose are shown in Table 2. XylB was very specific for D-xylose, having a specificity constant (k_{cat}/K_m) for D-xylose which is 400–600 fold higher than for L-arabinose at the physiological (pH 6.8) as well as in the optimum pH (pH 9).

3.2. Production of D-xylonate with the D-xylose dehydrogenase XylB

S. cerevisiae strains expressing *xylB* and *SUS2DD* genes coding for D-xylose dehydrogenases were grown in bioreactor cultures at

Table 2
Kinetic properties (k_{cat}, K_m for NAD^+, D-xylose or L-arabinose and specificity constant k_{cat}/K_m) of the N-terminally Strep tagged XylB produced in *S. cerevisiae* at pH 6.8 (IVL physiological buffer), pH 7 (50 mM BTP buffer) and pH 9 (50 mM BTP buffer). NAD^+ (5 mM) was used as cofactor to determine the properties with D-xylose and L-arabinose as substrate.

Substrate	Parameter	pH 6.8	pH 7	pH 9
NAD^+	k_{cat} (min^{-1})	1550 ± 55	2140 ± 65	1860 ± 65
	K_m (mM)	0.26 ± 0.04	0.31 ± 0.03	0.46 ± 0.05
	k_{cat}/K_m (mM^{-1} min^{-1})	6100 ± 1200	6900 ± 1000	4000 ± 650
D-Xylose	k_{cat} (min^{-1})	1570 ± 60	1820 ± 50	1360 ± 50
	K_m (mM)	0.40 ± 0.06	0.24 ± 0.03	0.08 ± 0.02
	k_{cat}/K_m (mM^{-1} min^{-1})	4000 ± 800	7500 ± 1100	16800 ± 4700
L-Arabinose	k_{cat} (min^{-1})	1335 ± 45	1755 ± 50	1455 ± 40
	K_m (mM)	180 ± 15	100 ± 10	40 ± 5
	k_{cat}/K_m (mM^{-1} min^{-1})	7 ± 1	17 ± 3	36 ± 6

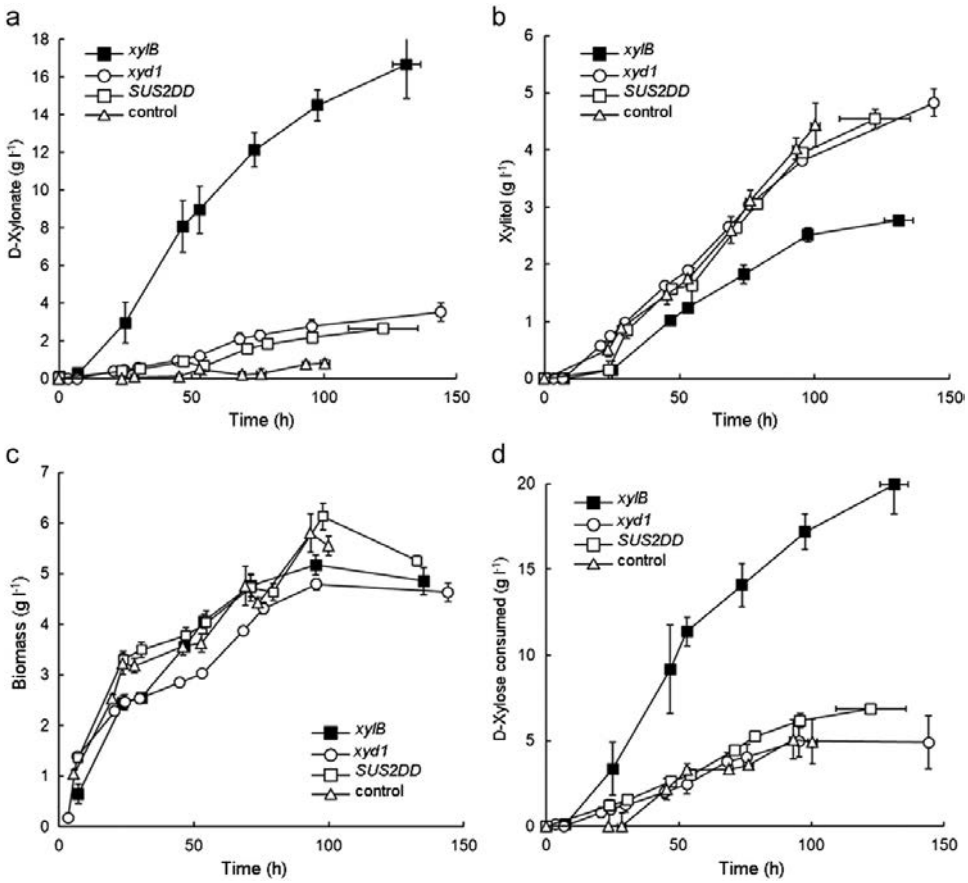

Fig. 1. D-xylonate (a), xylitol (b) and biomass (c) production and D-xylose consumed (d) by *S. cerevisiae* expressing *xylB* (solid square), *xyd1* (open circle, Toivari et al., 2010), or *SUS2DD* (open square), or containing an empty vector (open triangle). Cells were grown in bioreactors with 23 ± 1 g D-xylose l^{-1} and 9 ± 1 g D-glucose l^{-1}, pH 5.5, 30 °C, supplemented with 5 ± 1 g ethanol l^{-1} after ~54 h. Cultures were agitated at 500 rpm with 1 vvm aeration. Error bars show SEM (n=2 or 3).

pH 5.5 as described by Toivari et al. (2010). The strain expressing *SUS2DD* produced 2.7 ± 0.1 g D-xylonate l^{-1} and 4.5 ± 0.2 g xylitol l^{-1} when provided 23 ± 1 g D-xylose l^{-1} at pH 5.5 (Fig. 1), similar to that produced by the stain expressing *T. reesei xyd1* (Toivari et al., 2010). The strain expressing *xylB* produced 16.7 ± 1.8 g D-xylonate l^{-1} and 2.7 ± 0.1 g xylitol l^{-1} from 23 ± 1 g D-xylose l^{-1} (Fig. 1). The negative control produced no D-xylonate but similar amounts of xylitol (4.4 ± 0.4 g xylitol l^{-1}) as the strains expressing *SUS2DD* or *xyd1* (Toivari et al., 2010). *S. cerevisiae* expressing *xylB* produced D-xylonate at an approximately 10 times higher rate than observed in the strains expressing the $NADP^+$-dependent dehydrogenase encoding genes *xyd1* or *SUS2DD* (Table 3)

Table 3
Initial D-xylose consumption rate, D-xylonate and xylitol production rates, approximate yield of D-xylonate and xylitol on consumed D-xylose and specific D-xylonate production rate for recombinant *S. cerevisiae*. CEN.PK strains were grown in YSC 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. B67002 cells were grown in YP 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} after 47.4 h. Cultures were maintained at 30 °C, 500 rpm, 1 vvm aeration and pH 5.5, except as indicated. Values are mean ± SEM from 2 to 4 cultivations.

Strain	D-xylose consumption rate (g l^{-1} h^{-1})	D-xylonate production rate (g l^{-1} h^{-1})	D-xylitol production rate (g l^{-1} h^{-1})	Yield D-xylonate/ D-xylose (g g^{-1})	Yield D-xylitol/ D-xylose (g g^{-1})	Specific D-xylonate production rate (mg g^{-1} h^{-1})
control	0.06 ± 0.01	0	0.04 ± 0.00	0	0.9	0
xylB	0.23 ± 0.01	0.23 ± 0.02	0.03 ± 0.01	0.8	0.1	58
SUS2DD	0.07 ± 0.00	0.02 ± 0.00	0.05 ± 0.00	0.4	0.6	6
xyd1[a]	0.06 ± 0.01	0.03 ± 0.00	0.04 ± 0.00	0.4	0.6	7
gre3 xylB	0.21 ± 0.01	0.20 ± 0.01	0.01 ± 0.00	0.8	0.03	58
xylB xylC	0.27 ± 0.01	0.24 ± 0.01	0.04 ± 0.00	0.8	0.1	61
xylB pH 3	0.12 ± 0.02	0.08 ± 0.01	0.03 ± 0.00	0.8	0.2	28
xylB xylC pH 3	0.15 ± 0.02	0.13 ± 0.01	0.02 ± 0.00	0.8	0.1	48
B67002 0.5 vvm	0.48 ± 0.02	0.44 ± 0.01	0.11 ± 0.01	0.8	0.2	64

[a] Data from Toivari et al. (2010).

and was able to consume D-xylose 3 to 4 times faster than these strains or the parent which did not produce D-xylonate. Expression of *xylB* in *S. cerevisiae* did not affect biomass production (Fig. 1).

Deletion of the aldose reductase encoding gene *GRE3* reduced xylitol production to only 0.5 g xylitol l^{-1}, without reducing the D-xylose consumption rate or D-xylonate production rate in *S. cerevisiae* expressing *xylB* (Table 3). D-Xylonate production (15.8 ± 0.6 g l^{-1}) was similar to *S. cerevisiae xylB*, but biomass production (4.3 ± 0.1 g l^{-1}, compared to 5.2 ± 0.2 g l^{-1} for *S. cerevisiae xylB*, Fig. 1) was slightly reduced when *GRE3* was deleted.

The *xylB* gene was also integrated in two of several *GRE3* loci present in the industrial, hydrolysate tolerant *S. cerevisiae* strain B67002, which is polyploid or aneuploid. The strain was grown 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 (Fig. 2, Table 3). From 49 g D-xylose l^{-1} this strain produced 43 ± 1 g D-xylonate l^{-1}, and 8 g xylitol l^{-1} in 120 h, with an initial D-xylonate production rate of 0.44 g l^{-1} h^{-1} (Fig. 2, Table 3).

3.3. Effect of D-xylonolactone lactonase XylC on production of D-xylonate

The *xylB xylC* strain produced 13 ± 2 g D-xylonate l^{-1} at pH 5.5. It released more D-xylonate into the culture supernatant during the first 50 h cultivation than the *xylB* strain, although during the production phase the initial production rate (0.24 ± 0.01 g l^{-1} h^{-1}) was similar to that of *xylB* (0.23 ± 0.02 g l^{-1} h^{-1}; Fig. 3, Table 3). After ~50 h, D-xylonate production by *xylB xylC* essentially stopped, although D-xylose was still available. The *xylB xylC* strain produced less biomass than *S. cerevisiae xylB* (Fig. 3) and the specific D-xylonate production rate of the two strains was similar (Table 3). Since the lactone form is more stable at low pH, production of D-xylonic acid by *xylB* and *xylB xylC* was also compared at pH 3. Production of D-xylonic acid, xylitol and biomass were reduced for both strains at pH 3, compared to pH 5.5 (Fig. 3, Table 3). At pH 3, the *xylB xylC* strain produced more D-xylonic acid (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 (Table 3).

^{1}H NMR analysis demonstrated that in the culture supernatants of the *xylB*, *xylB xylC* and *xylB*-B67002 strains both linear D-xylonate and D-xylonolactone were present at pH 5.5 and pH 3. Spontaneous hydrolysis of D-xylonolactone in aqueous solution at pH 6.8 or 7.2 was relatively slow, with only 12% (pH 6.8) and 20% (pH 7.2) of the lactone hydrolysed spontaneously within 16 h. When gel filtered cell extract of the *xylB xylC* strain was used, the hydrolysis of D-xylonolactone was more rapid (100% in < 1 h) compared to similar cell extract from the *xylB* strain lacking *xylC* (40 to 90% hydrolysed in 16 to 24 h).

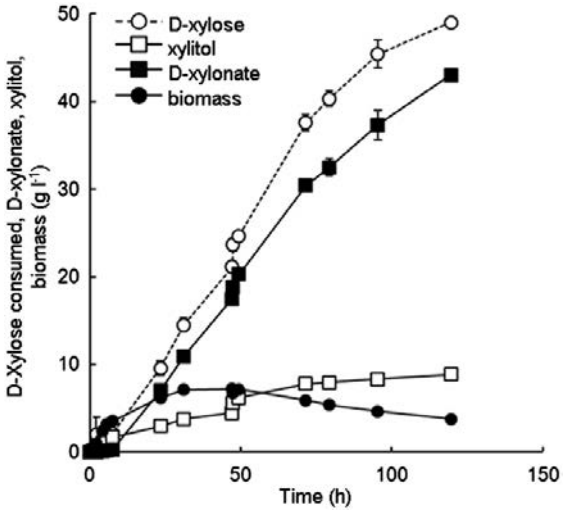

Fig. 2. D-xylonate (solid square), xylitol (open square) and biomass (solid circle) produced and D-xylose consumed (open circle) by *S. cerevisiae* B67002 *xylB* grown on YP with 8 g D-glucose l^{-1} and 21 g D-xylose l^{-1}, supplemented with 4 g D-glucose l^{-1} and 28 g D-xylose l^{-1} after 47.4 h. Cells were maintained at pH 5.5, 30 °C, with 500 rpm and 0.5 vvm aeration. Error bars show SEM for three cultivations and where not seen are smaller than the symbol.

3.4. Effect of D-xylonate production on cellular physiology

D-xylonate accumulated intracellularly to 40 to 80 mg g^{-1} (i.e., at least 20 to 40 g l^{-1}; Fig. 4(a)) in *S. cerevisiae* CEN.PK expressing D-xylose dehydrogenase (Fig. 1). Intracellular accumulation of D-xylonate did not reflect extracellular D-xylonate concentration. Xylitol also accumulated intracellularly (10 to 50 mg g^{-1}, Fig. 4(b) and (d)), and was generally correlated to the extracellular concentration (r^2 = 0.66 to 0.91). The intracellular xylitol concentration was lower in the *gre3 xylB* strain than in the strain with a functional Gre3p (Fig. 4(b)). Industrial strain B67002 had higher intracellular D-xylonate concentrations than the CEN.PK strains, maintaining ~170 mg D-xylonate [g dry biomass]$^{-1}$ in the cytoplasm after 48 h incubation in D-xylose containing medium (Fig. 4(a)). Intracellular D-xylonate was initially present in higher concentrations

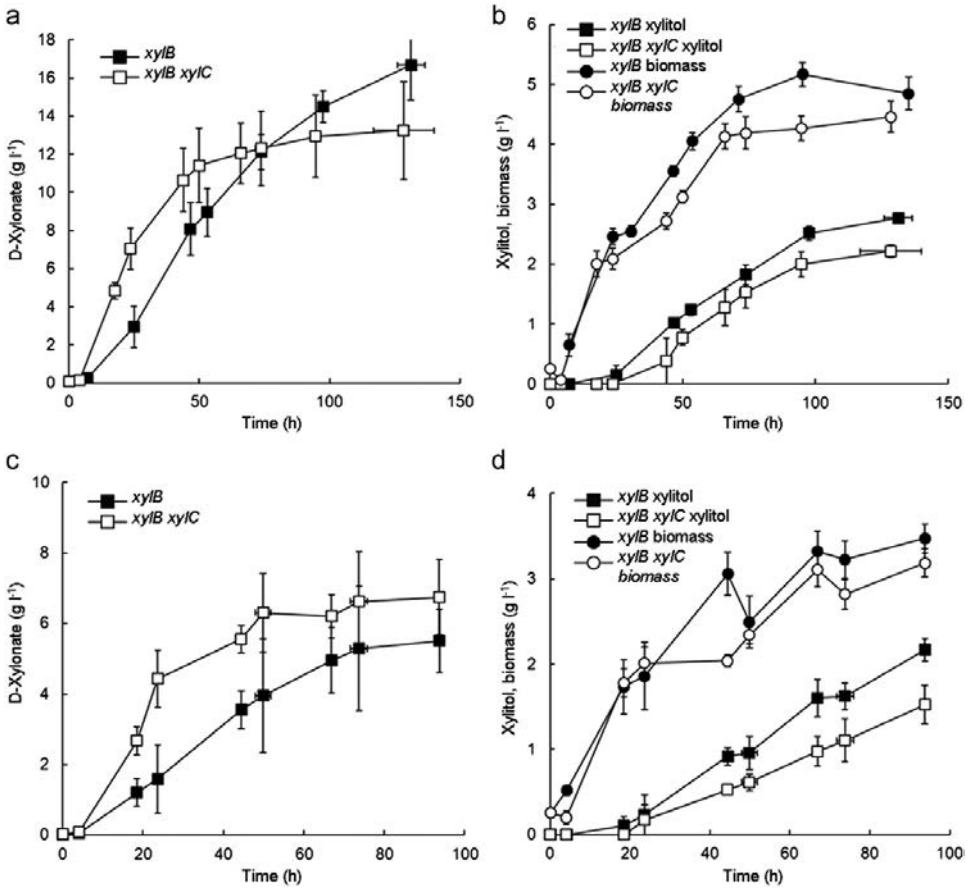

Fig. 3. Production of D-xylonate (a), (c), xylitol and biomass (b), (d) at pH 5.5 (a), (b) or at pH 3.0 (c), (d) by *S. cerevisiae xylB* (solid symbols) and *xylB xylC* (open symbols) strains in YSC medium with 9 ± 1 g D-glucose l^{-1} and 23 ± 1 g D-xylose l^{-1}, supplemented with 5 ± 1 g ethanol l^{-1} 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 for ($n=2$ to 4).

in the *xylB xylC* strain than in the *xylB* strain at both pH 5.5 and pH 3.0, but after ~50 h concentrations in *xylB xylC* were lower than in *xylB* (Fig. 4(c)). At pH 3, the intracellular D-xylonate concentration of both *xylB* and *xylB xylC* strains was lower compared to at pH 5.5 (Fig. 4(c)).

Production of D-xylonate resulted in a strong loss of vitality ($16 \pm 2\%$) for CEN.PK strains during the first 25 h cultivation (Fig. 5(a)). Subsequently, little or no cell death appeared to occur. Co-expression of pHluorin with *xylB* did not result in greater cell death (data not shown). In contrast with the CEN.PK strain, B67002 *xylB* did not show significant loss in vitality during the first 31 h of D-xylonate production (Fig. 5(a)), when 11 ± 0.3 g D-xylonate l^{-1} had been produced and 102 ± 9 mg D-xylonate [g biomass]$^{-1}$ was extracted from the cytoplasm. As extracellular D-xylonate continued to increase, inactive and empty cells began to accumulate until $77 \pm 1\%$ of the cells were no longer active at 120 h.

More inactive cells occurred in lactonase expressing cells than in the strain without *xylC* (Fig. 5(a) and (b)). More loss of vitality occurred at pH 3 than at pH 5.5 for both *xylB* and *xylB xylC* strains (Fig. 5(b)), and even small differences in pH (± 0.1 pH unit) had a large effect on the vitality of cells in replicate cultures at low pH.

Viable counts with B67002 *xylB* and CEN.PK *xylB* and *xylB xylC* strains at pH 3 showed that the loss in vitality reflected loss in viability (Fig. 5(c)). For simplicity, viability will be used in the discussion to refer to results from either staining or colony counting.

When strains expressing the pH sensitive green fluorescent protein pHluorin were grown at pH 5.5, both D-xylonate producing and non-producing cells had an initial average cytoplasmic pH of 7.5. Within 18 h, the average intracellular pH of the *xylB* strain (3 g D-xylonate l^{-1} produced) had decreased to 7.2, whereas that of the control cells was 7.4. Fewer cells remained fluorescent in the *xylB* than in the control strain.

