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2013

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Proefschrift

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus, prof. ir. K.C.A.M. Luyben,
voorzitter van het College voor Promoties,
in het openbaar te verdedigingen op
6 september 2013 om 12:30

door

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Ingenieur in Life Science and Technology
geboren te Zaltbommel.

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The research described in this thesis was performed in the Industrial Microbiology Section, Department of Biotechnology, Delft University of Technology, the Netherlands and financed by the Dutch Ministry of Economic Affairs via an EOS-LT grant. The Industrial Microbiology Section is part of the Kluyver Centre for Genomics of Industrial Fermentation, which is supported by the Netherlands Genomics Initiative.

Cover design by Nikki Vermeulen
ISBN: 978-90-5335-723-1

Table of Contents

Chapter 1

Introduction

Bioethanol, the fuel of the future?

Henry Ford believed ethanol (alcohol) was "the fuel of the future", and in the early 1900s designed his Model T to run on ethanol, which was to be produced from plant biomass such as corn or fruits (Kovarik, 1998). About 20 years later, Albert-Jan Kluyver, a famous Delft microbiology professor, also predicted that "carbohydrates -the sugars, starches and cell wall constituents- will become prominent as raw materials" (Van Maris *et al.*, 2006). It turned out they were ahead of their time. The 20th century was the era of fossil fuels and today's society is largely dependent on petrochemistry for the production of fuels and chemicals. However, concerns about finite fossil oil reserves, increasing oil prices, and environmental considerations, motivated the search for renewable fuels and rekindled the interest in biomass-derived fuels, such as ethanol. Ethanol can be used as fuel (often referred to as bioethanol), either pure or blended with gasoline.

Currently ethanol is the largest product in industrial biotechnology in terms of volume (Hong and Nielsen, 2012) and in terms of market value (Caesar, 2008). In 2011, the majority of ethanol was produced either in Brazil from sugar-cane-derived sucrose (19% of the world production) or in the USA from corn-derived starch (50%) (ethanolrfa.org). Sucrose is a dimer of D-glucose and D-fructose that can be hydrolysed by many micro-organisms. While many starch-degrading microorganisms are known, starch is usually hydrolysed to its D-glucose monomers before conversion in a fermentation process. In both cases, C6-sugar units are generated that are readily used by most microorganisms. However, there are several drawbacks of producing fuel ethanol from these feedstocks: first, the raw material costs are high, estimated to be 40%-70% of the production costs (Claassen *et al.*, 1999). Secondly, competition with food production may occur (Mueller *et al.*, 2011; Solomon, 2010). To avoid these drawbacks, waste streams or dedicated crops are promising alternative feedstocks for fuel ethanol production. Examples include agricultural residues like corn stover, wheat straw and bagasse (Cardona *et al.*, 2010; Kadam and McMillan, 2003; Talebnia *et al.*, 2010). Fast-growing trees and grasses like poplar and switch grass are examples of dedicated crops (Keshwani and Cheng, 2009; Sannigrahi *et al.*, 2010). These are low-maintenance and high-yield crops that can grow on marginal farmlands. These feedstocks consist primarily of cellulose, hemicellulose and lignin, and are therefore known as lignocellulosic feedstocks. To indicate the difference with the current state-of-the-art bioethanol production processes, these feedstocks are often also referred to as second-generation feedstocks (Hahn-Hägerdal *et al.*, 2006; Van Maris *et al.*, 2006; Weber *et al.*, 2010).

Challenges in ethanol production from lignocellulosic feedstocks

Lignocellulosic feedstocks consist largely of polymers that are highly resistant to degradation. In order to make these accessible for either enzymes or microorganisms, some form of pretreatment is needed (Figure 1), such as dilute acid-hydrolysis, steam pretreatment or ammonia fibre explosion (Galbe and Zacchi, 2007; Jørgensen *et al.*, 2007; Sun and Cheng, 2002). The method of pretreatment strongly affects the overall process by e.g. the extent of carbohydrate recovery and digestibility, and the formation of fermentation inhibitors (Galbe and Zacchi, 2007; Sun and Cheng, 2002). Enzymatic hydrolysis is the preferred method to hydrolyse