4. Discussion

The bacterial D-xylose dehydrogenase XylB from *C. crescentus* had > 30 fold higher activity in crude cell extracts with D-xylose as substrate, compared to the D-xylose dehydrogenase Xyd1 of *T. reesei* (Toivari et al., 2010) or the *SUS2DD* dehydrogenase from pig liver, when expressed in *S. cerevisiae*. The *S. cerevisiae* strain expressing the *xylB* gene had a more than 5 fold higher D-xylonate titre (Fig. 1) and 10 fold higher volumetric and specific production rates (Table 3), compared with the D-xylose dehydrogenases from *T. reesei* or pig liver. Even higher levels of D-xylonate production

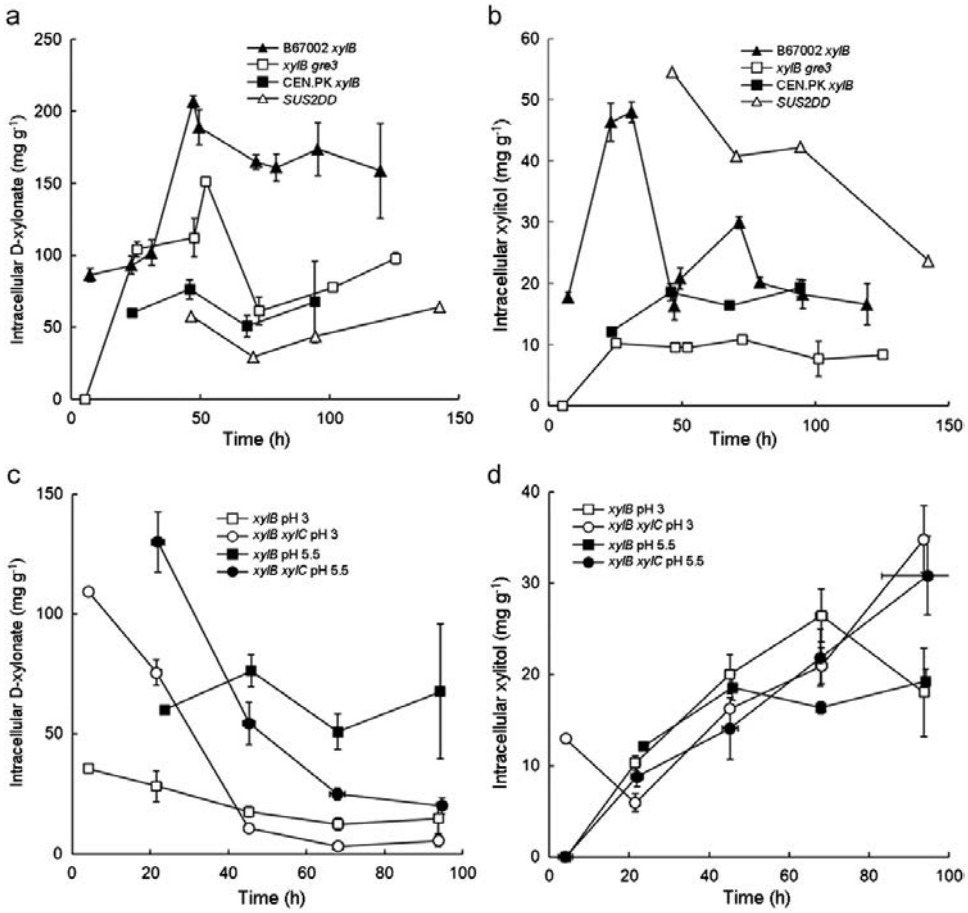

Fig. 4. Intracellular concentrations (expressed as mg per g dry biomass) of D-xylonate (a) and (c) and xylitol (b) and (d). Parts (a) and (b) show intracellular concentrations extracted from *S. cerevisiae xylB* (solid square), *SUS2DD* (open triangle), Gre3p-deficient *xylB* (open square) and B67002 *xylB* (solid triangle) grown on D-glucose and D-xylose as described in the Materials and Methods, and Figs. 2–4 at pH 5.5, 30 °C. Parts (c) and (d) show intracellular concentrations from *S. cerevisiae xylB* (squares) and *xylB xylC* (circles) grown at pH 3.0 (open symbols) or pH 5.5 (solid symbols). Error bars show SEM ($n=1$ to 3).

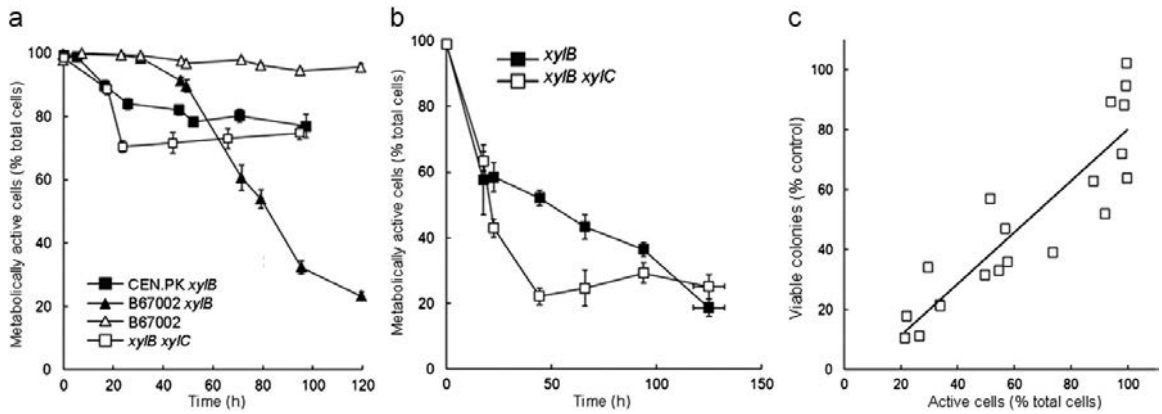

Fig. 5. Percentage of metabolically active cells, as determined by methylene blue staining, in populations of (a) *S. cerevisiae xylB* (solid squares), B67002 *xylB* (solid triangle), *xylB xylC* (open square) and B67002 (open triangle) grown in D-glucose and D-xylose containing medium at pH 5.5, as described in the Materials and Methods, and (b) *S. cerevisiae xylB* (solid squares) and *xylB xylC* (open squares) grown in D-glucose and D-xylose containing medium at pH 3.0. Extracellular D-xylonate concentrations are shown in Figs. 2 and 3 and intracellular D-xylonate concentrations in Fig. 4. The correlation between metabolically active cells and cell viability (expressed as the % viable colony forming units, CFU, relative to the non-D-xylonate-producing control strain, B67002) for B67002 *xylB* at pH 5.5 is presented in panel c.

(43 g D-xylonate l^{-1}) were obtained with an industrial, hydrolysate-tolerant strain expressing *xylB*.

NAD^+-dependency, kinetic properties and/or enzyme concentration presumably all contributed to enhanced D-xylonate production with *xylB* expressing *S. cerevisiae* strains. NADH generated by XylB activity can be efficiently reduced via oxidative phosphorylation in aerobic conditions, replenishing the NAD^+ supply and providing additional ATP for energy, whereas availability of $NADP^+$ would be mostly dependent on anabolic metabolic reactions.

Previously the best D-xylonate production levels with yeast have been reported for *K. lactis* expressing the *T. reesei xyd1* gene (Nygård et al., 2011). Compared to the *K. lactis xyd1* strain the *S. cerevisiae* CEN.PK *xylB* strain produced >2 fold more D-xylonate at a higher volumetric production rate, while producing less xylitol. Yield of D-xylonate on D-xylose consumed was thus also substantially improved with XylB in *S. cerevisiae*, compared to Xyd1 in *K. lactis*. Expression of *xylB* in *S. cerevisiae* CEN.PK resulted in a D-xylose consumption rate which was only slightly lower (0.23 g l^{-1} h^{-1}) than that of *K. lactis xyd1* (0.27 g l^{-1} h^{-1}; Nygård et al., 2011), demonstrating that the low natural D-xylose transport rates observed during ethanol production (typically less than 0.1 g l^{-1} h^{-1}; Pitkänen et al., 2005; van Vleet et al., 2008; Liu and Hu, 2010) can be increased with D-xylonate production.

The high specificity of XylB for D-xylose was confirmed (Table 2) (Stephens et al., 2007). The binding affinity for D-xylose (K_m=0.08–0.40 mM) is clearly higher than that of the *T. reesei* Xyd1 (K_m=43 mM when purified from *S. cerevisiae*; Berghäll et al., 2007) or the native aldose reductase Gre3p (K_m=14–28 mM; Jeong et al., 2002; Kuhn et al., 1995). The k_{cat} of XylB (1360–1820 min^{-1}) is also higher than that of Gre3p (k_{cat}=202–864 min^{-1}; Jeong et al., 2002; Kuhn et al., 1995). This facilitates D-xylose flux to D-xylonate rather than xylitol and strains expressing *xylB* produced less xylitol (Table 3), compared to the control strain or strains expressing *SUS2DD* or *xyd1* (Toivari et al., 2010). Deletion of *GRE3* in the *xylB* strain reduced xylitol production by ~80% compared to the *xylB* strain with *GRE3*, although its deletion generally reduces xylitol production by ~67% (Toivari et al., 2010; Träff et al., 2001).

D-Xylose dehydrogenases convert D-xylose to D-xylonolactone, which is subsequently hydrolysed either spontaneously or by a lactonase to yield D-xylonate. *S. cerevisiae* expressing the *C. crescentus* D-xylonolactone lactonase encoding gene *xylC* along with *xylB* excreted more D-xylonate earlier during the cultivation, both at pH 5.5 and at pH 3, than the strains lacking the lactonase. The greater initial reduction in viability of the *xylB xylC* strain, compared to the *xylB* strain, and the earlier stop in production at pH 5.5, suggested that in *S. cerevisiae* the linear form may be more toxic than the lactone form and that a more gradual hydrolysis of the lactone form may be advantageous to the cells.

The greatest loss in viability in CEN.PK *xylB* strains occurred during the first 24 h of cultivation when D-xylonate was accumulating within the cytoplasm, but extracellular concentrations remained low. This may suggest that intracellular accumulation of D-xylonate contributes to cell death, but that the cells which survive are able to adapt physiologically to the high intracellular D-xylonate concentrations. B67002 *xylB* cells were more viable than CEN.PK *xylB* cells with the same concentration of extracellular D-xylonate, but did not appear to adapt to the presence of D-xylonate. This probably reflects the continued D-xylonate production and/or the high intracellular D-xylonate concentration (Fig. 4). In vivo imaging of intracellular pH using the pH responsive protein pHluorin showed that the intracellular pH decreased more rapidly in D-xylonate producing CEN.PK strains than in control cells, as also observed for lactic acid producing *S. cerevisiae* using the pH responsive colour cSNARF-4f (Valli et al., 2006).

As has previously been observed with lactic acid production at low pH (Porro et al., 1999; van Maris et al., 2004a, b), less D-xylonic acid was produced at a lower rate at low compared to high pH. At low pH, maintenance of pH homeostasis may be more energy demanding than at pH values closer to the cytoplasmic pH. A larger supply of D-glucose/ethanol might improve low pH production by providing energy for cell maintenance.

Expression of the NAD^+ -specific *C. crescentus* XylB D-xylose dehydrogenase encoding gene in an industrial *S. cerevisiae* strain improved D-xylonate production to more than 40 g l^{-1}, and higher titres would be expected with provision of more D-xylose and optimisation of production conditions. However, the production rate is still 4 fold lower than observed with some bacteria (~1.7 g l^{-1} h^{-1}, Buchert, 1990) and a higher production rate would be needed for industrial production. Physiological studies indicate that export, maintenance energy and maintenance of cell viability are key issues for efficient production. Transcriptome and/or metabolome analysis of the industrial D-xylonic acid producing *S. cerevisiae* strain may provide information on how the cells adapt to D-xylonate production, which pathways are upregulated and whether specific factors are controlling cell death.

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PUBLICATION III

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MINI-REVIEW

Microbial D-xylonate production

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Abstract D-Xylonic acid is a versatile platform chemical with reported applications as complexing agent or chelator, in dispersal of concrete, and as a precursor for compounds such as co-polyamides, polyesters, hydrogels and 1,2,4-butanetriol. With increasing glucose prices, D-xylonic acid may provide a cheap, non-food derived alternative for gluconic acid, which is widely used (about 80 kton/year) in pharmaceuticals, food products, solvents, adhesives, dyes, paints and polishes. Large-scale production has not been developed, reflecting the current limited market for D-xylonate. D-Xylonic acid occurs naturally, being formed in the first step of oxidative metabolism of D-xylose by some archaea and bacteria via the action of D-xylose or D-glucose dehydrogenases. High extracellular concentrations of D-xylonate have been reported for various bacteria, in particular *Gluconobacter oxydans* and *Pseudomonas putida*. High yields of D-xylonate from D-xylose make *G. oxydans* an attractive choice for biotechnical production. *G. oxydans* is able to produce D-xylonate directly from plant biomass hydrolysates, but rates and yields are reduced because of sensitivity to hydrolysate inhibitors. Recently, D-xylonate has been produced by the genetically modified bacterium *Escherichia coli* and yeast *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Expression of NAD^+-dependent D-xylose dehydrogenase of *Caulobacter crescentus* in either *E. coli* or in a robust, hydrolysate-tolerant, industrial *Saccharomyces cerevisiae* strain has resulted in D-xylonate titres, which are comparable to those seen with *G. oxydans*, at a volumetric rate approximately 30 % of that observed with *G. oxydans*. With further development, genetically modified microbes may soon provide an alternative for production of D-xylonate at industrial scale.

Keywords D-Xylonate · D-Xylose · Oxidation · D-Xylose dehydrogenase · Lignocellulosic hydrolyzate

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Introduction

Sugar acids are currently generating considerable interest because of their potential as platform chemicals and particularly their use as precursors in the manufacture of biomass derived plastics. D-Xylonic acid (Fig. 1), derived from the hemicellulose sugar D-xylose, has applications similar to D-gluconic acid and could serve as a D-gluconic acid substitute, but would be produced from non-food carbohydrate. D-Xylonic acid has been used in dispersal of concrete (Chun et al. 2006), in the production of copolyamides (Zamora et al. 2000) and as a precursor for 1,2,4-butanetriol synthesis (Niu et al. 2003). Several other applications for D-xylonic acid have been patented.

Microbial production of D-xylonate was recognised already at the end of the nineteent century (Bertrand 1898, cited in Lockwood and Nelson 1946) and many species of *Pseudomonas*, *Acetobacter*, *Aerobacter*, *Gluconobacter*, *Erwinia* and related genera have been shown to produce D-xylonate (reviewed in Buchert 1990). Periplasmic D-xylose and D-glucose dehydrogenases use the pyrroloquinoline quinol (PQQ) prosthetic group to transfer electrons to cytochrome c in the respiratory chain, with a corresponding accumulation of D-xylonolactone or D-xylonate in the medium (Galar and Boiardi 1995; Hardy et al. 1993). D-Xylonolactone is the immediate product of the dehydrogenases, but the lactone generally opens spontaneously or with the aid of lactonase produced by the same species (Buchert and Viikari 1988). Some bacteria and also archaea metabolise D-xylonic acid

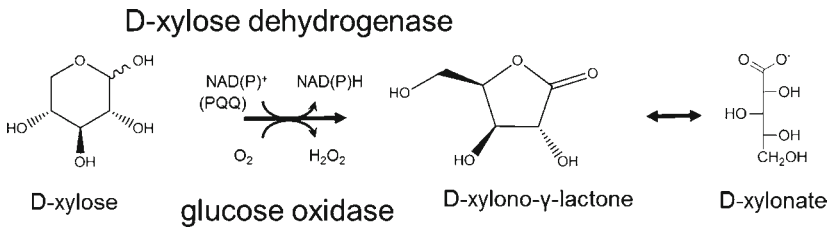

Fig. 1 Formation of D-xylonate from D-xylose by $NAD(P)^+$ or PQQ-dependent xylose dehydrogenases or glucose oxidase

further via non-phosphorylative D-xylose metabolic pathways (Weimberg 1961; Dahms 1974). Cytoplasmic $NAD(P)^+$-dependent D-xylose dehydrogenases oxidise D-xylose to D-xylonolactone (Johnsen and Schönheit 2004; Johnsen et al. 2009; Stephens et al. 2007), which is cleaved by lactonase to D-xylonate in the cytoplasm. D-Xylonate may be dehydrated to produce 2-keto-3-deoxy pentanoate, which is further dehydrated and reduced to α-ketoglutarate or cleaved by an aldolase to pyruvate and glycolaldehyde. There are also some reports of yeast and other fungi producing D-xylonic acid (Suzuki and Onishi 1973; Kiesling et al. 1962; Kanauchi and Bamforth 2003), although only one gene coding for D-xylose dehydrogenase has been identified in fungal species (Berghäll et al. 2007). Production of D-xylonate from D-xylose by D-glucose oxidase has also been described (Pezzotti and Therisod 2006; Chun et al. 2006) and *Aspergillus niger* produces D-xylonate when cultivated in suitable conditions (Fig. 2).

Recently, various yeast strains as well as the bacterium *Escherichia coli* have been engineered to produce D-xylonate, by the introduction of genes encoding D-xylose dehydrogenase (Toivari et al. 2010; Nygård et al. 2011; Liu et al. 2011). Gene sequences for several putative D-xylonolactonases have recently been identified (Johnsen et al. 2009; Stephens et al. 2007; Brouns et al. 2006), but the enzymes have not been studied. The mechanism of transport of either the linear or the lactone form of D-xylonate from strains with intracellular D-xylonate production is unknown.

In addition to the microbial production described in this review, D-xylonate can be produced via enzymatic (Pezzotti and Therisod 2006), electrochemical (Jokic et al. 1991) or chemical oxidation (Isbell and Hudson 1932). D-Xylonic acid can also be found in acid sulphite pulping liquor of hardwood (Samuelson and Simonson 1962; Pfister and Sjöström 1977). However, an efficient separation method to obtain D-xylonate from pulping liquor has not been established.

Although a variety of applications for D-xylonic acid have been patented, one of which includes a method for production of crude D-xylonic acid from plant biomass hydrolysate (Chun et al. 2003), bulk production of D-xylonic acid is limited. This review describes the current state in microbial production of D-xylonate with bacteria and fungi.

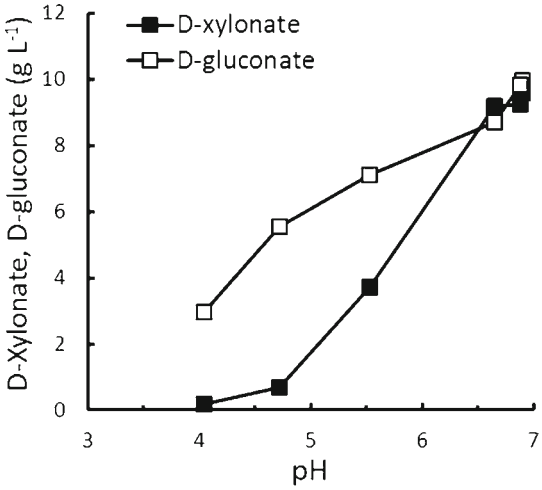

Fig. 2 Production of D-xylonate and D-gluconate by *Aspergillus niger* ATCC1015 after 79 h in defined medium with 45 g D-xylose l^{-1} and 10 g D-glucose l^{-1} as carbon source. Medium was buffered with 0.1 to 2.0 % (*w*/*v*) $CaCO_3$, and average pH over 79 h is shown

Bacterial D-xylonate production

Yields and conversion rates

Of the numerous bacteria described as producers of D-xylonate, species of *Pseudomonas* (Lockwood and Nelson 1946; Buchert et al. 1986), *Gluconobacter* (Buchert 1990), *Micrococcus* (Ohsugi et al. 1970) and *Enterobacter* (Ishizaki et al. 1973) have been the most productive (Table 1). High yields of D-xylonate are generally associated with poor or no conversion of D-xylose to biomass.

Although the pH optimum of the *Gluconobacter oxydans* D-xylose dehydrogenase is 6, D-xylonate has been produced at pH 4.5 (Buchert 1990) and even at pH 3.5 (Fig. 3, Table 1). Production rates are approximately 2 g D-xylonate l^{-1} h^{-1} at pH 4.5–6.5, even when biomass concentration is low (0.2 g biomass l^{-1}) and no cell growth occurs (Table 1, Buchert 1990). We have observed specific production rates up to 12 g D-xylonate (g biomass)$^{-1}h^{-1}$ at pH 5.6.

Since *G. oxydans* requires complex growth medium and efficiently converts most sugars to acids rather than biomass, other species may be more cost effective for D-xylonate

Table 1 D-Xylonate production with *G. oxydans*, *Pseudomonas* species, and *Enterobacter cloacea*, *A. niger*, and engineered strains of *Escherichia coli*, *S. cerevisiae* and *K. lactis*

Species	D-Xylose ($g\ l^{-1}$)	D-Xylonate ($g\ l^{-1}$)	$Yield_{P/S}$ ($g\ g^{-1}$)	Volumetric productivity ($g\ l^{-1}\ h^{-1}$)	Specific productivity [$g\ (g\ biomass)^{-1}\ h^{-1}$]	pH	Biomass ($g\ l^{-1}$)	Process	References
G. oxydans (ATCC621)	100	109	1.1	2.5	~1.5	5.5	1.7	Batch	Buchert (1990)
G. oxydans (ATCC621)	100	107	1.1	2.2	~1.5	4.5	1.3	Batch	Buchert (1990)
G. oxydans (ATCC621)	46	51	1.1	1.8	6	5.5	0.2	Batch	VTT
G. oxydans (ATCC621)	40	41	1.0	1.0	4	3.5	0.2	Batch	VTT
G. oxydans (ATCC621)	40	37	1.0	1.5	2.8	5.5	0.5	Continuous D=0.04 h^{-1}	VTT
P. fragi ATCC4973	150	162	1.1	1.4	0.2	6.5	6.9	Batch	Buchert and Viikari (1988)
P. putida	~0.4	~0.4	~1	~1.9	~0.7	6.8	2.9	Continuous D=0.2 h^{-1}	Hardy et al. (1993)
E. cloacea	200	190	~1	1.6		6.5	nd	Batch	Ishizaki et al. (1973)
E. coli	40	39	1.0	1.1	0.14	7.0	~8	Batch	Liu et al. (2011)
S. cerevisiae Xyd1	20	4	0.4	0.03	0.007	5.5	4.6	Batch	Toivari et al. (2010)
S. cerevisiae SUS2DD	23	3	0.4	0.02	0.006	5.5	5.3	Batch	Toivari et al. (2012)
S. cerevisiae xylB	23	17	0.8	0.23	0.06	5.5	5	Batch	Toivari et al. (2012)
S. cerevisiae B67002 *xylB*	49	43	0.8	0.44	0.06	5.5	7	Batch	Toivari et al. (2012)
K. lactis Xyd1	40	19	0.6	0.16	0.03	5.5	6	Batch	Nygård et al. (2011)
K. lactis Xyd1 Δ*XYL1*	23	8	0.4	0.13	0.01	5.5	9	Batch	Nygård et al. (2011)
A. niger ATCC1015	45	10	0.8	0.12		>5.5	nd	Batch	VTT

For production potential of other bacteria, see (Buchert 1990).

nd=no data, *VTT* unpublished data from VTT, M.G. Wiebe personal communication

production. *Pseudomonas fragi* ATCC4973 produces D-xylonate at similar volumetric rates to *G. oxydans*, but at lower specific rate (Table 1, Buchert and Viikari 1988), and production is more sensitive to pH and hydrolysate inhibitors (Buchert et al. 1986, 1988). Various other bacteria also produce D-xylonate and some such as *Gluconoacetobacter diazotrophicus*, which has been considered as an alternative to *Gluconobacter* for D-gluconate production because of its minimal nutritional requirements and low pH tolerance (Attwood et al. 1991) could also be considered for D-xylonate production.

The first example of bacteria engineered for D-xylonate production was recently described by Liu et al. (2011). By introducing a D-xylose dehydrogenase encoding gene, *xylB* from *Caulobacter crescentus*, into *E. coli* strain W3110 and by blocking the endogenous pathways for D-xylose and D-xylonate metabolism, they were able to produce 39 $g\ l^{-1}$ D-xylonic acid from 40 $g\ l^{-1}$ D-xylose in a batch process (Table 1).

Although continuous production of D-xylonate has not been reported, continuous production of D-gluconic (e.g. Attwood et al. 1991), 2-keto-L-gulonic (e.g. Takagi et al.

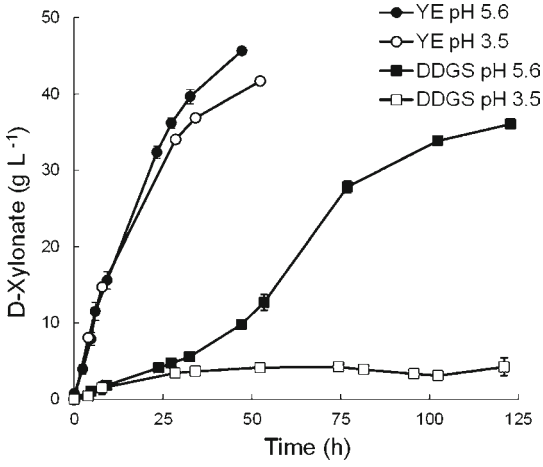

Fig. 3 D-Xylonate production by *Gluconobacter oxydans* ATCC621 from D-xylose in YE supplemented defined medium with 45 g D-xylose l^{-1} at pH 5.6 (*filled circle*) or pH 3.5 (*empty circle*) and from acid hydrolysed DDGS at pH 5.6 (*filled square*) or pH 3.5 (*empty square*). *Error bars* represent ±SEM for duplicate cultures

2009) and 2,5-diketogluconic (e.g. Buse et al. 1992a, b) acids have been described. Hardy et al. (1993) described the production of D-xylonate at a rate of ~4 mmol (g biomass)$^{-1}$ h^{-1} as a by-product for enhanced biomass yield of *Pseudomonas putida* on D-glucose or lactate at D=0.2 h^{-1}, pH 6.8 (Table 1). With *G. oxydans*, we have observed continuous production of D-xylonate with *G. oxydans* at a rate of 1.5 g D-xylonate l^{-1} h^{-1} at D=0.04 h^{-1} with 40 g D-xylose l^{-1} and 20 g D-glucose l^{-1} at pH 5.5 (Fig. 4, Table 1). In addition, D-gluconate, acetate and biomass were produced. Continuous production at pH 4.5 was also possible (Fig. 4). Conditions for D-xylonate production in fed-batch cultures have not been reported.

Hydrolysate

Lignocellulosic waste biomass would provide an economic raw material for D-xylonate production, and several studies have been carried out on the conversion of D-xylose to D-xylonate in hemicellulose hydrolysates (Chun et al. 2006; Buchert et al. 1988). These are summarised in Table 2. *G. oxydans* was found to be more tolerant to toxins in biomass hydrolysate than *P. fragi*, but growth and D-xylonate production were still inhibited by high concentrations of lignocellulosic hydrolysate. Pre-treatment by diethylether extraction, adsorption on mixed bed resin or ion exclusion chromatography enabled conversion of D-xylose in hydrolysate to D-xylonate by *G. oxydans*, with the biggest improvements seen when treated by ion exclusion chromatography (Table 2).

Turkia et al. (2010) also observed a low rate for the conversion of D-xylose to D-xylonate by *G. oxydans* ATCC621 (E97003) in a pentose-rich hydrolysate derived from wheat straw, with incomplete conversion of the D-xylose and a low yield of ~0.7 g D-xylonate (g D-xylose consumed)$^{-1}$. Overliming (Mohagheghi et al. 2006) the hydrolysate to remove some of the aromatic and aliphatic compounds was sufficient to enable full conversion of the hydrolysate [yield 1.0 g D-xylonate (g D-xylose consumed)$^{-1}$] and to improve the production rate to 1.1 g D-xylonate l^{-1} h^{-1} with an inoculum of only 1.0–1.5 g biomass l^{-1} (Fig. 5). The rate was thus only slightly lower than that observed with pure D-xylose and similar to that obtained by hydrolysate treated with ion exclusion chromatography. In contrast, overliming acid hydrolysed dried distillers grain solids (DDGS) from Abengoa Bioenergia Nuevas Tecnologias (ABNT, Spain) resulted in poorer conversion of the D-xylose to D-xylonate than in the untreated hydrolysate (Table 2).

Chun et al. (2006) found that *G. oxydans* was able to completely convert D-xylose in diluted spent sulphite liquor even though no cell growth occurred. Conversion rates with high cell density (~4.6 g biomass l^{-1}) were comparable to those in ether-extracted birchwood hydrolysate and untreated wheat straw hydrolysate (Table 2, Chun et al. 2006).

In continuous flow culture at D=0.03 h^{-1}, D-xylonate was produced by *G. oxydans* at a rate of 0.32 g D-xylonate l^{-1} h^{-1} for up to 3 days (Table 1). However, only ~50 % of the D-xylose in the hydrolysate was converted to D-xylonate, and the cells were being washed out. Approximately 76 % D-glucose in the hydrolysate was converted to D-gluconate.

Recombinant yeast for D-xylonate production

S. cerevisiae and the choice of D-xylose dehydrogenase

Toivari et al. (2010) described the production of D-xylonate by *S. cerevisiae* expressing an NADP$^+$-dependent xylose dehydrogenase from *T. reesei*. The engineered *S. cerevisiae* strains produced up to 3.8 g D-xylonate l^{-1} (Table 1). Xylitol (4.8 gl^{-1}) was the primary by-product and could be significantly reduced by deleting the aldose reductase encoded by *GRE3* (Toivari et al. 2010). Although this demonstrated the

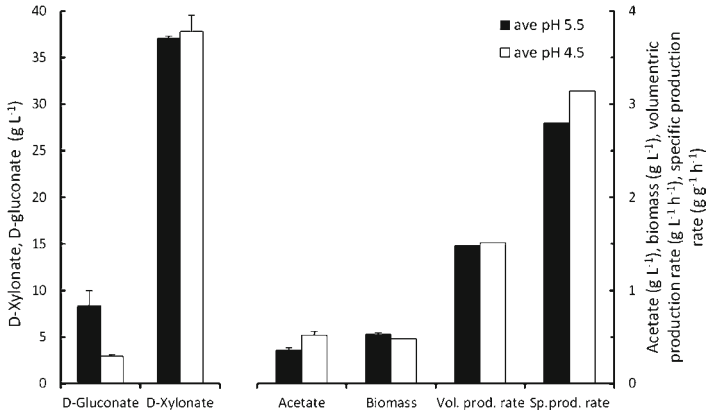

Fig. 4 D-Gluconate, D-xylonate, acetate and biomass production, volumetric D-xylonate production rate and specific D-xylonate production rate by *Gluconobacter oxydans* ATCC621 in chemostat culture with YE supplemented defined medium containing 10 g D-glucose l^{-1} and 40 g D-xylose l^{-1} at D=0.04 h^{-1}, pH 5.5 or 4.5. *Error bars* represent ±SEM for triplicate (pH 5.5) or duplicate (pH 4.5) samples

feasibility of producing D-xylonate with yeast, the titres and rates were low compared to those obtained with bacteria (see above) or for other acids produced in *S. cerevisiae*, e.g. lactate >100 gl^{-1} (Sauer et al. 2010).

Initial attempts at redox engineering to improve $NADP^+$-recycling did not improve D-xylonate production (Toivari et al. 2010), and the activity of alternative D-xylose dehydrogenases was assessed in *S. cerevisiae* (Toivari et al. 2012, Table 1). The *C. crescentus xylB* encoded NAD^+-dependent D-xylose dehydrogenase was found to have high activity in *S. cerevisiae*, and strains expressing *xylB* produced more D-xylonate (17±2 gl^{-1}) at a higher rate than the X*yd1* expressing strain (Table 1, Toivari et al. 2012). In addition to high activity, the xylB had high specificity for D-xylose (Toivari et al. 2012). This increase in D-xylonate production with *xylB* compared to *Xyd1* presumably reflects the higher activity of the dehydrogenase in the cytoplasm. However, it may also indicate that production of excess NADH, which can be oxidised in the electron transport chain to generate energy, is preferable to production of excess NADPH. Another $NADP^+$ requiring D-xylose dehydrogenase, SUS2DD from pig liver, which was successfully expressed in *S. cerevisiae*, showed similar activity and production characteristics to the *T. reesei Xyd1* (Table 1, Toivari et al. 2012).

Expression of *xylB* in the industrial *S. cerevisiae* strain B67002 enabled the production of higher concentrations of D-xylonate (e.g. 43 gl^{-1}) than with the lab strain, at rates approaching 0.5 gl^{-1} h^{-1}, i.e. 25–30 % those observed with *Gluconobacter* and *Pseudomonas* spp. (Table 1; Toivari et al. 2012).

Only limited research has been carried out on the environmental conditions that are required for good D-xylonate production by *S. cerevisiae*. Production has primarily been characterised at pH 5.5. D-Xylonate could also be produced at pH 3, but production and cell viability were reduced compared to that observed at pH 5.5 (Toivari et al. 2012). Similarly, low productivity at low pH has also been observed with lactic acid production (Porro et al. 1999). D-Xylonate accumulated intracellularly (Toivari et al. 2012), revealing a potential need to engineer D-xylonate transport. D-Xylonate production has been shown to be

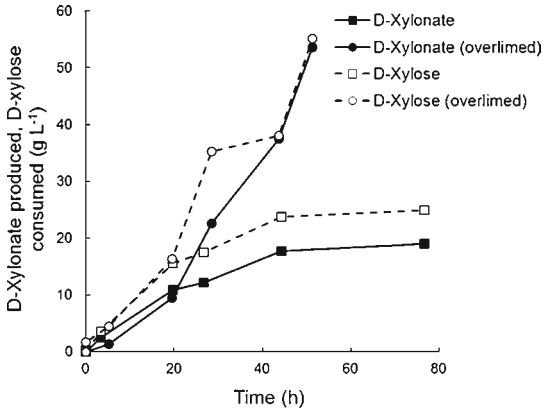

Fig. 5 D-Xylonate produced (solid symbols) and D-xylose consumed (*open symbols*) by *Gluconobacter oxydans* ATCC621 in pre-treated wheat straw derived hydrolysate (C5 fraction), with (*circles*) or without (*squares*) overliming, and supplemented with 5 g yeast extract l^{-1} at pH 5.6, 30 °C. The hydrolysate contained D-xylose, D-glucose, L-arabinose, and acetate. D-Xylonate measurements in untreated wheat straw hydrolysate are shown in Turkia et al. (2010)

Table 2 Production of D-xylonate from lignocellulosic hydrolysates with *G. oxydans* ATCC621

D-Xylose (g l^{-1})	D-Xylonate (g l^{-1})	Volumetric productivity (g D-xylonate l^{-1} h^{-1})	Hydrolysate	References
25	~13	0.2	Birchwood, steam	Buchert et al. (1988)
25	~22	0.3	Birchwood, steam, ether extracted	Buchert et al. (1988)
100	~88	1.2	Birchwood, steam, ion exclusion	Buchert et al. (1990)
~45	~48	~0.5	Birch spent sulphite liquor	Chun et al. (2006)
39	17	0.4	Wheat straw (ABNT), C5 fraction derived[a] from steam pre-treatment	Turkia et al. (2010)
na	54	1.1	Wheat straw (ABNT), C5 fraction derived[a] from steam pre-treatment, overlimed	VTT
35	35	0.6	DDGS (ABNT), acid hydrolysed	VTT
25	13	0.3	DDGS (ABNT), acid hydrolysed, continuous at D=0.03 h^{-1}	VTT
23	5	0.1	DDGS (ABNT), acid hydrolysed, overlimed, continuous at D=0.02 h^{-1}	VTT

When described, cultures were maintained at pH 5.5–6.5

ABNT Abengoa Bioenergia Nuevas Tecnologias, *VTT* unpublished data from VTT, M.G. Wiebe personal communication, *na* not available

[a] Provided by Dr. Robert Bakker, Wageningen University & Research Centre

an energy requiring process, with production essentially stopping once metabolisable carbon has been consumed, but resuming when additional co-substrate is added (Toivari et al. 2010). Since most biomass hydrolysates, even C5-enriched fractions, contain some C6 sugars, co-substrate would be at least partly provided from the biomass hydrolysate. Because D-xylonate production produces NADH (or NADPH), which needs to be oxidized, ultimately by channelling electrons to oxygen, the process must be aerobic. However, the energy produced in the reduction of oxygen will provide energy for the process, including D-xylonate transport (if active), pH homeostasis and cell maintenance.

When a putative D-xylono-lactone lactonase *xylC* from *C. crescentus* was expressed together with *xylB* in *S. cerevisiae*, increased lactonase activity was observed by NMR. More extracellular D-xylonate was initially produced than with cells lacking *xylC* at both pH 5.5 and 3 (Toivari et al. 2012). The lactonase-expressing strain also sustained higher production at pH 3. However, expression of the lactonase encoding gene decreased cell vitality and viability when D-xylonate was produced at pH 3.0 (Toivari et al. 2012).

Alternative yeast for D-xylonate production

D-Xylonate production has also been demonstrated with the D-xylose-utilising yeast *K. lactis*. D-Xylonate was produced in *K. lactis* with the Xyd1 enzyme from *T. reesei*, which is $NADP^+$-dependent and has relatively low activity. Although the activity levels were similar to those observed in *S. cerevisiae*, *K. lactis* produced more D-xylonate (6.3±0.1 g l^{-1}) at a higher rate (Nygård et al. 2011), compared to *S. cerevisiae* expressing the same gene. Increasing the substrate concentration led to higher productivity (19±2 $g l^{-1}$ at rates of 0.16±0.01 $g l^{-1} h^{-1}$, Table 1; Nygård et al. 2011), whereas the equivalent *S. cerevisiae* strains did not increase D-xylonate production when provided higher D-xylose concentrations. The natural ability to utilize D-xylose not only may benefit D-xylonate production by decreasing the need for added co-substrate but also decreases the overall yield and thus should be optimized to support good productivity without substantial loss in yield.

In *K. lactis* the deletion of the xylose reductase (encoded by *XYL1*) resulted not only in less xylitol production compared to the reductase containing strain but also increased D-xylonate production (Nygård et al. 2011), in contrast to the effect of deleting *GRE3* from *S. cerevisiae*, which only reduced xylitol production (Toivari et al. 2010). With *K. lactis*, oxygen provision affected the conversion of D-xylose to D-xylonate, xylitol or biomass. Metabolism of D-xylose was most efficient with high oxygen provision (12 mmol O_2 l^{-1} h^{-1}), but even with low oxygen concentration (6 mmol O_2 l^{-1} h^{-1}), no loss in D-xylonate titre or production rate occurred.

K. lactis is not particularly tolerant to biomass hydrolysates, but its good D-xylonate production ability demonstrates the potential benefit of producing D-xylonate in a D-xylose-utilising non-*Saccharomyces* yeast.