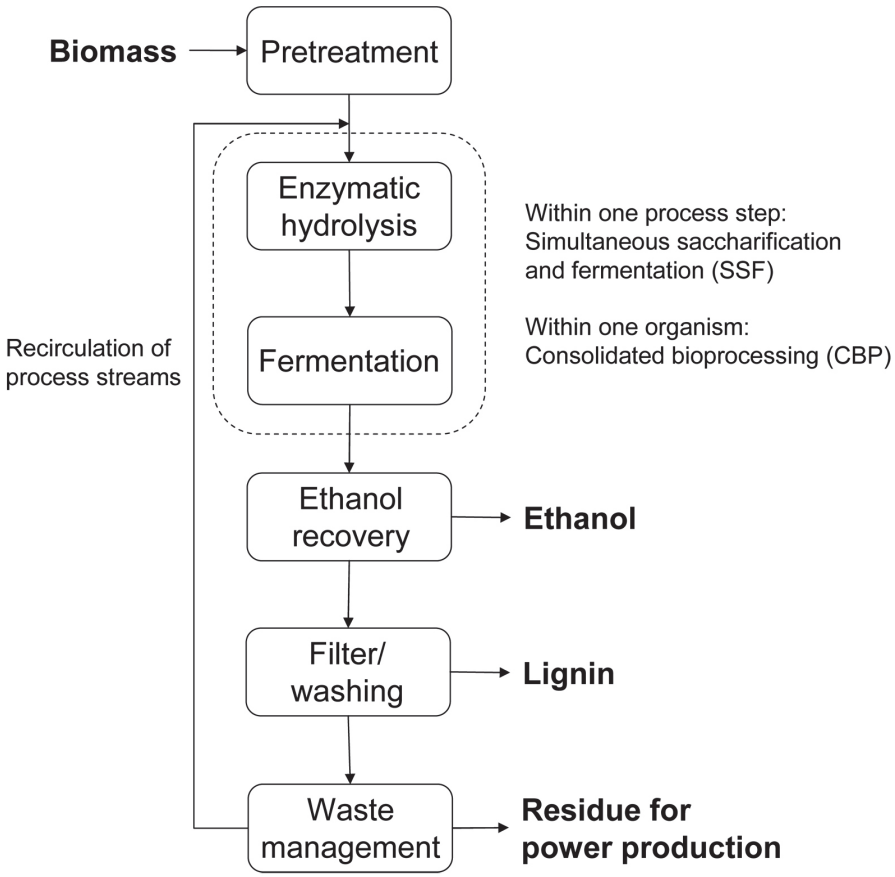

Figure 1. Simplified flow sheet for ethanol production from biomass, based on Galbe and Zacchi (2007). Biomass is pretreated to make it more accessible for either enzymes or microorganisms. Enzymatic hydrolysis liberates the monomeric sugars to enable fermentation of the sugar mixture to ethanol, which can be recovered by distillation.

the polymer after pretreatment and thereby release the monomeric sugars (i.e. saccharification) (Jørgensen *et al.*, 2007; Taherzadeh and Karimi, 2007): compared to chemical hydrolysis, enzymatic hydrolysis is performed at mild conditions (moderate temperature and pH) and does not require addition of chemicals that may be corrosive, or hamper the subsequent fermentation process (Taherzadeh and Karimi, 2007).

There are different process configurations possible for the saccharification and subsequent fermentation of the monomeric sugars. Enzymatic hydrolysis can be performed separately from the fermentation. Besides requiring either additional time or process vessels, another drawback of this process configuration is that the concentration of end-products, the monomeric sugars, increases. This may lead to end-product inhibition of the hydrolysing enzymes (Galbe and Zacchi, 2007). Alternatively, enzymatic hydrolysis and fermentation can be performed in a single step, which is called Simultaneous Saccharification and Fermentation (SSF) (Lynd, 1996). In an SSF process the yeast constantly consumes sugars that are released by the hydrolase enzymes, thereby reducing end-product inhibition on the enzymes and increasing hydrolysis rates and yields. However, at the same time this poses additional requirements on the process conditions due to differences in for instance the optimum temperature and pH of the enzymes and microorganisms (Doran-Peterson *et al.*, 2009; Olofsson *et al.*, 2008). Both of the process configurations mentioned above require the addition of externally produced enzymes, which has a drastic impact on the overall process economy (Lynd, 1996). An alternative process configuration combines both the production of the saccharolytic enzymes and the fermentation of the resulting sugar mixture in a single microorganism. This process configuration is called Consolidated BioProcessing (CBP) (Lynd *et al.*, 2005; Van Zyl *et al.*, 2007).

The choice of feedstock, upstream processing, method and configuration of saccharification also have a direct impact on the choice of the microorganism used in fermentation.

Baker's yeast for ethanol production

Baker's or brewer's yeast, *Saccharomyces cerevisiae*, is a unicellular eukaryotic organism that has been used for the leavening of bread and the production of fermented beverages for thousands of years (McGovern *et al.*, 2004; Samuel, 1996). Antonie van Leeuwenhoek, born in Delft, was the first to observe yeast using his famous microscopes in the 17th century. In the 1830s, three independent scientists, Charles

Cagniard-Latour, Friedrich Kützing and Theodor Schwann were the first to realize that yeast is a living organism (Barnett, 2003). Research on yeast was stimulated by its economic importance, and currently it is one of the best-studied eukaryotic model systems. In 1996 its genome sequence of about 12 megabases was published, containing roughly 6000 genes (Goffeau *et al.*, 1996). Availability of a complete and well-annotated genome sequence contributed to the expansion of the genetic toolbox. Today, high-efficiency transformation techniques (Gietz and Woods, 2001), specialized expression vectors (e.g. Baker Brachmann *et al.*, 1998; Christianson *et al.*, 1992; Mumberg *et al.*, 1995; Sikorski and Hieter, 1989), methods for introduction of gene deletions using recyclable markers (Güldener *et al.*, 1996; Güldener *et al.*, 2002; Solis-Escalante *et al.*, 2013) and clever use of the high efficiency of homologous recombination (Gibson *et al.*, 2008; Shao *et al.*, 2009) are just some of the tools that enable rapid genetic engineering of *S. cerevisiae*.