The prospects for future microbial D-xylonate production

Although the non-engineered bacteria are very efficient at producing D-xylonate, neither commercial production has been described, nor have cost effective, large-scale separation processes been developed. The current methods, e.g., precipitation or ion exchange, are for small-scale preparations from relatively pure solutions (Liu et al. 2011; Buchert et al. 1986; Devos and Huchette 1981) and would not be adequate for bulk production of D-xylonic acid required for use as a platform chemical, e.g., for polymer or hydrogel production. Since there is no historic market for D-xylonate, there has been no driving force to develop large-scale production. However, with the increasing need to replace petrochemicals and compounds derived from D-glucose, such as D-gluconate, with alternative chemicals, interest in large-scale production and purification of D-xylonate will grow.

Production of D-xylonate by *G. oxydans* has been limited by complex nutritional requirements and low biomass production, requiring costly inoculum development, even if D-xylonate could then be produced from biomass hydrolysates. Several patents improving biomass production by *G. oxydans* have been published (Zhao et al. 2011; Yuan et al. 2009; Shingoh 2009), but strategies that disrupt the peri- or cytoplasmic glucose dehydrogenases (Shingoh 2009) are expected to also reduce the D-xylonate production rate. *P. fragi* would provide more robust inoculum development, but would require more extensive treatment to remove inhibitors from the biomass hydrolysates than *G. oxydans* (Buchert et al. 1988). *G. diazotrophicus* may be a reasonable alternative (Attwood et al. 1991), but has not been evaluated in hydrolysate. Another major concern with these bacteria is the range of acidic products produced from the compounds present in lignocellulosic hydrolysates and their separation costs.

Genetically engineered bacteria and yeast now provide new alternatives to the non-engineered bacteria for large-scale production of D-xylonate and are likely to be developed further. The engineered *E. coli* strain provides benefits in having a fast specific growth rate, efficient generation of inoculum, and low nutrient requirements. Yeast such as *S. cerevisiae* and *K. lactis* also have good growth and low nutritional requirements. *S. cerevisiae* and several other yeast (e.g. Kwon et al. 2011) additionally offer good tolerance to the various inhibitors found in lignocellulosic hydrolysates, as well as tolerance to low pH conditions and even the capacity for acid production at low pH (cf. lactic acid production at pH 3, Suominen et al.

2009). The development of genetically engineered production strains opens new doors for the development of robust industrial processes for D-xylonic acid production.

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PUBLICATION IV

Low pH D-xylonate Production with *Pichia kudriavzevii*

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Low pH D-xylonate production with *Pichia kudriavzevii*

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HIGHLIGHTS

- *Pichia kudriavzevii* expressing *Caulobacter crescentus xylB* produced 146 g D-xylonate l^{-1} at pH 3.
- This *P. kudriavzevii* strain also produced 171 g D-xylonate l^{-1} at pH 5.5.
- D-Xylonate production was less toxic to *P. kudriavzevii* than to *Saccharomyces cerevisiae*.
- *P. kudriavzevii* is thus an excellent production organism for D-xylonic acid.

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ABSTRACT

D-Xylonic acid is one of the top 30 most desirable chemicals to be derived from biomass sugars identified by the US Department of Energy, being applicable as a non-food substitute for D-gluconic acid and as a platform chemical. We engineered the non-conventional yeast *Pichia kudriavzevii* VTT C-79090T to express a D-xylose dehydrogenase coding gene from *Caulobacter crescentus*. With this single modification the recombinant *P. kudriavzevii* strain produced up to 171 g L^{-1} of D-xylonate from 171 g L^{-1} D-xylose at a rate of 1.4 g L^{-1} h^{-1} and yield of 1.0 g [g substrate consumed]$^{-1}$, which was comparable with D-xylonate production by *Gluconobacter oxydans* or *Pseudomonas* sp. The productivity of the strain was also remarkable at low pH, producing 146 g L^{-1} D-xylonate at 1.2 g L^{-1} h^{-1} at pH 3.0. This is the best low pH production reported for D-xylonate. These results encourage further development towards industrial scale production.

1. Introduction

The current threats of increasing population, global warming and limited fossil resources have triggered a vision for a bioeconomy, i.e. sustainable production and conversion of biomass to products and energy, as exemplified in the EU white paper "The European Bioeconomy in 2030" and the "National Bioeconomy Blueprint" from the USA White House in 2012. Development of biorefineries to convert existing biomass, preferably non-utilized waste material, to usable products is a key element of the bioeconomy. Biorefineries will need to be economically viable, setting strict targets for the whole value chain. Microbes, when used as biocatalysts in the processes should be robust, tolerant to various inhibitors while maintaining high productivity, as well as being safe and economical to produce and use.

Several organic acids, such as gluconic acid (80 kton $year^{-1}$), acetic acid (150 kton $year^{-1}$) and citric acid (1600 kton $year^{-1}$), are already produced by microbes as bulk chemicals with wide application ranges (Sauer et al., 2008). Organic acids have traditionally been used as chelators, buffers and preservatives. They can also be important platform chemicals: around half of the current lists of desirable sugar-derived platform chemicals which could be produced using biotechnology are organic or amino acids (Bozell and Petersen, 2010; OECD, 2011 http://dx.doi.org/10.1787/9789264126633-en; Werpy and Petersen, 2004). The number of organic acids produced from biomass sugars with microbes is increasing. Production of lactic acid, malic acid, succinic acid, and itaconic acid has been considerably improved during the last years (Lee et al., 2011; Erickson et al., 2012; Sauer et al., 2010) and the feasibility of production has been demonstrated for e.g. glucaric acid (Lee et al., 2011), D-xylonic acid (Toivari et al., 2012a) and galactaric acid (Mojzita et al., 2010). Some organic acids like citric acid are produced in low pH processes, but most are produced at pH values closer to neutral because of physiological constraints, even though low pH processes would reduce costs during both production (less use of neutralizing base) and product separation (less acid addition and less salt produced as byproduct, Sauer et al., 2008).

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The 5-carbon sugar D-xylose is a major component of hemicellulose in lignocellosic materials. It is thus abundant, but currently much less used than lignocellulosic D-glucose. Major efforts have been made to ferment the pentose sugars D-xylose and D-arabinose to ethanol (Van Vleet and Jeffries, 2009), however, the efficiency of C5 conversion is still low compared with D-glucose conversion. D-Xylose can also be reduced to xylitol or oxidized to D-xylonic acid, compounds with a variety of applications. D-Xylonic acid can be used as complexing agent and chelator in dispersal of concrete (Chun et al., 2006). It has also been used in co-polyamides, polyesters and hydrogels, and as a precursor for compounds such as 1,2,4-butanetriol or ethylene glycol (Liu et al., 2012b; Niu et al., 2003; reviewed in Toivari et al., 2012a). With increasing D-glucose prices, D-xylonic acid may provide a cheaper, non-food-derived alternative for gluconic acid which is widely used in pharmaceuticals, food products, solvents, adhesives, dyes, paints and polishes.

D-Xylonic acid is formed in the oxidative metabolism of D-xylose by some archaea and bacteria and may accumulate in high extracellular concentrations when sufficient D-xylose is provided to bacteria such as *Gluconobacter oxydans* or *Pseudomonas* sp. (reviewed in Toivari et al., 2012a). Recently D-xylonate has also been produced by genetically modified organisms, including the bacterium *Escherichia coli* (Liu et al., 2012a) and yeast *Saccharomyces cerevisiae* (Toivari et al., 2010, 2012b) and *Kluyveromyces lactis* (Nygård et al., 2011), to provide alternative, more robust production hosts which could be suitable for large scale production. D-Xylonate titres up to 43 g L^{-1} have been obtained, but the production rates were low compared to those observed with *G. oxydans* (Toivari et al., 2012a, b) and these strains have not been useful for D-xylonate production at low pH (Liu et al., 2012a; Toivari et al., 2012b), with its associated benefits in reducing contamination risk, reducing use of neutralisation agent, generating new options for down stream purification, and the corresponding reductions in costs.

While *S. cerevisiae* and *E. coli* are well known and extensively used production organisms, other production organisms may be superior in production of organic acids. The yeast *Pichia kudriavzevii* (previously named as *Issatchenkia orientalis*) has recently been described as a multi-stress-tolerant, robust organism with tolerance to low pH, high salt concentrations, and temperatures as high as 42 °C (Gallardo et al., 2011; Isono et al., 2012; Kitagawa et al., 2010; Kwon et al., 2011). The potential of *P. kudriavzevii* for the production of bioethanol at 40 °C has already been demonstrated (Dhaliwal et al., 2011). Examples of genetic modifications of *P. kudriavzevii* (*I. orientalis*) are still scarce: it has been engineered for production of β-glucosidase and examples of its engineering for L-lactic acid production can be found in patents (Suominen et al., 2009; Kitagawa et al., 2010).

The purpose of this study was to assess the possibility of using *P. kudriavzevii* to produce D-xylonic acid at low pH.

2. Methods

2.1. Strains, plasmids and strain construction

Pichia kudriavzevii (formerly *Issatchenkia orientalis* (Kurtzman et al., 2008), also referred to as *Candida krusei*) strain VTT C-79090T (ATCC 32196, isolated from cabbage waste, Kofu, Japan), VTT C-05705 (ATCC 60585, isolated from rye sour dough starter, Germany) and VTT C-75010 (isolated from a sample of commercial baker's yeast, Finland), and *S. cerevisiae* VTT B-67002 were obtained from the VTT Culture Collection (http://culturecollection.vtt.fi/). An industrial *K. lactis* strain (GG799) was obtained from New England Biolab (MA). All strains were maintained as streaks on YPD agar or in 15% v/v glycerol at −80 °C.

A synthetic gene for the D-xylose dehydrogenase from *Caulobacter crescentus* (*xylB*, CC_0821, Gene ID: 941308, NCBI) codon optimized for *S. cerevisiae* was obtained from Gene Art (Germany). The gene was cloned under the *P. kudriavzevii PGK1* promoter (*IoPGK1*) and was introduced as a single copy into the *P. kudriavzevii* genome with targeted integration into the *PDC1* locus. The primers used for the PCR amplification of sequences from the genomic DNA of the diploid strain *P.kudriavzevii* VTT C-79090T were previously described in Suominen et al. (2009). The *IoPGK1* promoter, 624 bp 5′ of *ATG*, was amplified with primers IopPGKfrw2 5′TCCCCCGGGCGGATCCTTG CTGCAACGGCAACATCA ATG3' and IopPGKrev2 5′CCCAAGCTTGGAAGATCTTGTTGTTGTT GTTGTCGTTG TTTTTG3′. The 833 bp 5′ and 746 bp 3′ flanking regions of the *P. kudriavzevii PDC1*(*IoPDC1*) gene were amplified with primer pairs IoPDC 5′flank frw 5′ATAAGAATGCGGCCGCACTGCAG AGTATATGGAATTGACG GCTCATC3′/IoPDC 5′flank rev 5′ACTGAC GCGTCGACGGATCCGTATCATTTGTAGCCCACGC CACC3′ and IoPDC 3′flank frw 5′GGA ATTGATATCGACTAGTCTTGGCTACCCACTTACCAA GAGAT3′/IoPDC 3′flank rev 5′ATAAGAATGCGGCCGCAATAGAGA GTGACCTATCCAAGCT3′.

α-Galactosidase activity derived from melibiase coded by *S. cerevisiae MEL5* (Gene ID: 547463, NCBI) was used for selection of transformants. The double expression cassette for expression of *xylB* and *MEL5* was constructed in the plasmid pSP72 (Promega) and the final construct, pMLV100A, contained the *IoPDC1* flank (5′) – [*IoPGK1* promoter – *S. cerevisiae MEL5*-*S. cerevisiae MEL5* terminator] – [*IoPGK1* promoter – *xylB* – *S. cerevisie ADH1* terminator] – *IoPDC1* flank (3′). The double expression cassette with *PDC1* regions was released from pMLV100A with *NotI* and introduced into *P. kudriavzevii* cells using the lithium acetate transformation protocol (Gietz et al., 1992). The transformants were selected based on blue colour formation on YPD plates containing 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (*X-α-Gal*). A transformant containing a functional copy of the *xylB* was deposited as VTT-C-12903 in the VTT Culture Collection.

2.2. Media and culture conditions

YPD plates contained 10 g yeast extract L^{-1}, 20 g bacto-peptone L^{-1}, 20 g D-glucose L^{-1} and 15 g agar L^{-1}, and were supplemented with 40 μg ml^{-1} *X-α-Gal* for selection of transformants.

Medium for flask and bioreactor cultures contained yeast extract and peptone (YP, 10 g yeast extract L^{-1}, 20 g bacto-peptone L^{-1}) with D-glucose (12.5–20 g L^{-1}) and/or D-xylose (20–171 g L^{-1}) for production of D-xylonate. Concentrations of added carbon source (D-glucose, D-xylose) are indicated in the text.

The defined medium described by Verduyn et al. (1992) was used to assess growth in the Bioscreen analyser (Bioscreen C MBR automated turbidometric analyser, Growth Curves Ltd., Finland). To determine specific growth rates at different pH values, the medium was buffered with potassium hydrogen phthalate ($KHC_8H_4O_4$, pH 2.2–5.5) or monopotassium phosphate (KH_2PO_4, pH 6.2–8). Bioscreen microtiter plates (100-Well Honeycomb plate) containing 270 μL medium were inoculated with 30 μL cell suspension to an initial OD of 0.05. Growth at 30 °C with continuous, extra intensive shaking was measured as optical density at 600 nm (OD_{600}) at 30 min intervals for up to 24 h. The pH of the medium was measured at the end of the exponential growth phase. The buffering capacity of the buffers used was adequate to maintain a specific pH during the logarithmic growth phase and thus for determination of the relationship of specific growth rate and pH. Specific growth rates were determined from the exponential phase after the OD_{600} was 0.2 or higher. Each condition assessed in the Bioscreen was performed in at least four replicates. Growth at pH 6.2 was also assessed in bioreactors, using 200 mL defined medium (Verduyn et al., 1992), inoculated to an OD of

0.05. The experiments were done without pH control in medium buffered with potassium hydrogen phthalate for direct comparison with the Bioscreen measurement, and also in medium lacking potassium hydrogen phthalate at constant pH, maintained by addition of 2 M NaOH or 1 M H_2PO_4. Logarthimic growth was monitored by hourly measurement of OD_{600} and by measurement of CO_2 production. The gas concentration (CO_2, $^{13}CO_2$, O_2, N_2 and Ar) was analysed in an Omnistar quadrupole mass spectrometer (Balzers AG, Liechtenstein), calibrated with 3% CO_2 in Ar.

Flask cultures were carried out in 50 mL YP medium in 250 mL Erlenmeyer flasks, at 250 rpm, 30 °C. Multifors bioreactors (max. working volume 500 ml, Infors HT, Switzerland) were used to obtain pH controlled cultivations. Yeast were grown in 500 mL YP medium at pH 3.0 or 5.5, 30 °C, 1–1.8 volume air [volume culture]$^{-1}$ min^{-1} (vvm) and 800–1000 rpm agitation with 2 Rushton turbine impellors, as previously described (Toivari et al., 2010). The pH was maintained constant by addition of 2 M NaOH or 1 M H_2PO_4. Clerol antifoaming agent (Cognis, France, 0.08–0.10 μL L^{-1}) or mixed molecular weight polypropylene glycol was added to prevent foam formation.

2.3. Analytical methods

2.3.1. Measurement of biomass

Biomass was measured as OD_{600} or as dry weight. For dry weight, samples were collected in 2 mL pre-dried, pre-weighed microcentrifuge tubes, washed twice with equal volume distilled water and dried at 100 °C.

2.3.2. Chemical analyses

To determine intracellular D-xylonate and xylitol amounts, cells were collected from 10 mL culture, washed and extracted as described by (Nygård et al., 2011). The intracellular concentration is given as mg per g dry biomass. A conservative estimate of intracellular concentrations can be derived by assuming that 1 g dry cell weight corresponds to 2 mL cell volume, as with *S. cerevisiae* (Nygård et al., 2011).

D-Xylonic acid and/or D-xylonolactone, ethanol, glycerol, pyruvate and acetate, D-glucose and D-xylose concentrations from intra and extracellular samples were analysed by HPLC, as described previously (Toivari et al., 2010). Extracellular D-xylonic acid concentrations were also measured as the lactone using the hydroxymate method (Toivari et al., 2010).

Xylose dehydrogenase activity was measured as described in Toivari et al. (2012b).

2.3.3. Determination of vitality and viability of D-xylonate producing cells

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) staining. Viability was determined by comparing the number of viable colony forming units (CFU) on YPD to the total cell number determined microscopically. Viability determined as CFU correlated well with the per cent of metabolically active cells determined microscopically, although microscopic observation generally indicated slightly higher vitality/viability than CFU determination (data not shown).

3. Results and discussion

3.1. Effect of pH on specific growth rate for S. cerevisiae, K. lactis and P. kudriavzevii growing on D-glucose

D-Xylonate production has previously been studied with yeast *S. cerevisiae* (Toivari et al., 2012b) and *K. lactis* (Nygård et al., 2011). The specific growth rates of these strains growing on D-glucose were compared with those of *P. kudriavzevii* strains for pH values from 2 to 8. *S. cerevisiae* VTT B-67002 ($\mu = 0.57 \pm 0.01$ h^{-1}), *K. lactis* GG799 ($\mu = 0.29 \pm 0.02$ h^{-1}), and *P. kudriavzevii* VTT C-79090T ($\mu = 0.48 \pm 0.01$ h^{-1}), VTT C-05705 ($\mu = 0.56 \pm 0.02$ h^{-1}) and VTT-C-75010 ($\mu = 0.50 \pm 0.00$ h^{-1}) all had maximal specific growth rates at pH 6 when grown in defined medium in Bioscreen micro titre wells (Fig. 1). *K. lactis* GG799 was the least tolerant of these species to low pH (Fig. 1). Both *P. kudriavzevii* and *S. cerevisiae* strains grew at rates greater than 0.30 h^{-1} at pH values as low as 3.8, and with slower growth at pH 3.0 (Fig. 1). At pH 3 the specific growth rates of the *P. kudriavzevii* strains were about threefold higher than those of *S. cerevisiae* and *K. lactis* strains, and only *P. kudriavzevii* VTT C-79090T and VTT C-05705 grew at pH 2.2 ($\mu = 0.30 \pm 0.01$; Fig. 1). *P. kudriavzevii* (*C. krusei*) 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, VTT C-05705 and VTT-C-75010 (pH 3 only) at pH 2.2–3, suggested that *P. kudriavzevii* is a potential host for D-xylonate production.

3.2. Construction and characterization of D-xylonate producing P. kudriavzevii

P. kudriavzevii has been described as unable to utilise D-xylose, based on API-ID32C screening (Gallardo et. al., 2011). However, *P. kudriavzevii* VTT-C-75010 consumed D-xylose at a rate of 0.28 ± 0.02 g L^{-1} h^{-1} in flasks with 7 g D-glucose L^{-1} and 20 g D-xylose L^{-1} on YP media, which was higher than the D-xylose consumption rate of the D-xylose utilising yeast *K. lactis* (0.22 g L^{-1} h^{-1}, Nygård et al., 2011). The yield of xylitol on D-xylose was only 0.22 ± 0.01 g xylitol [g D-xylose]$^{-1}$, whereas the yield of biomass on D-xylose was initially about 0.5 g g^{-1}, decreasing in older cultures (~0.14 g [g biomass]$^{-1}$), confirming that *P. kudriavzevii* VTT-C-75010 was able to metabolize D-xylose. The genes encoding the three enzymes needed for D-xylose utilization, D-xylose reductase, xylitol dehydrogenase and xylulokinase, are present in the genome of *P. kudriavzevii* (Chan et al., 2012) and we demonstrate that they are active in at least some *P. kudriavzevii* strains. In contrast, *P. kudriavzevii* VTT-C-75010 did not utilise D-xylonate as a carbon source at pH 5 in either defined or complex medium, with or without D-glucose present, even after 138 h

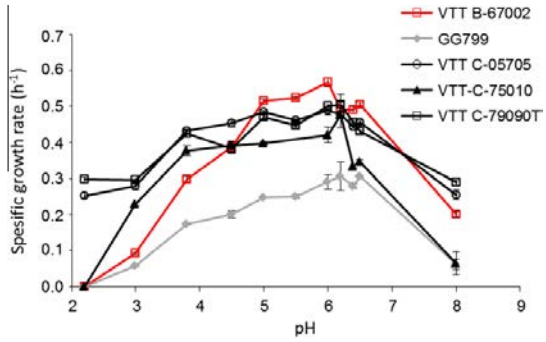

Fig. 1. Comparison of specific growth rates of *S. cerevisiae* VTT B-67002 (red square), *K. lactis* GG799 (grey diamond) and *P. kudriavzevii* VTT-C-75010 (black triangle), VTT C-79090T (open square), and VTT C-05705 (open circle) at pH 2–8 in defined medium with D-glucose as carbon source, 30 °C. Cells were grown in 300 μL microtitre wells and OD_{600} measured in a Bioscreen C MBR automated analyser. The specific growth rate of *K. lactis*, VTT B-67002 and VTT C-75010 was also determined from biomass and CO_2 production in a bioreactor at pH 6.2. Error bars represent ± SEM (n = 4–10).

incubation (data not shown). Externally added D-xylonate (100 g L^{-1} at pH 3.5 or pH 4.5) did not inhibit growth of VTT-C-75010, nor cause an increase in loss of cell vitality (<5% non-vital cells in cultures with and without D-xylonate added).

Although *P. kudriavzevii* (*I. orientalis*) is increasingly being recognised as a robust production organism, e.g. for ethanol production (Dhaliwal et al., 2011; Isono et al., 2012; Kitagawa et al., 2010; Kwon et al., 2011), reports on genetic manipulation of this organism are scarce (Kitagawa et al., 2010). No episomal vectors are known for *P. kudriavzevii*. Kitagawa et al. (2010) noticed integration of the *S. cerevisiae* 2μ episomal plasmid into the *P. kudriavzevii* (*I. orientalis*) genome. Our D-xylose dehydrogenase expression cassette was integrated into the genome, with the native *PGK* promoter driving expression of both the *MEL5* marker gene and the D-xylose dehydrogenase encoding gene *xylB*. Transformation frequencies of 1–3 colonies per μg DNA were obtained for both *P. kudriavzevii* VTT C-79090T and VTT-C-75010.

Several *xylB* transformants of VTT C-79090T and VTT-C-75010 were assessed for production of D-xylonate in medium containing D-glucose and D-xylose. No variation between transformants of the same strain was seen. VTT C-79090T *xylB* produced 50 g D-xylonate L^{-1} in 48 h when grown in unbuffered YP medium (final pH 2.96) in flasks with 20 g D-glucose L^{-1} and 50 g D-xylose L^{-1}, whereas VTT-C-75010 *xylB* produced only 42 ± 1 g D-xylonate L^{-1} (final pH 3.05) in the same conditions. Thus, the better growth of VTT C-79090T, compared with VTT-C-75010, at low pH (Fig. 1) was reflected in production of more D-xylonic acid by its transformants. A representative *xylB* transformant of VTT C-79090T was deposited in the VTT Culture Collection as strain VTT-C-12903 and used in bioreactor cultivations. D-xylose dehydrogenase activity in crude cell extract of this strain after ~20 h growth in YPD was 2.0 ± 0.2 nkat [mg protein]$^{-1}$, which was much lower than has been reported for the *S. cerevisiae* CEN.PK *xylB* transformant (45 nkat [mg protein]$^{-1}$, Toivari et al., 2012b), which may reflect the difference in copy number (a single copy of the gene in *P. kudriavzevii*, multiple copies on plasmids in *S. cerevisiae*), differences in promoter strength or suitability of *S. cerevisiae* codon optimisation for expression in *P. kudriavzevii*. The activity was none-the-less adequate for good D-xylonate production.

3.3. Production of D-xylonate by *P. kudriavzevii* VTT-C-12903 at pH 5.5

VTT-C-12903, expressing the D-xylose dehydrogenase encoding gene *xylB*, was grown in YP medium with D-glucose and D-xylose under conditions similar to those used to produce D-xylonate with *S. cerevisiae* VTT B-67002 *xylB* (Toivari et al., 2012b), but with higher concentrations of D-xylose. D-Glucose was provided to obtain rapid biomass production during the initial stage of the cultures, as in *S. cerevisiae* cultures (Toivari et al., 2010, 2012b), and was consumed at a rate of 3.6 ± 0.02 g L^{-1} h^{-1}. Ethanol, produced from D-glucose, was consumed at 0.34 ± 0.02 g L^{-1} h^{-1}. In some cultures additional D-glucose was provided after 28.4 h, since addition of ethanol or D-glucose helped sustain D-xylonate production in *S. cerevisiae* (Toivari et al., 2010, 2012b). Cells grown at pH 5.5, produced 88 ± 0.4 g D-xylonate L^{-1} from 98 ± 1 g D-xylose L^{-1}, at an initial rate of 1.4 ± 0.02 g L^{-1} h^{-1} (Table 1). Up to 8.2 ± 0.6 g xylitol L^{-1} were produced, but xylitol was consumed after 74 h at a rate ⩾0.36 ± 0.02 g L^{-1} h^{-1} when D-xylose was no longer present in the supernatant. Further cultures were provided a total of 171 ± 5 g D-xylose L^{-1}, in pulses approximately every 24 h (Fig. 2), and 171 ± 5 g D-xylonate L^{-1} were produced (Table 1). Only 17.1 ± 1.5 g xylitol L^{-1} were produced, which was being consumed after 146 h (Fig. 2) Production of D-xylonate at the rate of 1.4 ± 0.03 g L^{-1} h^{-1} was sustained for the first 100 h, after which the amounts of D-xylose provided limited the D-xylonate production rate and should be optimised. The initial rate was comparable to that of *Pseudomonas fragi* (1.4 g L^{-1} h^{-1}) and *Enterobacter cloacea* (1.6 g L^{-1} h^{-1}) producing similar concentrations of D-xylonate (150–200 g L^{-1}, pH 6.5; Table 1; Toivari et al., 2012a) at similar yield (Table 1). In contrast with *P. fragi* and *E. cloacea*, which produced these concentrations of D-xylonate from initial D-xylose concentrations of 150–200 g L^{-1}, *P. kudriavzevii* produced only 103 ± 1 g D-xylonate L^{-1} (yield 0.48 g D-xylonate [g D-xylose consumed]$^{-1}$) in 213 h when the initial concentration of D-xylose was high (251 g L^{-1}, data not shown), even though this concentration of D-xylose would not normally be inhibitory for *P. kudriavzevii* (unpublished data), most of the D-xylose (215 ± 12 g L^{-1}) was consumed, and only 40 ± 0.4 g xylitol L^{-1} (yield 0.19 g [g D-xylose]$^{-1}$) were produced. Thus, pulsed feeding, as used here, or a fed-batch or continuous culture system are more appropriate for production of D-xylonate with *P. kudriavzevii* than the batch cultures used with bacteria. Although different production systems were used, the results demonstrate that *P. kudriavzevii* can perform as well (in titre, yield and volumetric rate) as the best reported bacteria for D-xylonate production, while its high stress tolerance (Kitagawa et al., 2010) makes it a good choice for D-xylonate production from complex plant biomass hydrolysates.

The approximate yield of D-xylonate on D-xylose was 0.92 ± 0.04 g g^{-1} (0.83 mol mol^{-1}) in the cultures fed 98 g D-xylose L^{-1}, and 1.0 ± 0.0 g g^{-1} (0.90 mol mol^{-1}) in cultures fed 171 g D-xylose

Table 1
Production of D-xylonate with *P. kudriavzevii* VTT-C-12903 and *S. cerevisiae* VTT B-67002 *xylB* at pH 5.5 or pH 3.0, with data from *P. fragi* and *E. cloacea* (pH 6.5), and *G. oxydans* (pH 3.5) for comparison. D-Glucose was used as a co-substrate.

Species	pH	D-Xylose provided (g L^{-1})	Co-substrate provided (g L^{-1})	D-Xylonate (g L^{-1})	$Yield_{P/S}$ (approx., g g^{-1})	Volumetric production rate (initial) (g L^{-1} h^{-1})	Biomass (g L^{-1})	Time (h)	Process
P. kudriavzevii VTT-C-12903	5.5	96 ± 1	12.5	88 ± 1	0.9	1.4 ± 0.1	12.1 ± 0.2	97	Fed-batch [c]
P. kudriavzevii VTT-C-12903	5.5	99 ± 1	20.5	87 ± 0	0.9	1.4 ± 0.0	15.3 ± 0.2	97	Fed-batch [c]
P. kudriavzevii VTT-C-12903	5.5	171 ± 5	24.7	171 ± 5	1.0	1.4 ± 0.0	12.6 ± 0.3	170	Fed-batch [c]
S. cerevisiae VTT B-67002 *xylB* [a]	5.5	49	12	43	0.8	0.44	7	120	Fed-batch [c]
P. fragi [b]	6.5	150	0	162	1.1	1.4	6.9	120	Batch
E. cloacea [b]	6.5	200	<2	190	1.0	1.6	nd	120	Batch
P. kudriavzevii VTT-C-12903	3.0	153 ± 1	24.1	146 ± 5	1.0	1.2 ± 0.0	10.7 ± 0.1	170	Fed-batch [c]
P. kudriavzevii VTT-C-12903	3.0	55 ± 1	12.5	57 ± 0	1.0	1.2 ± 0.0	9.1 ± 0.1	74	Batch
S. cerevisiae VTT B-67002 xylB	3.0	60	12.6	13 ± 0.3	0.7	0.2 ± 0.0	5.0 ± 0.2	117	Fed-batch [c]
G. oxydans (ATCC621) [b]	3.5	40	2.3	41	1.0	1.0	0.2	52	Batch

nd, no data.
[a] Toivari et al. (2012b).
[b] See Toivari et al. (2012a).
[c] With discontinuous addition of substrate, see Figs. 2 and 4.

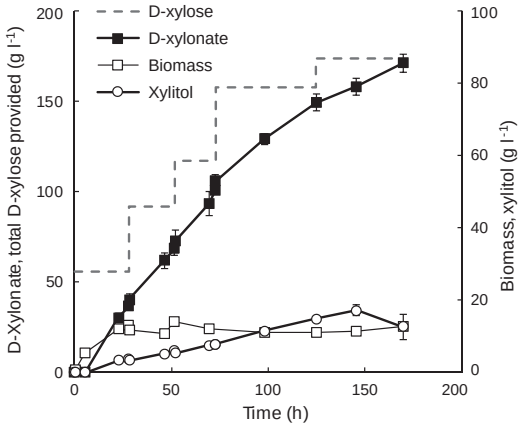

Fig. 2. D-Xylonate (solid square), xylitol (open circle) and biomass (open square) produced by *P. kudriavzevii* VTT-C-12903 at pH 5.5 in cultures provided a total 171 g D-xylose in pulses at ~24 h intervals (indicated by grey line). YP medium initially contained 15.4 g L^{-1} D-glucose and 54.0 g D-xylose L^{-1}. D-Glucose (9.4 g D-glucose L^{-1}) was also added at 28.4 h. Error bars represent SEM for two cultures and where not visible are smaller than the size of the symbol.

L^{-1} (Table 1). Biomass, as well as D-xylonate and xylitol (yield prior to consumption 0.09 ± 0.01 mol mol^{-1}), was produced from D-xylose mainly during the first 50 h cultivation, resulting in lower yields of D-xylonate on D-xylose for shorter cultures. Yield was not affected ($p > 0.05$, Table 1) by the amount of D-glucose added and D-glucose is probably not needed as co-substrate, other than for the initial rapid production of biomass. Further, production of 171 g D-xylonate L^{-1} did not require the consumption of more D-xylose as an energy source than production of 88 g D-xylonate L^{-1}, probably because ATP was provided by the regeneration of NAD^+ through the respiratory chain.

Cell viability and/or vitality were monitored at intervals throughout the cultivations and was similar in all cultures with pulsed D-xylose feed (Fig. 3). Cell vitality was strongly correlated (R^2 0.86 to 0.96) with the extracellular D-xylonate concentration (Fig. 3b). Even after 146 h (extracellular D-xylonate = 158 ± 5 g L^{-1}), 61 ± 2% of the population remained metabolically active (52 ± 2% viable; Fig. 3a) in the cultures provided 171 g D-xylose L^{-1}. In contrast, only 23% of *S. cerevisiae* VTT B-67002 *xylB* cultures which had produced 43 g D-xylose L^{-1} in similar production conditions remained active (Toivari et al. 2012b). The ability of *P. kudriavzevii* to utilise D-xylose/xylitol as an energy/carbon source, may contribute to its maintenance of viability while producing D-xylonate even though only a small proportion of D-xylose (<10%) was consumed for biomass production. Because 60% of the cells were metabolically active after 146 h, we expect that much higher concentrations of D-xylonate could be produced if more D-xylose were provided.

In *G. oxydans*, *P. fragi* and other natural D-xylonate producers, D-xylonate is typically produced in the periplasm and accumulation of high cytoplasmic concentrations has not been addressed beyond the observation that the lactone form of D-xylonate may be more toxic than the linear form (Buchert and Viikari, 1988; Meijnen et al., 2009). In D-xylonate producing yeast, D-xylonate is produced in the cytoplasm, where it may accumulate, depending on the cell's capacity to export it. Thus the ability to tolerate intracellular acid concentrations is important and may be correlated with viability/vitality. In *P. kudriavzevii* VTT-C-12903 cultures at pH 5.5, intracellular D-xylonate concentration was high (115–143 mg D-xylonate [g biomass]$^{-1}$) within 6–9 h of providing D-xylose to the culture (Fig. 4), even though the extracellular D-xylonate concentration was very low (0.1–7 g L^{-1}). High initial D-xylonate concentrations (94 ± 4 mg [g biomass]$^{-1}$) were also observed in *S. cerevisiae* VTT B-67002 *xylB* (Toivari et al. 2012b), but unlike in B-67002, the intracellular concentration in *P. kudriavzevii* decreased during the first 40 h of D-xylonate production to a concentration of 63 ± 2 mg [g biomass]$^{-1}$, remaining more-or-less constant until D-xylose had been consumed (Fig. 4). Thus intracellular D-xylonate concentrations in *P. kudriavzevii* were much lower than those observed in *S. cerevisiae* VTT B-67002 *xylB* (~170 mg [g biomass]$^{-1}$, Toivari et al., 2012b). *P. kudriavzevii* was exporting D-xylonate (either free or dissociated acid) against a concentration gradient from the intracellular to the extracellular environment after the first 50 h, whereas the opposite was generally the case for *S. cerevisiae* VTT B-67002 *xylB*. D-Xylonate is not naturally produced by these yeast and nothing is known about its export from the cells, but it is interesting to note that the yeast which can naturally

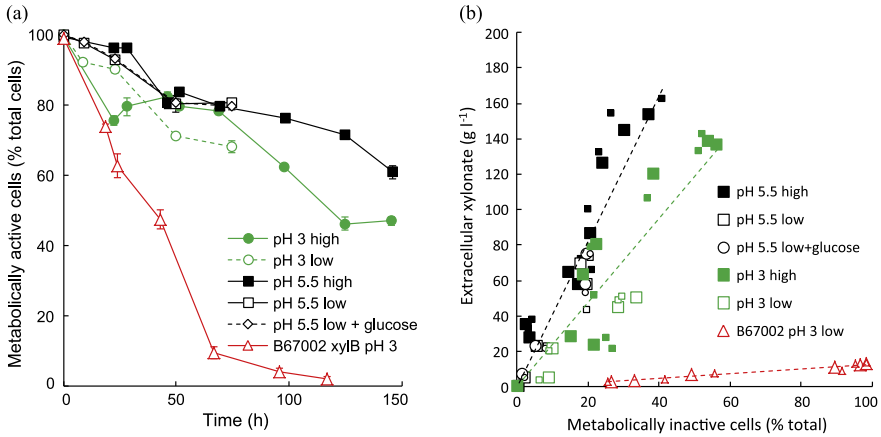

Fig. 3. (a) Metabolically active cells (as % total cells) in cultures of *P. kudriavzevii* VTT-C-12903 at pH 5.5 (squares, diamond) or 3.0 (circles) and *S. cerevisiae* VTT B-67002 *xylB* at pH 3.0 (triangle) in YP medium. (b) The relationship between the amount of extracellular D-xylonate in the culture supernatant and the percentage of cells which have lost metabolic activity, as determined by staining with methylene blue. Data from replicate cultures are indicated with large and small symbols in part b and the dashed straight lines represent the best fit linear regression for all cultures of VTT-C-12903 at pH 5.5 (R^2 = 0.86) or pH 3.0 (R^2 = 0. 82) and of VTT B-67002 *xylB* at pH 3 (R^2 = 0.93). Cultures of VTT-C-12903 received 98 (low) or 171 (high) g D-xylose L^{-1} at pH 5.5 and 55 (low) or 153 (high) g D-xylose L^{-1} at pH 3.0 (see Figs. 2 and 5), with 12.5 (pH 5.5 low, pH 3 low), 20.7 (pH 5.5 low + glucose) or 24 (pH 5.5 high) g D-glucose L^{-1}. VTT B-67002 *xylB* received 60.5 g D-xylose L^{-1} and 12.6 g D-glucose L^{-1}.

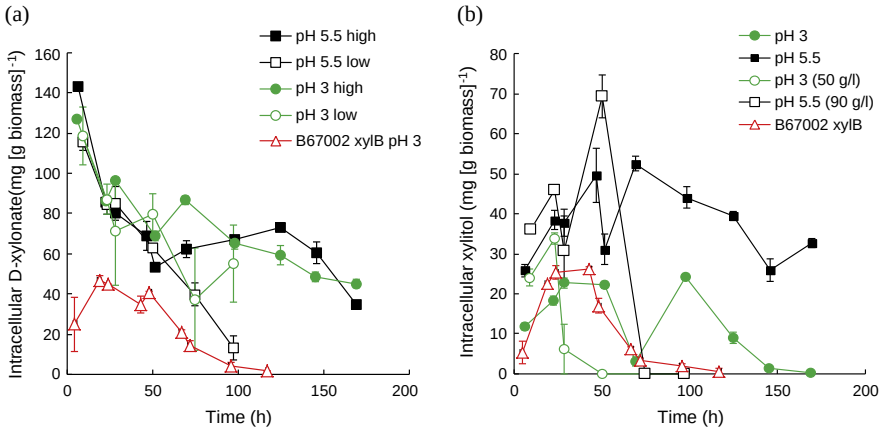

Fig. 4. Intracellular (a) D-xylonate and (b) xylitol in VTT-C-12903 at pH 5.5 (squares) or pH 3.0 (circles) and VTT B-67002 *xylB* at pH 3.0 (triangles) when provided with high (171 g L^{-1}) or low (98 g L^{-1} for VTT-C-12903; 55 g L^{-1} for VTT B-67002 *xylB*) amounts of D-xylose in YP medium with D-glucose as co-substrate, as described in the text. Error bars represent SEM.