Although the requirements differ for its many industrial applications, various attributes make *S. cerevisiae* well-suited for industrial use: it is insensitive to bacteriophages, and its acid-tolerance enables fermentations at low pH, which limits the chance of bacterial infections. The ability of *S. cerevisiae* to grow under strictly anaerobic conditions, its high-rate and high-yield ethanol production, tolerance to relatively high concentrations of ethanol and inhibitors contribute to its attractiveness. Moreover, *S. cerevisiae* is osmotolerant, which enables fermentations at high substrate concentrations. These traits made *S. cerevisiae* the organism of choice for the current industrial ethanol production from sucrose or starch, and a promising microorganism for fuel ethanol production from lignocellulosic feedstocks (Van Maris *et al.*, 2006).

Yeast as a metabolic engineering platform

"Metabolic engineering is the directed improvement of cellular properties through the modification of specific biochemical reactions or the introduction of new ones, with the use of recombinant DNA technology" (Stephanopoulos, 1999). The genetic accessibility of *S. cerevisiae* and the rapid developments in synthetic biology (Endy, 2005; Krivoruchko *et al.*, 2011), contributed to the use of this yeast as metabolic engineering platform for the production of a large and increasing number of other compounds besides ethanol. This topic has been excellently reviewed by Nevoigt (2008) and, more recently, by Hong and Nielsen (2012). Succinic acid, human insulin and artemisinic acid are just some examples of the spectrum of products that are already produced at pilot or full scale using *S. cerevisiae*. Furthermore, there are many academic studies exploring this yeast for the

production of fuels, bulk chemicals, fine chemicals and pharmaceuticals. To give a complete overview is outside the scope of this introduction, and the reader is referred to the above-mentioned reviews (Hong and Nielsen, 2012; Nevoigt, 2008).

An important aspect of yeast as a metabolic engineering platform is the choice of yeast strain. Ideally, a strain combines a number of traits such as a wide substrate range for carbon and nitrogen sources, no auxotrophic requirements, high biomass yield, good growth under aerobic as well as fully anaerobic conditions and good genetic accessibility and stability (Van Dijken *et al.*, 2000). Strains of the CEN.PK family (Entian and Kötter, 2007) combine good growth characteristics with good genetic accessibility and are therefore very popular. Moreover, the genome of CEN.PK113-7D was recently sequenced and annotated (Nijkamp *et al.*, 2012), which further facilitates the use of this strain family.

Physiological challenges in ethanol production from lignocellulose

Even though ethanol production with *S. cerevisiae* is an established industry, this is not (yet) the case for second-generation bioethanol production. There were, and still are, a number of challenges to be conquered. Below, the physiological challenges will be discussed in three sections: (1) expansion of substrate range, (2) mixed substrates and (3) robustness, although obviously there is considerable overlap and interplay between these subjects. The challenges will be discussed in the context of ethanol production from lignocellulose. However, also when *S. cerevisiae* is used for the production of other products from second-generation feedstocks, these challenges and concepts are relevant.

Expansion of substrate range

The main sugar monomers present in lignocellulosic hydrolysates are D-glucose, D-xylose and L-arabinose (Hahn-Hägerdal *et al.*, 2006; Van Maris *et al.*, 2006; Weber *et al.*, 2010). While D-glucose is a preferred substrate for *S. cerevisiae*, it is unable to either grow on or consume the pentose sugars D-xylose and L-arabinose. The expansion of the substrate range of *S. cerevisiae* is therefore essential to achieve economically viable ethanol production from lignocellulosic feedstocks. Significant progress has now been made in metabolic engineering of *S. cerevisiae* for the utilization of D-xylose and L-arabinose (Hahn-Hägerdal *et al.*, 2007; Weber *et al.*, 2010; Wisselink *et al.*, 2009).

Two routes for D-xylose metabolism have been considered for metabolic engineering of *S. cerevisiae*, that both convert D-xylose to D-xylulose and thereby link D-xylose metabolism to the pentose phosphate pathway via xylulokinase (Figure 2):