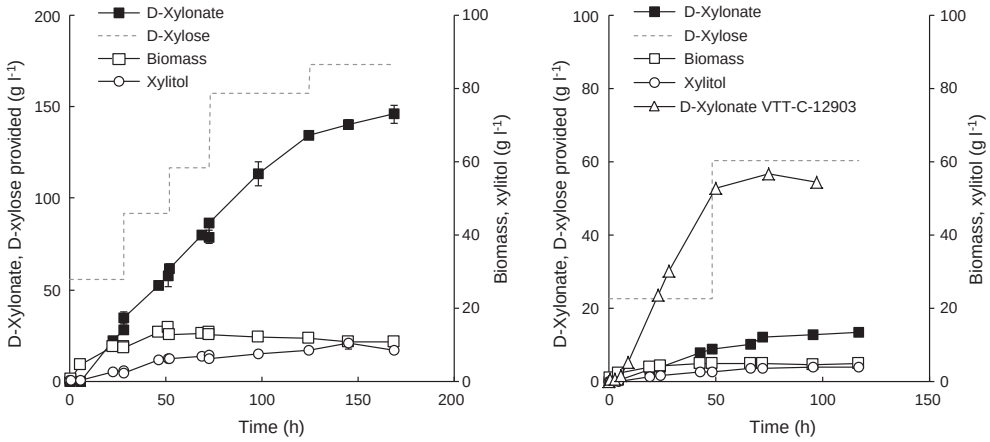

Fig. 5. D-Xylonate, biomass and xylitol production by *P. kudriavzevii* VTT C-12903 (left) and *S. cerevisiae* VTT B-67002 *xylB* (right)at pH 3.0 in YP medium from 60 (VTT B-67002 *xylB*) or 153 (VTT-C-12903) g D-xylose L^{-1}, provided in pulses as shown (dashed lines). VTT-C-12903 cultures initially contained 15 g L^{-1} D-glucose and 53.2 ± 0.8 g D-xylose L^{-1} and received 9.1 g D-glucose L^{-1} at 28.4 h. VTT B-67002 *xylB* cultures initially contained 8.2 g D-glucose L^{-1} and 22.7 g D-xylose L^{-1} and were provided 4.4 g D-glucose L^{-1} and 37.7 g D-xylose L^{-1} at 48 h. Data for D-xylonate production from VTT-C-12903 in batch culture (56 g D-xylose L^{-1} with 12.5 g D-glucose L^{-1}) are shown with the VTT B-67002 *xylB* data for comparison. Error bars represent SEM for two cultures and where not visible are smaller than the size of the symbol.

utilise D-xylose (*P. kudriavzevii*, as presented here, and *K. lactis*, Nygård et al., 2011) appear able to sustain lower intracellular concentrations than *S. cerevisiae* VTT B-67002.

The intracellular xylitol concentrations in *P. kudriavzevii* (25–55 mg [g biomass]$^{-1}$, Fig. 4) were similar to those of *S. cerevisiae* VTT B-67002 *xylB* in YP with D-glucose and D-xylose at pH 5.5 (20 to 50 mg [g biomass]$^{-1}$, Toivari et al. 2012b), but lower than those of *K. lactis xyd1* strains (50–150 mg [g biomass]$^{-1}$) (Nygård et al. 2011). Intracellular xylitol concentrations decreased prior to measureable decreases in extracellular concentrations (*cf.* Figs. 2 and 4).

3.4. Production of D-xylonate by *P. kudriavzevii* VTT-C-12903 and *S. cerevisiae* VTT B-67002 xylB at pH 3

P. kudriavzevii VTT-C-12903 efficiently produced D-xylonic acid at pH 3 (Fig. 5) when D-xylose was provided in pulses, as with cultures at pH 5.5. *P. kudriavzevii* VTT-C-12903 produced 146 ± 5 g D-xylonic acid L^{-1} from 153 ± 1 g D-xylose L^{-1} at a rate of 1.2 ± 0.03 g L^{-1} h^{-1} and approximate yield of 0.95 ± 0.04 g [g D-xylose consumed]$^{-1}$ (Table 1, 0.86 mol mol^{-1}). There was more cell death at pH 3.0 than at pH 5.5, but 47 ± 1% of the population remained metabolically active (30% viable) after 145 h (Fig. 3). With nearly half of the population still metabolically active it is probable that higher concentrations of D-xylonic acid could have been produced if more D-xylose had been provided. As at pH 5.5, vitality was negatively correlated with extracellular D-xylonic acid concentration (R^2 = 0.89, Fig. 3b). Intracellular accumulation of D-xylonate was similar at pH 3.0 and pH 5.5 (Fig. 4). Less xylitol (10.5 ± 1.7 g L^{-1}, yield 0.06 ± 0.00 mol mol^{-1}, Fig. 5) was produced at pH 3.0 than at pH 5.5. As at pH 5.5, high initial D-xylose concentration was inhibitory (data not shown).

In contrast, the robust *S. cerevisiae* VTT B-67002 *xylB* produced only 13 ± 0.3 g D-xylonic acid L^{-1} at pH 3.0 (Fig. 5, Table 1). Xylitol

(4 g L^{-1}) was also produced. Cell vitality decreased rapidly, so that less than 10% of the population remained metabolically active after 67 h cultivation in the presence of D-xylose and D-xylonic acid (Fig. 3a). Vitality of *S. cerevisiae* VTT B-67002 *xylB* was negatively correlated to the extracellular D-xylonic acid concentration (R^2 = 0.94, Fig. 3b). D-Xylonate accumulation within the cytoplasm (Fig. 4) was lower than at pH 5.5 (Toivari et al., 2012b) or in *P. kudriavzevii* VTT-C-12903 and decreased with time. A similar decrease in intracellular xylitol concentration was observed (Fig. 4) (R^2 = 0.77), indicating that this release of D-xylonate (and xylitol) to the supernatant resulted from cell death and lysis. In contrast, only 7–45% of the changes in intracellular xylitol were reflected in changes in the intracellular D-xylonate concentration in VTT-C-12903 at pH 3, indicating that cell lysis was not primarily responsible.

Production of D-xylonate at pH 3.0 has previously been reported for *S. cerevisiae* CEN.PK *xylB* expressing strains, but these produced only 5–7 g D-xylonate L^{-1} (Toivari et al., 2012b). The viability of CEN.PK strains was severely affected by production of D-xylonic acid at pH 3.0 (Toivari et al., 2012b). Liu et al. (2012a) reported that D-xylonic acid could not be produced by recombinant *E. coli* if the medium pH was below 4.0. *P. fragi* did not produce D-xylonate at pH values below 5 (Buchert et al., 1986). *G. oxydans* can produce D-xylonate at pH 3.5 at a production rate of 1.0 g L^{-1} h^{-1} (Toivari et al., 2012a), but pH values below 3.5 have not been tested and concentrations above 40 g L^{-1} have not been produced (Toivari et al., 2012a).

Thus *P. kudriavzevii* is able to produce over 10-fold more D-xylonic acid at pH 3.0 than any other reported strain producing D-xylonic acid, at rates which are almost as high as those at pH 5.5. While some acids such as citric and itaconic acid have traditionally been produced at pH values of 2–3 (Magnuson and Lasure, 2004), it has been more common to produce acids at higher pH values, either because of enzymatic constraints, as in the case of gluconic acid production by *A. niger*, or because of physiological constraints, as in the production of acetic, lactic, gluconic, butyric and other acids with bacteria which are generally unable to grow at pH values below 4 or 4.5. Recent interest in developing processes for the production of lactic (Sauer et al., 2010; Suominen et al., 2009) and succinic (Yuzbashev et al., 2010) acid at low pH to reduce subsequent downstream processing steps has highlighted both the potential and the challenges of producing organic acids at low pH.

P. kudriavzevii is one of the yeast which has been successfully engineered to produce lactic acid at low pH, producing 67–70 g L^{-1} at pH 3.0 (Suominen et al., 2009). The underlying mechanisms of tolerance of low pH are not well understood (Mira et al., 2010; Warnecke and Gill, 2005), nor are acid export mechanisms. However, it is clear that the ability to maintain intracellular pH at near-neutral level is important (Mira et al., 2010; Warnecke and Gill, 2005). Halm et al. (2004) measured intracellular pH in *C. krusei* (synonym of *I. orientalis*, and thus *P. kudriavzevii*) to demonstrate that *P. kudriavzevii* had better pH homeostasis than *S. cerevisiae* at pH 2.5 in the presence of lactic acid. This was measured in the presence of exogenous lactic acid. The intracellular pH of *S. cerevisiae* has also been shown to decrease in response to synthesising L-lactic acid (Valli et al., 2006), and possibly D-xylonate (Toivari et al., 2012b), but equivalent studies have not been carried out with *P. kudriavzevii*.

In addition to maintaining intracellular pH more effectively than *S. cerevisiae*, *P. kudriavzevii* may also have better methods of exporting organic acids than *S. cerevisiae*, since VTT-C-12903 maintained lower intracellular D-xylonate levels than *S. cerevisiae* VTT B-67002 (Toivari et al., 2012b) at pH 5.5. Thus *P. kudriavzevii* is an interesting subject for comparative genomic studies, while transcriptomic studies of the D-xylonate producing strain would also be of interest. With the recent publication of a *P. kudriavzevii* genome (Chan et al., 2012), such investigations have become possible. Meanwhile, the ability of *P. kudriavzevii* to produce D-xylonate in hemicellulose hydrolysates also needs to be addressed.

4. Conclusions

P. kudriavzevii is an excellent production organism not only for traditional products such as ethanol, but also for novel products like D-xylonic acid. By introducing a D-xylose dehydrogenase gene we produced 171 g D-xylonate L^{-1} at pH 5.5 and 146 g D-xylonic acid L^{-1} at pH 3.0, at rates of 1.2–1.4 g L^{-1} h^{-1}. VTT-C-12903 produces D-xylonic acid as well or better than natural D-xylonate producers, such as *G. oxydans*, and has the benefit of also producing it very efficiently at low pH. Choice of strain, gene and culture conditions have led to significant improvements in D-xylonate production.

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PUBLICATION V

The diverse role of Pdr12 in resistance to weak organic acids

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Research Article

The diverse role of Pdr12 in resistance to weak organic acids

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Abstract

Resistance to weak organic acids is important relative to both weak organic acid preservatives and the development of inhibitor tolerant yeast as industrial production organisms. The ABC transporter Pdr12 is important for resistance to sorbic and propionic acid, but its role in tolerance to other weak organic acids with industrial relevance is not well established. In this study, yeast strains with altered expression of *PDR12* and/or *CMK1*, a protein kinase associated with post-transcriptional negative regulation of Pdr12, were exposed to seven weak organic acids: acetic, formic, glycolic, lactic, propionic, sorbic and levulinic acid. These are widely used as preservatives, present in lignocellulosic hydrolysates or attractive as chemical precursors. Overexpression of *PDR12* increased tolerance to acids with longer chain length, such as sorbic, propionic and levulinic acid, whereas deletion of *PDR12* increased tolerance to the shorter acetic and formic acid. The viability of all strains decreased dramatically in acetic or propionic acid, but the Δ*pdr12* strains recovered more rapidly than other strains in acetic acid. Furthermore, our results indicated that Cmk1 plays a role in weak organic acid tolerance, beyond its role in regulation of Pdr12, since deletion of both Cmk1 and Pdr12 resulted in different responses to exposure to acids than were explained by deletion of Pdr12 alone. Copyright © 2014 John Wiley & Sons, Ltd.

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Keywords: **Saccharomyces cerevisiae; weak organic acid tolerance; Pdr12; plasma membrane transporters; Cmk1**

Introduction

Weak organic acid tolerance is a key trait in resistance of fungi to preservatives such as sorbic acid, and in tolerance towards inhibitors present in lignocellulosic hydrolysates, which are being used in second-generation bioprocesses. In addition, increasing the biotechnological production of organic acids, which are useful as industrial precursors for bio-based polymers, requires production hosts that are tolerant towards the acids produced. Several yeasts, including *Saccharomyces cerevisiae*, have been genetically engineered to produce organic acids, such as lactic (Skory, 2003), xylonic (Toivari *et al*., 2010; Toivari *et al*., 2012) and glycolic acid (Koivistoinen *et al*., 2013). In order to engineer fungal production hosts with improved weak organic acid tolerance, acid tolerance needs to be better understood.

The inhibition of growth by weak organic acids may result from alterations in the structure of the plasma membrane or cell wall, from challenges to cellular energy balance while maintaining the intracellular pH, and from intracellular accumulation of anions or decrease in intracellular pH (Ray, 1996; Bracey *et al*., 1998; Piper *et al*., 2001; Ullah *et al*., 2013). For example, Pma1 is the major cytosolic proton pump in yeast (Serrano *et al*., 1986) and an increase in H^+-ATPase activity occurred in cells challenged with sorbic acid at low pH (Holyoak *et al*., 1996). Maintenance of a constant cytosolic pH via the export of protons is crucial for sorbic acid adaptation (Holyoak *et al*.,

1996), and ATP depletion has been observed in acetic or sorbic acid-stressed *S. cerevisiae* cells, particularly in concentrations which reduced, rather than completely inhibited, growth (Piper *et al.*, 1997; Ullah *et al.*, 2013). Bracey *et al.* (1998) suggested that the inhibitory action of sorbic acid resulted from the activation of energy-consuming mechanisms to maintain pH homeostasis, and thus less energy was available for cell growth. In line with this, ATP depletion during sorbic acid stress resulted from sorbate anion pumping, rather than proton-pumping activity (Ullah *et al.*, 2013). Sorbic and benzoic acid have also been shown to cause oxidative stress and increase free radical formation (Peter, 1999). Nonetheless, Stratford and Anslow (1998) proposed that sorbic acid acts primarily as a membrane active compound, inhibiting yeast in a manner comparable to that of sorbic alcohol and sorbic aldehyde, unlike inhibition by other weak acid preservatives, such as propionic and acetic acid.

Pdr12 is an ATP-binding cassette (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). *PDR12* is highly induced by moderately lipophilic acids, such as sorbic and benzoic acid, and also by low pH (Piper *et al.*, 2001; Hatzixanthis *et al.*, 2003), but not by acetic or formic acid (Hatzixanthis *et al.*, 2003). Exposure to sorbic acid and/or low pH dramatically increased the cellular level of Pdr12 (Piper *et al.*, 1998), which is one of the most abundant proteins in cells adapted to sorbic acid stress (Piper *et al.*, 2001). Holyoak *et al.* (1999) showed that Pdr12 exported fluorescein from the cytosol by an energy-dependent mechanism, which was inhibited by sorbic or benzoic acid. They concluded that Pdr12 transported water-soluble weak organic acids with short chain length (C_1–C_7) and that its activity led to exhaustion of intracellular ATP (Holyoak *et al.*, 1999).

An important feature in organic acid tolerance is adaptation; after adaptation, cells are more tolerant to subsequent acid stress. Piper *et al.* (2001) concluded that cells adapted to growth in the presence of weak organic acids limit the diffusional entry of the acid. This response mechanism is poorly understood. Cell wall mannoproteins are known to limit the porosity of the yeast cell wall (De Nobel and Barnett, 1991) and a large number of cell wall-related proteins play a role in the resistance to organic acids by yeast (Fernandes *et al.*, 2005; Simões *et al.*, 2006; Mira *et al.*, 2009, 2010). The Pdr10 transporter acts as a negative regulator of the microenvironment of Pdr12 (Rockwell *et al.*, 2009). Cells lacking Pdr10 had increased resistance to sorbic acid and increased amounts of Pdr12 located in the detergent-resistant membrane fraction (lipid rafts) (Rockwell *et al.*, 2009). Although the expression and abundance of Pdr12 in the presence of sorbic, propionic or acetic acid has been studied, less is known about its role in the tolerance to other industrially relevant acids. In addition, the effect of Pdr12 deletion on tolerance to weak organic acids is still being investigated. A recent patent application claims that deletion of Pdr12 improved isobutanol production (Dundon *et al.*, 2013).

Pdr12 is associated with negative, post-transcriptional regulation by Cmk1 and deletion of *CMK1* can improve resistance to weak organic acid stress by shortening the lag phase of cells in their presence (Holyoak *et al.*, 2000). Cmk1 is a Ca^{2+}–calmodulin-dependent protein kinase with broad substrate specificity (Londesborough and Nuutinen, 1987). The calmodulin–Ca^{2+} complex regulates a large variety of cellular functions (reviewed by Cyert, 2001, among others). There are three genes homologous to *CMK1* in *S. cerevisiae*: *CMK2* (Ohya *et al.*, 1991; Pausch *et al.*, 1991), *RCK2* (alias *CLK1* and *CMK3*) and *RCK1* (Melcher and Thorner, 1996). Deletion of all four genes, including *CMK1*, encoding these Ca^{2+}–calmodulin-activated protein kinase (CaMK) homologues was not detrimental, even though they are thought to play important roles in adaptation to environmental stress (Melcher and Thorner, 1996).

In the present study, we investigated the role of Pdr12 and Cmk1 in resistance to seven 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 (Table 1). These acids differ in size (C_1–C_6) and pK_a (3.77–4.87). Pdr12 and Cmk1 encoding genes were deleted either individually or from the same strain to address their additive effect on weak acid resistance. Additionally, *PDR12* was overexpressed in the parental and Cmk1-deficient strains. The effect of pre-adaptation to sorbic or acetic acid on growth was also assessed.

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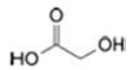
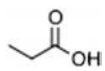
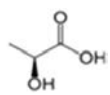
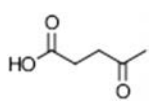
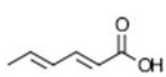
Table 1. Description of the acids studied

Compound	Chemical structure	Molecular formula	pK_a
Formic acid		$C_1H_2O_2$	3.77
Acetic acid		$C_2H_4O_2$	4.79
Glycolic acid		$C_2H_4O_3$	3.84
Propionic acid		$C_3H_6O_2$	4.87
Lactic acid		$C_3H_6O_3$	3.86
Levulinic acid		$C_5H_8O_3$	4.62
Sorbic acid		$C_6H_8O_2$	4.76

Materials and methods

Strains and strain construction

S. cerevisiae CEN.PK2-1D (VW-1B, H1346) (MAT*α*, *leu*2-3/112 *ura* 3–52 *trpl*-289 *his*3Δ1 MAL2-8^{c}SUC2; Boles *et al.*, 1996) was used as the parental strain. All yeast strains used in the study are listed in Table 2. Strains from which *PDR12* was deleted gained a genomic *LEU2* gene, whereas the other strains carried the *LEU2* gene in the modified pYX242 vector B2158 (R&D Systems, UK; modifications described by Toivari *et al.*, 2010). All yeast transformations were carried out using the method described by Gietz *et al.* (1992).

For the *PDR12* overexpression plasmid (p*PDR12*), two ~2500 bp, partially overlapping, PCR fragments of *PDR12* were amplified from a genomic DNA template using primers (Table 3) Pdr12_pYX_F and Pdr12_rec1_R for the 5′-proximal part of the gene and Pdr12_rec2_F and Pdr12_pYX_R for the 3′-proximal part of the gene. The modified pYX242 plasmid was linearized with

Table 2. Strains used in the study

Strain	Modifications
Control	CEN.PK2-1D, *TRP1* + modified pYX242
Δ*cmk1*	Δ*cmk1*::*TRP1*
Δ*cmk1* + p*PDR12*	Δ*cmk1*::*TRP1* + p*PDR12*
Ctrl + p*PDR12*	CEN.PK2-1D, *TRP1* + p*PDR12*
Δ*pdr12*	Δ*pdr12*::*LEU2, TRP1*
Δ*pdr12*Δ*cmk1*	Δ*pdr12*::*LEU2*;Δ*cmk1*::*TRP1*

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Table 3. Primers used in the study

Primer	Sequence (5'→3')
Pdr12_pYX_F	TTTTGTTTGTATTCTTTTCTTGCTTAAATCTATAACTACAAAAA ACACATACAGGAATTCATGTCTTCGACTGACGAACATATTG
Pdr12_rec1_R	ATATAACAGCAAGTCACCTC
Pdr12_rec2_F	GTGTGAACATTGTGTGGAC
Pdr12_pYX_R	AGTTAGCTAGCTGAGCTCGAGATATCATGCGTAGTCAGGCACA TCATACGGATACCCGGGTTATTTCTTCGTGATTTTATTTTCG
Pdr12_Leu2_F	TAATTTTCACTTAAAAAAAAGGTTTACAGATTTATTGTTA TTGTTCTTATTAATAAAAAATCGAGGAGAACTTCTAGTAT
Pdr12_Leu2_R	AAAATTGAAAATAAAAATTGTGTGTTAAACCACGAAATA CAAATATATTTGCTTGCTTGT TCGACTACGTCGTTAAG
Cmk1_Trp1_F	TAGAGAACTCACAATTATATAATATTGGAAGACACCAGA AAAAATAACGAGTCAATTACTTAAAACTTTTCAAACGGC
Cmk1_Trp1_R	TTCGAGCTTACAAAGTCATTGAAGATTTATTCGCCAGT GTGGTAAAACGGCATACTGTTATTATATACTAAGCTGCCGG
Trp1_F	TAAAACTTTTCAAACGGC
Trp1_R	TTATATACTAAGCTGCCGG

SmaI (New England Biolabs, USA) and introduced, together with the PCR products, with overlapping flanks into the parental strain H1346 to combine the fragments with homologous recombination, placing the *PDR12* gene between the *TPI1* promoter and terminator. The modified pYX242 plasmid with the *PDR12* insert was named p*PDR12* and the parental strain overexpressing *PDR12* was annotated as Ctrl + p*PDR12*. Overexpression of *PDR12* was verified by quantitative PCR.

Deletion of the *PDR12* gene was achieved by replacement of the ORF by the *LEU2* gene. The *LEU2* gene, with its endogenous promoter and terminator sequences, was amplified from plasmid p425MET25 (Mumberg *et al*., 1994), using the Pdr12_Leu2_F and Pdr12_Leu2_R primers, which have flanks overlapping the 5′-and 3′-regions of the *PDR12* gene (Table 3). The parental strain was transformed with the PCR product, generating the Δ*pdr12*::*LEU2* strain, here referred to as Δ*pdr12* (Table 2).

Deletion of the *CMK1* gene was achieved by replacement of the ORF by the *TRP1* gene. The *TRP1* gene was amplified with endogenous promoter and terminator sequences, using genomic DNA (of a CEN.PK strain) as template. The Cmk1_Trp1_F and Cmk1_Trp1_R primers were used for amplification of the *TRP1* gene (Table 3). The primers were designed with flanks overlapping sequences in the 5′- and 3′-regions of the *CMK1* gene. The PCR products were then introduced to the parental strain, *Δpdr12*, or the parental strain overexpressing p*PDR12*, generating strains *Δcmk1*::*TRP1*, *Δpdr12*::LEU2;*Δcmk1*::*TRP1* and *Δcmk1*::*TRP1* + p*PDR12* (Table 2). All deletions were verified by PCR.

The parental strain and the parental strain + p*PDR12* were cured to tryptophan prototrophy by transforming with a *TRP1* fragment, amplified from genomic DNA template using the Trp1_F and Trp1_R primers (Table 3). Transformants were selected by growth in medium lacking tryptophan.

The modified pYX242 plasmid without an insert was introduced into all strains except those with deleted *PDR12* and those containing plasmid p*PDR12* to obtain leucine prototrophic strains. The parental strain cured to tryptophan prototrophy and containing the empty, modified pYX242 plasmid is referred to as the control strain (Ctrl).

Media and culture conditions

Modified synthetic complete (SC) medium (Sherman *et al*., 1983; Richard *et al*., 2000), lacking leucine, with 20 g/l D-glucose (SCD – leu medium) was used for cultivations. Overnight precultures (20 or 50 ml) were grown in 100 or 250 ml Erlenmeyer flasks at 250 rpm and 30°C. To test the effect of prior induction of the endogenous *PDR12* gene, 0.45 mM sorbic acid was added to the preculture medium. This concentration of sorbic acid should induce *PDR12* without affecting growth (Holyoak *et al*., 2000); all strains grew to OD 8–9 during 16 h of incubation. The effect of adaptation

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to growth in the presence of acetic acid was studied by using precultures supplemented with 50 mM acetic acid. Growth in 50 mM acetic acid was severely inhibited. Although all strains were growing after 16 h incubation, the ODs were only 1.6–3.

SCD – leu medium with various acids added was used for growth assays in a Bioscreen apparatus (Bioscreen C MBR automated turbidometric analyser, Growth Curves Ltd, Finland). Formic, acetic, lactic, glycolic, propionic, sorbic and levulinic acid were prepared as 200 mM stocks with no pH adjustment. Medium (270 μl), containing medium components and concentrations of the acid leading to final acid concentrations as described in the results and undiluted media, was added to the wells of a Bioscreen microtitre plate (100-Well Honeycomb plate). Cells from precultures were diluted into deionized water to OD = 0.5 and 30 μl cell suspension was inoculated into the wells containing the medium, to give initial OD = 0.10. Growth at 30° C with continuous, extra-intensive shaking was monitored by measuring the OD at 600 nm. The pH of the medium without added acid was 6.0, whereas the pH of acid-containing media was < 4, except for the medium containing sorbic acid (5–15 mM), in which the pH was 6.0. At pH < 4, at least 50% of the acids studied here would have been undissociated, whereas at pH 6, sorbic acid would be mostly dissociated. Since these acids are toxic at different concentrations at different pH values, they were not compared under the same conditions. Rather, we assessed growth in several concentrations of each acid to determine the concentrations which were inhibitory for the control and compared all strains to the control under these conditions. Results in representative, inhibitory concentrations are shown. The effect of inorganic acid on growth was assessed at pH 4.5 by addition of HCl.

Cells were defined as growing from the time when the OD of the suspension exceeded 0.2. Each condition assessed in the Bioscreen was performed in at least four replicates and statistical differences between the control and other strains were assessed using Student's *t*-test.

Determination of viability of cells exposed to acetic or propionic acid

The viability of cells exposed to acids was determined by comparing the number of viable colony-forming units (CFUs) from Bioscreen cultures which grew within 48 h on agar-solidified SCD – leu medium to the total cell number determined microscopically, using a Cellometer Auto T4 cell counter (Nexcelom Bioscience LLC, USA).

Results

Modulated expression of *PDR12* or *CMK1* affects growth

Overexpression of *PDR12* had a negative impact on the specific growth rate in both the control and the Δ*cmk1* background, even in the absence of acid (Table 4). Deletion of *CMK1* also reduced the specific growth rate on D-glucose, compared to the control strain (Table 4). Strains in which *PDR12* had been deleted by the integration of *LEU2* had slightly higher specific growth rates

Table 4. Specific growth rate of *S. cerevisiae* strains in SCD – leu medium with 20 g/l D-glucose, 300 μl cultures in Bioscreen microtitre plates

Strain	Initial pH 6	Initial pH 4.5	Pregrowth with sorbic acid, initial pH 6	Pregrowth with acetic acid, initial pH 6
Control	0.32 ± 0.004	0.34 ± 0.004	0.31 ± 0.002	0.32 ± 0.001
Δ*cmk1*	0.26* ± 0.004	0.29* ± 0.002	0.30 ± 0.008	0.32*,† ± 0.001
Δ*cmk1* + p*PDR12*	0.25* ± 0.004	0.26* ± 0.005	0.24* ± 0.003	0.25* ± 0.002
Ctrl + p*PDR12*	0.25* ± 0.004	0.26* ± 0.001	0.24* ± 0.003	0.21* ± 0.001
Δ*pdr12*	0.34* ± 0.003	0.36* ± 0.004	0.35* ± 0.003	0.35* ± 0.001
Δ*pdr12*Δ*cmk1*	0.34* ± 0.004	0.36 ± 0.004	0.34* ± 0.003	0.36* ± 0.001

Strains were pregrown in SCD – leu medium or in SCD – leu medium with 0.45 mM sorbic acid or 50 mM acetic acid. Data shown are mean ± SEM for four to five or 31 (initial pH 6) cultures. pH refers to the pH of the medium at the time of inoculation.

*Significantly different ($p < 0.05$, Student's *t*-test) from the control strain in the given condition.

†Grows 2.7% slower than the control strain.

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(~6%; $p < 0.05$) than the control strain, probably reflecting the reduced metabolic burden of having *LEU2* integrated, rather than on a plasmid (Karim *et al*., 2013). Interestingly, deletion of *CMK1* did not reduce the specific growth rate in the *pdr12* background (Table 4). When determining the effect of the various acids on Δ*pdr12* strains, the improved growth in the absence of acids was taken into account, i.e. improved growth was attributed to the deletion when > 6% higher than the control, while reduced growth was always attributed to the deletion.

When inocula were grown in media containing 0.45 mM sorbic or 50 mM acetic acid to allow adaptation to weak organic acid stress, the specific growth rate on D-glucose was generally not affected, even though growth had been inhibited in 50 mM acetic acid. However, the growth rate of the Δ*cmk1* strain was comparable to that of the control strain ($p > 0.05$; Table 4). Likewise, when the pH of the medium was adjusted to 4.5, the specific growth rate of the Δ*cmk1* strain was greater than at initial pH 6.0 ($p < 0.05$), but the specific growth rates of the other strains were not affected.

Overexpression of *PDR12* improved tolerance, whereas deletion of *PDR12* decreased tolerance to sorbic, propionic and levulinic acid

Strains overexpressing *PDR12* (Δ*cmk1* + p*PDR12*, $\mu = 0.08$ h^{-1}, and Ctrl + p*PDR12*, $\mu = 0.09$ h^{-1}) had higher specific growth rates ($p < 0.05$) than the control strain ($\mu = 0.05$ h^{-1}) and reached a higher final OD (> 0.5) compared to the control strain (final OD < 0.4) when exposed to 20 mM sorbic acid (Figure 1a). The strains overexpressing *PDR12* also had shorter lag phases, compared to the control strain, in the presence of 35 or 40 mM propionic acid (Figure 1b, c) or 130 mM levulinic acid (Figure 1d).

Deletion of *PDR12* resulted in strains unable to grow in the presence of 70 (data not shown) to 130 mM levulinic acid (Figure 1d), although the control did. When exposed to lower concentrations of levulinic acid (10–60 mM), the Δ*pdr12* and Δ*pdr12*Δ*cmk1* strains had longer lag phases and lower specific growth rates than the control strain (data not shown). The Δ*pdr12*Δ*cmk1* strain started to grow 1.5 and 3.5 h before the Δ*pdr12* strain in the presence of 30 and 40 mM levulinic acid, respectively. In these conditions, the difference between the Δ*pdr12*Δ*cmk1* and the control strain was 5.5 h.

When exposed to 40 mM propionic acid, the difference in the lag phase between the control and the Δ*pdr12* strains was 5 h (Figure 1c). No growth occurred during 100 h of incubation when the Pdr12-deficient strains were exposed to 45–50 mM propionic acid. The Pdr12-deficient strains barely doubled their biomass when exposed to 20 mM sorbic acid (Figure 1a).

Deletion of *CMK1* increased resistance to propionic and levulinic acid, but decreased tolerance to other acids

The deletion of *CMK1* alone improved the resistance to propionic and levulinic acid (Fig. 1b, d). The Δ*cmk1* cells resumed growth earlier than the control cells when exposed to 30 (data not shown) or 35 mM propionic acid (Figure 1b), but not when exposed to 40 mM propionic acid (Figure 1c). In the presence of 130 mM levulinic acid, the Δ*cmk1* strain and the *PDR12*-overexpressing strains had similar lag phases, which were ~20 h shorter than for the control strain (Figure 1d). The difference in the lag phase between Δ*cmk1* and the control strain increased with increasing concentration of levulinic acid (data not shown).

In the presence of 20 mM sorbic acid the strain with *CMK1* deleted barely doubled its biomass during 70 h (Fig. 1a).

In contrast, deletion of *CMK1* had a negative effect on resistance to acetic (100 mM), glycolic (120 mM) and, in particular, to formic acid (25 mM), in which the lag phase of Δ*cmk1* was increased ~15 h compared to the control strain (Figure 2b). With 20 mM formic acid the difference in lag phase between the Δ*cmk1* strain and the control strain was 5 h but with 15 mM formic acid no difference in lag phase was seen. Acetic acid did not affect the length of the lag phase at concentrations < 100 mM.

Improved tolerance to propionic acid by overexpression of *PDR12* was further enhanced by deletion of *CMK1*

The Cmk1-deficient strain overexpressing *PDR12* (Δ*cmk1* + p*PDR12*) was more tolerant to 35–40 mM propionic acid than the strain only overexpressing *PDR12* (Ctrl + p*PDR12*) (Figure 1b, c). The difference between overexpression of *PDR12* in the control strain, compared to overexpression in

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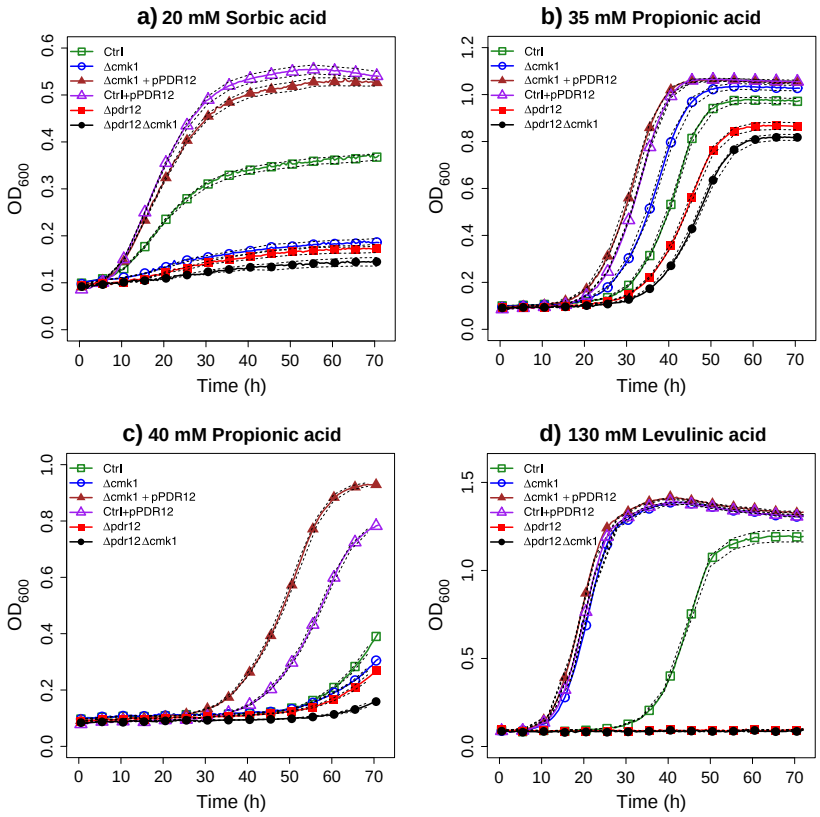

Figure 1. Measurement of biomass in bioscreen cultures of Ctrl (open squares), Δ*cmk1* (open circles), Δ*cmk1* + p*PDR12* (solid triangles), Ctrl + p*PDR12*, (open triangles), Δ*pdr12* (solid squares) and Δ*pdr12*Δ*cmk1* (solid circles) grown in SCD − leu medium containing 20 g/l D-glucose in the presence of: (a) 20 mM sorbic acid, pH 6.0; (b) 35 mM propionic acid, pH 3.9; (c) 40 mM propionic acid, pH 3.9; and (d) 130 mM levulinic acid, pH 3.1. Symbols show every 10th measurement; dashed lines represent SEM of five replicates

the Δ*cmk1* strain, was more pronounced in 40 mM propionic acid (8 h difference in lag phase) than in 35 mM. Even when exposed to 35 mM propionic acid, the *Δcmk1* strain overexpressing *PDR12* resumed growth 2 h earlier than the strain only overexpressing *PDR12* and 5 h earlier than the Cmk1-deficient strain (Figure 1b). This additive effect of *PDR12* overexpression in the Δ*cmk1* strain on tolerance was seen with propionic acid, but not with the other acids tested.

Effect of *PDR12* deletion or overexpression on resistance to acetic, formic, lactic and glycolic acid

In contrast to the sensitivity of the Δ*pdr12* and Δ*pdr12*Δ*cmk1* strains to propionic, sorbic and levulinic acid, these strains were more tolerant to acetic and formic acid than the control strain (Figure 2a, b), with shorter lag phases in the presence of 100 mM acetic or 25 mM formic acid (Figure 2a, b) and higher final biomass concentrations within the 70 h experiments. Cells growing in 100 mM acetic acid did not consume the acetic acid (as measured by HPLC; Toivari *et al.*, 2010; data not shown). The Δ*pdr12*Δ*cmk1* strain also showed a somewhat improved tolerance to glycolic acid when compared to the control strain (Figure 2c).

Overexpression of *PDR12* made cells more sensitive to acetic and formic, and also to lactic and glycolic acid (Figure 2a–d), both in the control and Cmk1-deficient backgrounds, in contrast to their improved tolerance to propionic, sorbic or levulinic acid. The specific growth rate in the

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presence of 100 mM acetic acid, 25 mM formic acid, 120 mM glycolic acid or 140 mM lactic acid was lower and the lag phase longer than that of the control strain, and the length of the lag phases increased with increasing acid concentration (data not shown).

Exposure to inhibitory concentrations of acetic or propionic acid resulted in reduced strain viability

When exposed to 100 mM acetic acid or 40 mM propionic acid, the viability of all the strains studied was reduced; < 1% of the cells were viable within 24 h of incubation (Figure 3). The percentage of viable cells of the control, Δ*pdr12* and Δ*pdr12*Δ*cmk1* strains had increased within 40 h in the presence of 100 mM acetic acid (Figure 3a). The increase in viability corresponded to the length of the lag phase, for the latter two strains in particular (Figure 2a). After 72 h, when Δ*pdr12* and Δ*pdr12*Δ*cmk1* had grown to OD = 0.7–0.9 (Figure 2a), approximately 70% of the cells of these strains were viable. In contrast, the Cmk1-deficient strains and the control strain were more viable after 72 h in 40 mM propionic acid than either of the Δ*pdr12* strains or the strain overexpressing *PDR12* (Figure 3b).