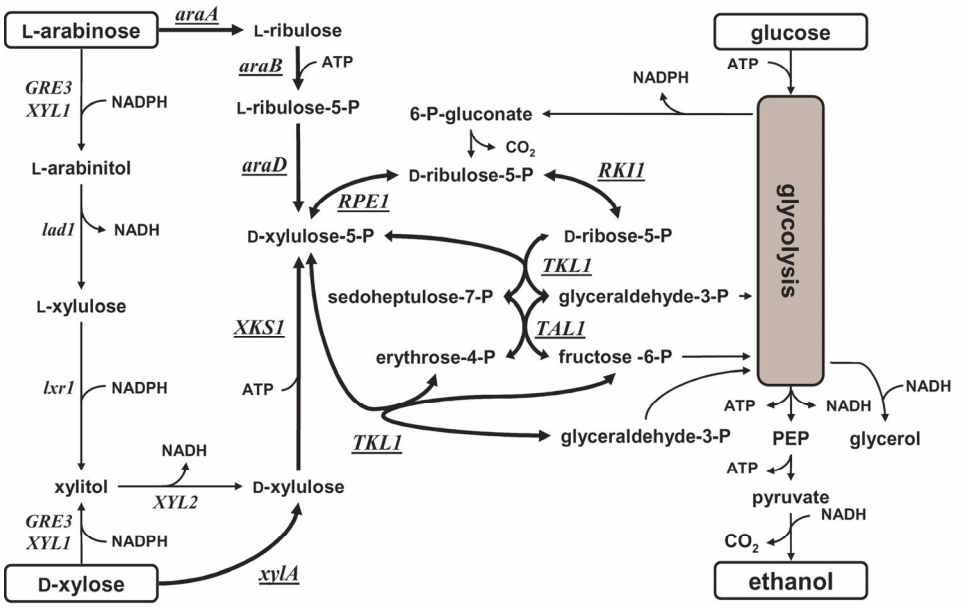

Figure 2. From Wisselink *et al.* (2007). Schematic representation of D-xylose and L-arabinose catabolism in metabolically engineered *S. cerevisiae* strains described in the literature. Components of the catabolism are as follows: aldose/xylose reductase (encoded by *GRE3*/*XYL1*), xylitol dehydrogenase (*XYL2*), xylulokinase (*XKS1*), D-xylose isomerase (*xylA*), arabinitol 4-dehydrogenase (*lad1*), L-xylulose reductase (*lxr1*), L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), L-ribulose-5-phosphate 4-epimerase (*araD*), transaldolase (*TAL1*), transketolase (*TKL1*), D-ribulose-5-phosphate 3-epimerase (*RPE1*), and ribose-5-phosphate ketol-isomerase (*RKI1*). PEP, phosphoenolpyruvate.

in the first route, D-xylose reductase (EC 1.1.1.21, encoded by *XYL1*) reduces D-xylose to xylitol. Subsequently, xylitol dehydrogenase (EC 1.1.1.9, *XYL2*) oxidizes this to D-xylulose. Usually these enzymes have different cofactor preferences, which results in the production of by-products and a decreased ethanol yield under anaerobic conditions (Van Maris *et al.*, 2007) to maintain the redox cofactor balance (Bruinenberg *et al.*, 1983). The second route proceeds via D-xylose isomerase (EC 5.3.1.5, encoded by *xylA*), which directly converts D-xylose to D-xylulose, thus circumventing the problem of maintaining the redox cofactor balance (Bruinenberg *et al.*, 1983). However, functional expression of a D-xylose isomerase in *S. cerevisiae* proved to be a significant challenge (Amore *et al.*, 1989; Gárdonyi and Hahn-Hägerdal, 2003; Moes *et al.*, 1996; Sarthy *et al.*, 1987; Van Maris *et al.*, 2007; Walfridsson *et al.*, 1996). The first D-xylose isomerase gene that was eventually

successfully expressed in *S. cerevisiae* – in the sense that it enabled high levels of D-xylose isomerase activity under physiologically relevant conditions - originated from the anaerobic fungus *Piromyces* sp. E2. This enabled *S. cerevisiae* to grow on D-xylose (Kuyper *et al.*, 2003), albeit at a low specific growth rate (0.005 h^{-1} in shake flask cultures). In addition to the introduction of a functional D-xylose isomerase, further metabolic engineering improved the resulting strains. A combination of overexpression of the non-oxidative part of the pentose phosphate pathway and deletion of the non-specific aldose reductase *GRE3* resulted in strains with an anaerobic specific growth rate of 0.09 h^{-1} on D-xylose (Kuyper *et al.*, 2005a). Subsequently, evolutionary engineering was used to further improve the resulting strain. A final isolate (RWB218) had a specific growth rate of 0.12 h^{-1} and a specific xylose consumption rate 1.4 $g \cdot g^{-1} \cdot h^{-1}$ when grown anaerobically on D-xylose as the sole carbon source (Kuyper *et al.*, 2005b). Although *S. cerevisiae* is generally accepted to be unable to utilize D-xylose, Attfield and Bell (2006) managed to isolate non-recombinant strains able to use D-xylose as sole carbon source under aerobic conditions after selection on xylose-containing medium for 1463 days (roughly 4 years). The resulting strains demonstrated D-xylose reductase and xylitol dehydrogenase activities and are therefore probably not very attractive for anaerobic bioethanol production.

Although L-arabinose is less abundant in most biomass hydrolysates than D-xylose, conversion of the smaller L-arabinose fraction can contribute to economic feasibility of fuel ethanol from second generation feedstocks (Grohmann and Bothast, 1994). Also for L-arabinose, two metabolic routes have been considered for metabolic engineering of *S. cerevisiae* (Figure 2): the first occurs in fungi, and involves a series of oxidation and reduction reactions that, analogously to the situation for D-xylose, results in byproduct formation and/or yield decrease to maintain the redox cofactor balance (Dien *et al.*, 1996; Van Maris *et al.*, 2006). This pathway was introduced in *S. cerevisiae*, but the resulting strain only produced ethanol from L-arabinose at a very low rate in anaerobic batch fermentations (Richard *et al.*, 2003). In another study, a strain where this pathway was introduced, was able to produce 10 $g \cdot l^{-1}$ ethanol from 45 $g \cdot l^{-1}$ L-arabinose under micro-aerobic conditions, but a considerable amount of arabinitol was produced (Bera *et al.*, 2010). The second pathway is found in bacteria and converts arabinose via L-arabinose isomerase (EC 5.3.1.4, encoded by *araA*), L-ribulokinase (EC 2.7.1.16, *araB*), and L-ribulose-5-phosphate 4-epimerase (EC 5.1.3.4, *araD*) to D-xylulose-5-phosphate. As this pathway circumvents the challenges in maintaining the redox cofactor balance, several laboratories tried to functionally express these enzymes in *S. cerevisiae* (Becker and Boles, 2003; Sedlak and Ho, 2001). However, using genes originating from *Escherichia coli*, no ethanol was produced from L-arabinose and no growth was

observed under aerobic conditions (Sedlak and Ho, 2001). Using *Bacillus subtilis araA* and the *E. coli araB* and *araD*, combined with an overexpression of the *S. cerevisiae GAL2* gene, Becker and Boles (2003) achieved a specific growth rate of 0.09 h^{-1} and a specific ethanol production rate of approximately 0.07 $g \cdot g^{-1} \cdot h^{-1}$ under oxygen-limiting conditions. In another study, efficient anaerobic fermentation of L-arabinose was accomplished using *araA*, *araB* and *araD* from *Lactobacillus plantarum*, and overexpression of the native *S. cerevisiae* genes encoding the enzymes of the non-oxidative part of the pentose phosphate pathway (Wisselink *et al.*, 2007). After extensive evolutionary engineering the resulting strain (IMS0010) demonstrated a maximum specific arabinose consumption rate of 0.53 $g \cdot g^{-1} \cdot h^{-1}$ (Wisselink *et al.*, 2009).

Mixed substrates

S. cerevisiae frequently encounters mixed substrates in an industrial setting: grape must contains a mixture of D-glucose and D-fructose, and beer wort a mixture of maltose, sucrose, D-glucose and D-fructose. Also fuel ethanol production from hydrolysates of lignocellulosic feedstocks involves the use of mixed substrates containing hexoses and pentoses. Glucose and sucrose are preferred substrates of *S. cerevisiae*, and are always consumed first by repression of genes involved in uptake and/or consumption of other substrates (Gancedo, 1998; Verstrepen *et al.*, 2004). Multiple substrates are therefore sequentially consumed, which is known as diauxic growth (Monod, 1945). As fast consumption of substrates is a prerequisite for economically viable ethanol production from lignocellulosic hydrolysates, it is highly desirable to have simultaneous consumption of mixed substrates or to have fast sequential consumption with no or very short lag phases between the consumption of the different substrates.

There are examples of metabolic engineering strategies to improve mixed-sugar fermentation by *S. cerevisiae*: deletion of *HXK2*, encoding hexokinase II, relieved glucose repression and thus enables co-consumption of glucose and sucrose, glucose and galactose, glucose and maltose and even glucose and ethanol in aerobic batch cultivations (Raamsdonk *et al.*, 2001). Also mutations in *MIG1* and *MIG2* relieved glucose repression, resulting in co-consumption of glucose and sucrose, and shortened lag-times in glucose/galactose mixtures (Klein *et al.*, 1999). Similarly, overexpression of a truncated version of *TUP1* (a general transcription repressor also involved in glucose repression) resulted in a shortened lag-time in the glucose/galactose switch (Lee *et al.*, 2011).

When *S. cerevisiae* is engineered for the consumption of non-natural sugars such as D-xylose and L-arabinose, diauxic growth is also observed (Kuyper *et al.*, 2005a; Wisselink *et al.*, 2009). For a D-xylose-consuming strain (RWB217), this was

quite extreme: after rapid exponential growth on glucose, most xylose was consumed in the second phase. This xylose-consumption phase was slow and non-exponential, which was caused by poor kinetics for D-xylose transport (Kuyper *et al.*, 2005b). Evolutionary engineering (Sauer, 2001) by prolonged cultivation in a chemostat under xylose-limited conditions resulted in improved uptake kinetics and subsequent cultivation in an anaerobic sequencing batch reactor (SBR) improved mixed-sugar utilization of the two sugars (Kuyper *et al.*, 2005b). Using a clever evolution strategy, based on an equal distribution of the number of generations on each sugar, Wisselink *et al.* managed to drastically improve the consumption of mixtures of three sugars: D-glucose, D-xylose and L-arabinose (Wisselink *et al.*, 2009). This resulted in a strain (IMS0010) able to consume a mixture of 30 $g \cdot l^{-1}$ D-glucose, 15 $g \cdot l^{-1}$ D-xylose and 15 $g \cdot l^{-1}$ L-arabinose in approx. 35 h (Wisselink *et al.*, 2009). After rapidly consuming all glucose, in a second phase both xylose and arabinose were consumed simultaneously and fast, with a maximum specific xylose consumption rate of 0.35 $g \cdot g^{-1} \cdot h^{-1}$ and a maximum specific arabinose consumption rate of 0.53 $g \cdot g^{-1} \cdot h^{-1}$.

Engineering of mixed substrate utilization is not always as straightforward as the previous examples suggest. Sanchez *et al.* (2010) evaluated the beneficial effects of two metabolic engineering strategies alone and in combination. *PGM2* overexpression benefits D-galactose utilization. A combination of the deletion of *GRE3*, overexpression of xylulokinase and overexpression of pentose phosphate pathway genes benefits xylose utilization in strains engineered for D-xylose consumption. However, they found that whereas *GRE3* overexpression improved both D-xylose and D-galactose utilization, the combination *GRE3* and *PGM2* overexpression had a negative effect on D-galactose fermentation. This research illustrated that metabolic engineering of mixed-sugar metabolism can lead to unexpected results and requires further research (Sanchez *et al.*, 2010).