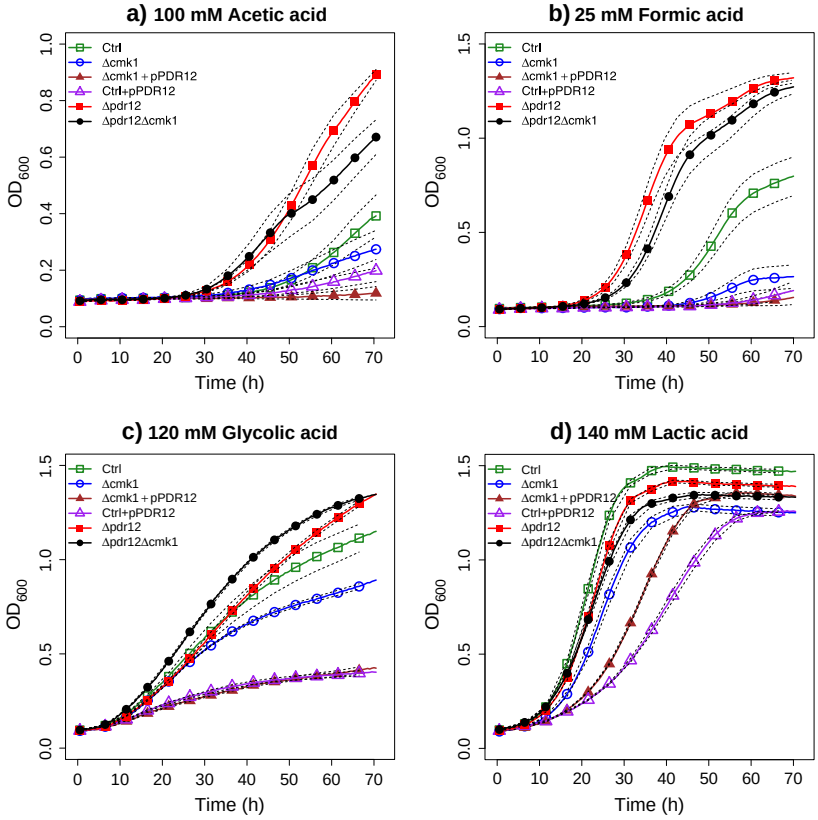

Figure 2. Measurement of biomass in bioscreen cultures of Ctrl (open squares), Δ*cmk1* (open circles), Δ*cmk1* + p*PDR12* (solid triangles), Ctrl + p*PDR12*, (open triangles), Δ*pdr12* (solid squares) and Δ*pdr12*Δ*cmk1* (solid circles) grown in SCD – leu medium containing 20 g/l D-glucose in the presence of: (a) 100 mM acetic acid, pH 3.3; (b) 25 mM formic acid, pH 3.1; (c) 120 mM glycolic acid, pH 3.1; and (d) 140 mM lactic acid, pH 3.0. Symbols show every 10th measurement; dashed lines represent SEM of (a) 15, (b) 20 or (c, d) five replicates

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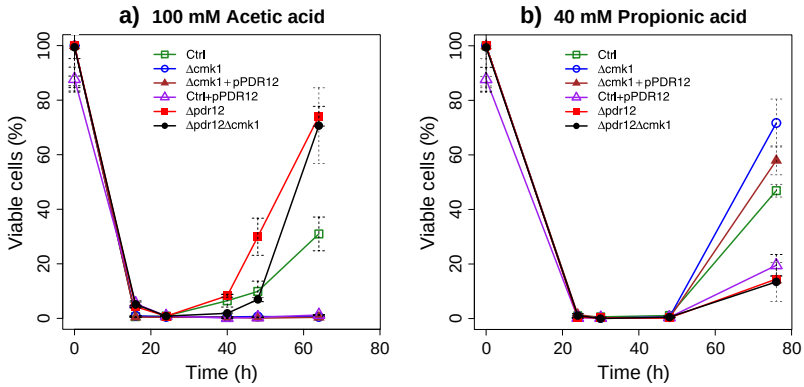

Figure 3. Percentage of viable cells in populations of cells grown in SCD – leu medium containing 20 g/l D-glucose in the presence of: (a) 100 mM acetic acid, pH 3.3; or (b) 40 mM propionic acid, pH 3.9, expressed as the percentage of colony-forming units (CFU) relative to the total cell number determined using a Cellometer Auto T4 cell counter. Error bars show SEM for two biological replicates and reflect the error in both the CFU determination and the estimation of total cell number

Effect of adaptation in sorbic or acetic acid on tolerance to weak organic acids

Exposure of precultures to 0.45 mM sorbic acid did not affect the growth of control cells in 20 mM sorbic acid, but reduced the growth of all the other strains in medium supplemented with sorbic acid (Figure 4). When strains expressing *PDR12* were pregrown in the presence of 0.45 mM sorbic acid, they adapted more rapidly to growth in the presence of 40 mM propionic (~12–40 h faster, depending on the strain) or 100 and 120 mM acetic acid (at least 25 h faster) compared to the same strains pregrown in a medium without added acid (Table 5, Figures 1b, c, 2a, 4b, d, e). The control and the Cmk1-deficient strain that had been grown in 0.45 mM sorbic acid were as resistant to propionic acid as the strains overexpressing *PDR12* (Δ*cmk1* + p*PDR12* and Ctrl + p*PDR12*), which had shown the highest tolerance when not pre-adapted (Figures. 1b, c, 4b). The same phenomenon was observed when cells were transferred to 140 mM levulinic acid; the control strain resumed growth at the same time as the strains overexpressing *PDR12* and the Δ*cmk1* strain (Figures 1d, 4c). Pregrowth in 0.45 mM sorbic acid had a negative impact on the subsequent growth of strains from which *PDR12* was deleted. When these cells were transferred to medium with 40 mM propionic acid they did not grow (Figure 4b, Table 5). The Cmk1-deficient strain produced slightly more biomass in 20 mM sorbic acid when the cells were first exposed to 0.45 mM sorbic acid (Figures 1a, 4a).

The lag phase of all strains in medium supplemented with 100 mM acetic acid was shorter after pregrowth in the presence of 0.45 mM sorbic acid (cf. Figures 2, 4). None of the strains were able to grow in 110 mM acetic acid without pregrowth in 0.45 mM sorbic acid (data not shown). Only Δ*pdr12* and the Δ*pdr12*Δ*cmk1* cells were able to grow in the presence of 120 mM acetic acid after sorbic acid pretreatment, the Δ*pdr12*Δ*cmk1* strain having a shorter lag phase than the Δ*pdr12* strain (Figure 4e).

When sorbic acid pregrown cells were exposed to 25 mM formic acid, the Δ*pdr12*Δ*cmk1* strain had a slightly shorter lag phase compared to cells pregrown without sorbic acid. The Δ*pdr12* and the control strains had longer lag phases and produced less biomass within 72 h after pregrowth in sorbic acid, compared to pregrowth without sorbic acid. Again, the strains overexpressing *PDR12* did not grow within this time frame (Figures 2b, 4f).

When the strains were pregrown in the presence of 50 mM acetic acid, only the Pdr12-deficient strains (Δ*pdr12* and Δ*pdr12*Δ*cmk1*) had improved growth (~25 h shorter lag phase) when subsequently exposed to 100 mM acetic acid (Figures 2a, 5a). The Δ*pdr12*Δ*cmk1* strain also had higher tolerance to 25 mM formic acid (10 h shorter lag phase) when the cells were pregrown in the presence of 50 mM acetic acid, compared to cells pregrown in a medium without added acetic acid (Figures. 2b, 5b). Other strains did not grow in 100 mM acetic acid after incubation in 50 mM acetic acid or 25 mM formic

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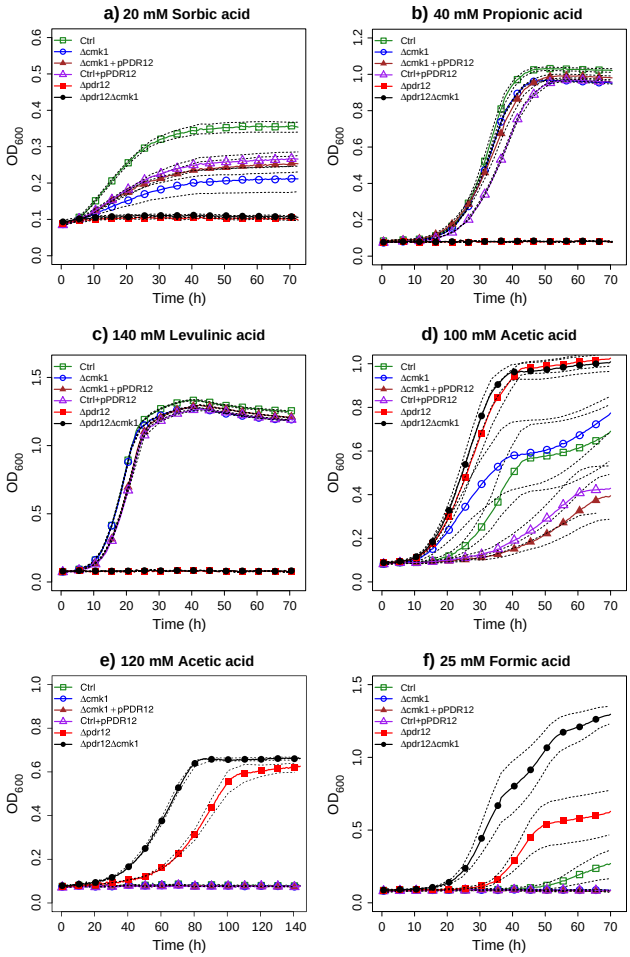

Figure 4. Measurement of biomass in bioscreen cultures of cells pregrown in SCD – leu medium supplemented with 0.45 mM sorbic acid. Ctrl (open squares), Δ*cmk1* (open circles), Δ*cmk1* + p*PDR12* (solid triangles), Ctrl + p*PDR12*, (open triangles), Δ*pdr12* (solid squares) and Δ*pdr12*Δ*cmk1* (solid circles) cells were subsequently grown in SCD – leu medium containing 20 g/l D-glucose in the presence of: (a) 20 mM sorbic acid, pH 6.0; (b) 40 mM propionic acid, pH 3.9; (c) 140 mM levulinic acid, pH 3.1; (d) 100 mM acetic acid, pH 3.3; (e) 120 mM acetic acid, pH 3.3; and (f) 25 mM formic acid, pH 3.1. Symbols show every 10th measurement; dashed lines represent SEM of 4–10 replicates

acid. The Pdr12-deficient strains did not grow in propionic or sorbic acid after exposure to acetic acid.

Less than 1% of the populations were viable after 8 h of incubation in medium supplemented with 100 mM acetic acid, when precultures were grown in 50 mM acetic acid (40–100% viable at the time of inoculation). The Δ*pdr12* strains had regained viability within ~24 h and the Cmk1-deficient strain within ~72 h, whereas the control and *PDR12*-overexpressing cultures contained essentially no viable cells during the interval studied.

Discussion

Previous studies of the Pdr12 ABC transporter have focused on tolerance/sensitivity to only one or a few acids. In the current study, we expanded

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Table 5. Effect of adaptation in sorbic or acetic acid on tolerance to weak organic acids

	0.45 mM sorbic acid adaptation					50 mM acetic acid adaptation			
Strain	**FA**	**AA**	**PA**	**LA**	**SA**	**FA**	**AA**	**PA**	**SA**
Control	–	+	+	+	no	–	–	–	–
Δ*cmk1*	–	+	+	+	+	–	–	–	–
Δ*cmk1* + p*PDR12*	–	+	+	+	–	–	–	–	–
Ctrl + p*PDR12*	–	+	+	no	–	–	–	–	–
Δ*pdr12*	–	+	–	no	–	+	+	–	–
Δ*pdr12*Δ*cmk1*	+	+	–	no	–	+	+	–	–

Cells were pregrown in SCD – leu medium supplemented with 0.45 mM sorbic acid or 50 mM acetic acid for 16 h and then transferred to new medium supplemented with acid.
FA=25 mM formic acid; AA=100 mM acetic acid; PA=40 mM propionic acid; LA=140 mM levulinic acid; SA=20 mM sorbic acid.
+, Pre-adaptation increased tolerance to the acid; –, tolerance was decreased; no, tolerance was not affected.

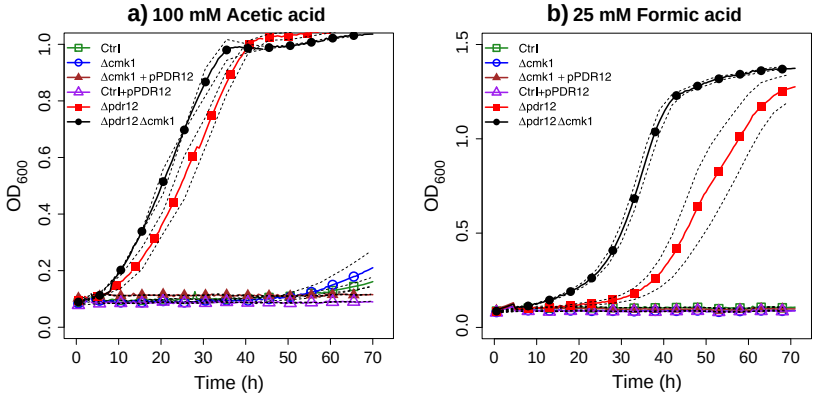

Figure 5. Measurement of biomass in bioscreen cultures of cells pregrown in SCD – leu medium supplemented with 50 mM acetic acid. Ctrl (open squares), Δ*cmk1* (open circles), Δ*cmk1* + p*PDR12* (solid triangles), Ctrl + p*PDR12*, (open triangles), Δ*pdr12* (solid squares) and Δ*pdr12*Δ*cmk1* (solid circles) cells were subsequently grown in SCD – leu medium containing 20 g/l D-glucose in the presence of: (a) 100 acetic acid, pH 3.3; and (b) 25 mM formic acid, pH 3.1. Symbols show every 10th measurement; dashed lines represent SEM of (a) eight or (b) four replicates

the portfolio of acids (Table 1), tested both deletion and overexpression of *PDR12* and evaluated the role of Cmk1 in weak organic acid tolerance. The role of Pdr12 in response to weak organic acid stress was dependent on the acid. The effect of Pdr12 deletion or overexpression on tolerance/sensitivity to the short-chain, hydrophilic acids, formic, acetic, lactic and glycolic acid, was to a large extent opposite to that of the longer sorbic, propionic and levulinic acid (Table 1). The differing effects of pre-adaptation to sorbic and acetic acid was consistent with this.

Overexpression of *PDR12* has been challenging to achieve, but when constitutively expressed, the cells have improved tolerance to propionate (Kuchler *et al.*, 1999). We found that overexpression of *PDR12* improved tolerance to sorbic, propionic and levulinic acid, suggesting that the protein was functional and that the level of protein had been increased in the overexpression strains, even though integration of additional Pdr12 in the cell membranes was not confirmed. As previously shown, deletion of *PDR12* was detrimental for growth in the presence of sorbic (Bauer

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et al., 2003; Ullah *et al.*, 2012) and propionic acid (Holyoak *et al.*, 1999). This also applied to levulinic acid (Figure 1d). The deletion of Cmk1 from the ΔPdr12 strain further decreased the tolerance to sorbic, propionic and levulinic acid (Figure 1 and unpublished data).

Cmk1 is a negative regulator of Pdr12 (Holyoak *et al.*, 2000), so deletion of *CMK1* was expected to generate a phenotype similar to that of *PDR12* overexpression. Deletion of *CMK1* led to decreased resistance to formic, acetic, glycolic and lactic acid, as was seen with strains overexpressing *PDR12*, and improved growth in the presence of levulinic and 35 mM propionic acid, but not 40 mM propionic or sorbic acid (Figure 1). Overexpression of *PDR12* in the Δ*cmk1* strain further increased tolerance to propionic acid (Figure 1c). Holyoak *et al.* (2000) only compared the growth of a tryptophan prototrophic *CMK1* deletion strain with the tryptophan auxotrophic parent, which would be more sensitive to propionic acid (Bauer *et al.*, 2003). We found that tryptophan prototrophy increased tolerance to all the acids we studied (data not shown).

Although some early reports suggested that deletion of *PDR12* would increase sensitivity to acetic acid, this was later shown to be an artefact from the use of a *trp1*-deficient strain, since the uptake of tryptophan can be inhibited by sorbic or acetic acid (Bauer *et al.*, 2003). Leucine uptake has also been shown to be inhibited by acetic acid, whereas histidine and uracil transport was not expected to be affected (Guillem *et al.*, 2012). We used leucine and tryptophan prototrophic strains and medium lacking leucine to avoid secondary effects from its transport. We confirmed that Pdr12 was undesirable for growth in acetic acid and showed that this also applied to formic and glycolic acid.

Overexpression of *PDR12* reduced the tolerance of cells to acetic, formic, glycolic and lactic acid. This low tolerance to acetic and other small acids was partly diminished by sorbic acid adaptation (incubation of the cells in 0.45 mM sorbic acid, prior to exposure to acetic acid) of the cells, similar to the effect of incubation at pH 3 with HCl (Bauer *et al.*, 2003), even though incubation in 0.45 mM sorbic acid was expected to induce *PDR12* (Holyoak *et al.*, 2000). Ullah *et al.* (2013) suggested that adaptation to sorbic acid also reduced diffusional entry of acids to the cell by altering the plasma membrane or cell wall composition, or by improving intracellular buffering. Alterations of the cell wall and plasma membrane in response to adaptation to weak organic acids (Stratford and Anslow, 1998) may be affected by the overexpression of *PDR12*. Thus, altering the expression of *PDR12* may affect the tolerance to acids indirectly, by altering the composition of the cell membrane. It will also affect the ATP demand of the Pdr12 pump, depleting (overexpression) or conserving (deletion) energy for other cellular processes, including the ATP-utilizing Pma1 proton pump, which is important in tolerance to acetic acid (Ullah *et al.*, 2012). Similarly, an increased production of Pdr12 could create a metabolic burden which would contribute to acid intolerance (i.e. to acetic or formic acid), but this should affect cells equally in the presence of propionic, levulinic or sorbic acid, which it did not.

Acetic and propionic acid caused cell death (Giannattasio *et al.*, 2005; Lourenço *et al.*, 2011; and Figure 3). Deletion of *PDR12* did not reduce cell death in the presence of acetic acid, as suggested by Bauer *et al.* (2003), but rather increased the rate of recovery of the small surviving population on acetic, but not on propionic, acid. Based on the specific growth rates of strains which grew, $< 3\%$ of the original population ($\sim 1 \times 10^5$ cells) survived in these populations, which corresponded well with the survival observed in the viability tests.

Holyoak *et al.* (2000) claimed that the improved resistance to acids of the Δ*cmk1* strain was dependent on the activity of Pdr12. However, improved tolerance to acetic or formic acid in the Δ*pdr12* strain was further improved by deletion of *CMK1*, in cells which had been precultured in the presence of either 0.45 mM sorbic acid or 50 mM acetic acid (Figure 4d–f). Thus, it is clear that the role of Cmk1 in acetic or formic acid tolerance is not solely linked to regulation of Pdr12. Cmk1 apparently acts as a negative regulator of other defence mechanisms involved in resistance to acetic or formic acid. The improved tolerance to propionic acid seen in the Cmk1-deficient strain overexpressing *PDR12* may also result from combined effects of non-Pdr12-related regulation and the removal of regulation of Pdr12 by Cmk1.

The improved tolerance to acetic and formic acid in the Δ*pdr12* and Δ*pdr12*Δ*cmk1* strains makes these strains interesting for use in conversion of sugars from lignocellulosic hydrolysates to

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biofuels or chemicals. Acetic (e.g. 27–73 mM) and formic (30–67 mM) acids are the most common acids in most lignocellulosic hydrolysates (Almeida *et al*. 2007) and growth inhibition due to presence of these acids has been linked to reductions in, for example, ethanol production (Larsson *et al*., 1999). Deletion of Pdr12 and/or Cmk1 may thus provide a novel approach to improve productivity in lignocellulosic hydrolysates and should be investigated further. The data also suggest that deletion of *PDR12* could be beneficial in glycolic or lactic acid production processes.

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PUBLICATION VI

Single cell and *in vivo* analyses elucidate the effect of xylC lactonase during production of D-xylonate in *Saccharomyces cerevisiae*

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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.
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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.
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Production of D-xylonate and organic acid tolerance in yeast

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. 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 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.

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