Transport is an important aspect when dealing with expansion of the substrate range, and (subsequently) consumption of mixed substrates. It was already observed in the 1960s that *S. cerevisiae* is able to transport D-xylose (Kotyk, 1967), and Hxt4p, Hxt5p, Hxt7p and the galactose transporter Gal2p are able to import this pentose (Hamacher *et al.*, 2002; Saloheimo *et al.*, 2007; Sedlak and Ho, 2004; Young *et al.*, 2011). Gal2p is also responsible for arabinose uptake in engineered *S. cerevisiae* strains (Becker and Boles, 2003; Kou *et al.*, 1970; Wisselink *et al.*, 2010; Young *et al.*, 2011). In fact, Gal2p was reported to have a very broad substrate range, encompassing also D-fucose, D-glucose, D-fructose, D-mannose and D-ribose (Kou *et al.*, 1970; Young *et al.*, 2011). In the RWB218 strain, which was engineered for D-xylose consumption and subsequently evolved for glucose/xylose co-consumption, transcriptome analysis with DNA microarrays indicated an

upregulation of the hexose transporters *HXT1*, *HXT2* and *HXT4*, and improved xylose uptake kinetics compared to the ancestral strain were confirmed by zero trans-influx experiments (Kuyper *et al.*, 2005b; Van Maris *et al.*, 2007). Although pentose uptake kinetics have been improved by heterologous expression of pentose transporters (Runquist *et al.*, 2010; Sedlak and Ho, 2004; Subtil and Boles, 2011) or evolutionary engineering (Kuyper *et al.*, 2005b), all pentose-fermenting strains growing on mixtures of glucose and pentoses still display a diauxic growth pattern. Inhibition of pentose uptake by glucose, which is caused by competition for the hexose uptake system (Subtil and Boles, 2012), plays an important role in this growth pattern. Dedicated pentose transporters might alleviate this.

Robustness

Robustness is an important aspect in developing yeast strains for industrial purposes. In industrial processes, *S. cerevisiae* encounters many stresses, such as exposure to inhibitory compounds, high osmotic pressure, stress due to growth media deficient in certain nutrients, sheer stress, temperature stress (e.g. cold stress during storage) and microbial contaminants and their metabolic products. This may cause problems in the process, such as long lag phases, stuck fermentations and process hiccups, which is undesirable from a process-economy point of view.

Exposure to inhibitors is specifically relevant in bioethanol production from second-generation feedstocks with *S. cerevisiae*, where inhibitors are formed during pretreatment and enzymatic hydrolysis. The choice of the method of pretreatment influences the amount and types of inhibitors that are formed. There are three main classes of inhibitors: furaldehydes such as furfural and hydroxymethylfurfural (HMF), phenolics and weak acids. Inhibitors and their mechanism of action have been extensively reviewed (Almeida *et al.*, 2007; Klinke *et al.*, 2004; Palmqvist and Hahn-Hägerdal, 2000). There are basically four strategies to reduce the impact of inhibitors on fermentation (Almeida *et al.*, 2007). First, preventing the formation of inhibitors seems an obvious and the most desirable option, but unfortunately this is often not possible. For instance, acetic acid is released during hydrolysis of lignocellulosic feedstock because the plant polymers are acetylated. The second option is to detoxify the hydrolysate prior to fermentation. However, for economic reasons hydrolysate detoxification is often unfavourable (Sivers *et al.*, 1994). Third, clever control of the fermentation process can decrease the impact of inhibitors significantly, e.g. by using high biomass concentrations to increase inhibitor conversion rates, pH control or fed-batch operation to keep inhibitor concentrations low (Almeida *et al.*, 2007). Finally, inhibitor tolerance can vary between microorganisms and even between strains, which makes using yeast strains with superior inhibitor tolerance an option (Almeida *et al.*, 2007). In addition it was

shown that it is possible to engineer strains for improved resistance towards various inhibitors, such as acetic acid (Wright *et al.*, 2011), the furaldehydes furfural and HMF (Gorsich *et al.*, 2006; Petersson *et al.*, 2006) and phenolics (Larsson *et al.*, 2001a; Larsson *et al.*, 2001b).

There has been a lot of research on the effect of weak organic acids on the physiology and fermentation performance of yeasts like *S. cerevisiae* for two main reasons: first, the resistance mechanisms used by spoilage yeasts towards weak organic acids that are used as food preservatives (acetate, propionate, sorbate and benzoate) are of major interest for large-scale food and beverage preservation (reviewed in Piper *et al.*, 2001; Piper, 2011). Secondly, as mentioned above, acetic acid is an inevitable inhibitor in hydrolysates of second-generation feedstocks. Although acetic acid is taken as an example here, other weak organic acids also occur in lignocellulosic processes: for example, formic and levulinic acids, which are breakdown products of HMF, or lactic acid, which may occur in contaminated feedstocks or fermentation processes.

Acetic acid, which has a pKa of 4.75, is especially toxic at the lower pH values relevant for industrial fermentations. At these pH values, the acid is present in its undissociated form, and able to freely diffuse into the cell. At the higher intracellular pH value, acetic acid will dissociate into the acetate anion and a proton. As the acetate anion is charged, it is less likely to diffuse over the membrane and will therefore accumulate in the cytosol. The accumulation of protons will cause intracellular acidification. To maintain cellular homeostasis, both species therefore have to be exported. For the proton, this is mediated by the H^+-ATPase. Also the acid anion is extruded by weak acid efflux pumps like Pdr12p, which plays an essential role in the resistance of *S. cerevisiae* to a wide range of lipophilic weak acids, including the food preservatives benzoic acid, sorbic acid and propionic acid (Hazelwood *et al.*, 2006; Piper *et al.*, 1998). Both processes occur at the expense of ATP, which is reflected by the lower biomass yield in cultures grown in the presence of weak organic acids (Abbott *et al.*, 2007; Piper *et al.*, 2001; Piper, 2011).

Measuring robustness can be experimentally challenging as there are many ways to define robustness (strain stability, toxic level of a compound, fermentation performance, etc.). Viability measurements are often used to measure robustness. However, even in viability measurements there are various options (Davey, 2011): first, the traditional way is to define viability as the ability to reproduce. By relating the total cell count (either achieved using a counting chamber under the microscope or by using e.g. a Coulter counter) to the number of colonies that appear on plate, the viable percentage of the culture is obtained. Second, integrity of the cell membrane can be used as measure for viability. Propidium iodide staining is a well-known method to evaluate this, as it diffuses over compromised cell membranes

and yields a red fluorescence which can be measured using a fluorescent microscope or a flow cytometer. Finally, viability may be defined as the cells being metabolically active or not. This can, for example, be measured using the acetoxymethyl ester of 5-carboxyfluorescein diacetate (CFDA, AM). This compound diffuses over the membrane, and can be cleaved by cytosolic non-specific esterases, thereby yielding green fluorescence which can also be measured microscopically or using a flow cytometer.

The impact of inhibitors on mixed substrate fermentations may differ from that on single-sugar fermentations. For multiple engineered *S. cerevisiae* strains it has been observed that acetic acid, an inhibitor commonly present in lignocellulosic hydrolysates, strongly impacts specifically the xylose consumption phase (Bellissimi *et al.*, 2009; Casey *et al.*, 2010). At pH 3.5, xylose consumption by the D-xylose-fermenting strain *S. cerevisiae* RWB218 slowed down in the presence of 3 $g \cdot l^{-1}$ acetic acid, leaving half of the xylose unused 48 h into the fermentation. The lower rate of ATP production on xylose compared to glucose, combined with an increased ATP requirement to maintain cellular homeostasis was the proposed cause for the observed effects (Bellissimi *et al.*, 2009).

The pectin challenge

In addition to cellulose and hemicellulose, plant biomass contains pectin as a third major carbohydrate polymer. In some potential feedstocks, such as citrus peel and sugar beet pulp, the pectin fraction can be up to around 20% (weight percent of dry total solids) (Grohmann and Bothast, 1994). These pectin-rich feedstocks are abundantly available and high in carbohydrates but are currently sold as cattle feed, a low-value product. Using these feedstocks for fuel ethanol production would therefore be economically beneficial and at the same time expand the total biomass volume available for fuel ethanol production (Doran *et al.*, 2000; Edwards and Doran-Peterson, 2012; Grohmann and Bothast, 1994). Both citrus peel and sugar beet pulp originate from production plants, and these materials are therefore already stocked, which would decrease transportation and collection costs if ethanol would be produced locally (Doran *et al.*, 2000).

Pectin is a highly complex polymer with a backbone of α-1,4-linked D-galacturonic acid and side chains containing rhamnose residues and neutral sugars like arabinose and galactose (Mohnen, 2008). Pectin hydrolysis is well-established in fruit juice and textile industries, but due to the complexity of the molecule, complete saccharification requires a large range of hydrolytic enzymes and is therefore not

Table 1. Composition of the cell-wall fraction of pectin-rich feedstocks (data adapted from Grohmann and Bothast (1994)).

	Sugar beet pulp	Orange peel
Carbohydrate (%)		
Glucose	24.1	23.7
Galactose	4.6	8.2
Mannose	0.9	-
Arabinose	18.2	14.2
Xylose	1.5	<5
Rhamnose	1.6	<2
Galacturonic acid	20.7	26.0
Non-carbohydrate (%)		
Lignin	1.5	3.0
Protein	5.8	6.3
Ash	8.2	4.0

straightforward (Kashyap *et al.*, 2001). Once hydrolysed, pectin-rich feedstocks such as sugar-beet pulp yield a mixture high in D-glucose, L-arabinose, and D-galacturonic acid (Micard *et al.*, 1996) (Table 1). Although many organisms are able to utilise these carbon sources, none have been described that can efficiently convert all to ethanol (Edwards and Doran-Peterson, 2012; Grohmann and Bothast, 1994).

The most promising results have been obtained using genetically engineered bacteria (Doran *et al.*, 2000; Edwards and Doran-Peterson, 2012; Sutton and Peterson, 2001). However, bacteria generally have low tolerance to acidic conditions, ethanol and other inhibitors. Consequently, the necessity to run fermentations at high pH and low substrate concentration (to ensure low inhibitor concentration as well as a sufficiently low final ethanol concentration) will make it difficult to avoid contamination. *S. cerevisiae* does not suffer from these drawbacks. Moreover, D-glucose and L-arabinose, two of the predominant sugars present in hydrolysates of pectin-rich feedstocks, can be fermented to ethanol with high rate and yield by engineered yeast strains (Wisselink *et al.*, 2009). However, *S. cerevisiae* naturally neither grows on nor consumes D-galacturonic acid (Barnett *et al.*, 1990) and therefore metabolic engineering is required to achieve this.

Physiological challenges in ethanol production from pectin-rich materials

Expansion of substrate range: microbial D-galacturonic acid metabolism

In order to design a metabolic engineering strategy to achieve D-galacturonic acid fermentation by *S. cerevisiae*, it is essential to explore the natural diversity in microbial D-galacturonic acid metabolism.

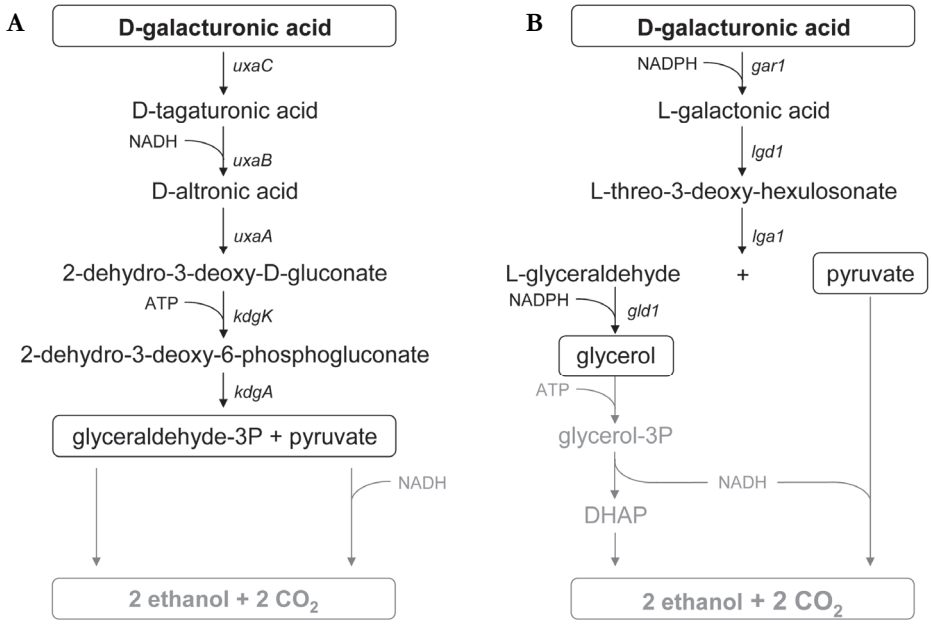

Figure 3. Schematic representation of the pathways for bacterial (panel A) and fungal (panel B) metabolism of D-galacturonic acid which were considered for heterologous expression in *S. cerevisiae*. Codes indicate the following genes and enzyme reactions: panel A: *uxaC* gene, encoding D-galacturonate isomerase (EC 5.3.1.12) *uxaB* gene, encoding D-tagaturonate reductase (EC 1.1.1.58); *uxaA* gene, encoding D-altronate dehydratase (EC 4.2.1.7); *kdgK* gene, encoding 2-keto-3-deoxygluconate kinase (EC 2.7.1.45); *kdgA* gene, encoding 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14). Panel B: *gar1* gene, encoding D-galacturonate reductase; *lgd1* gene, encoding L-galactonic acid dehydratase; *lga1* gene, encoding L-threo-3-deoxy-hexulosonate aldolase; *gld1* gene, encoding glycerol dehydrogenase (EC 1.1.1.72). Reactions in grey indicate conversion of the central metabolites glycerol, pyruvate and glyceraldehyde-3-phosphate to ethanol via the central metabolism of *S. cerevisiae* when these pathways would be implemented in this yeast.

Of the several routes for D-galacturonic acid metabolism known in nature (reviewed in Richard and Hilditch, 2009), the most well-known bacterial pathway (the "isomerase pathway") was identified in the 1950s and `60s. The isomerase pathway involves five enzyme-catalysed reactions (Figure 3A). First, D-galacturonic acid is converted to D-tagaturonic acid by uronate isomerase (Ashwell *et al.*, 1960). D-Tagaturonic acid is subsequently reduced to D-altronic acid in an NADH-dependent manner (Hickman and Ashwell, 1960). D-altronic acid is then dehydrated to 2-dehydro-3-deoxy-D-gluconic acid (KDG) (Smiley and Ashwell, 1960). Phosphorylation of KDG then yields 2-keto-3-deoxy-6-phosphogluconic acid (KDPG) (Cynkin and Ashwell, 1960). Finally, an aldolase splits KDPG to yield pyruvate and glyceraldehyde-3-phosphate (Kovachevich and Wood, 1955), thereby linking this pathway to glycolysis.

Some bacteria, such as *Agrobacterium tumefaciens* and *Pseudomonas* use an alternative pathway for D-galacturonic acid metabolism (the "oxidative pathway"). In this pathway, D-galacturonic acid is first oxidised to galactaro-1,5-lactone by the enzyme uronate dehydrogenase (EC 1.1.1.203) (Chang and Feingold, 1969; Parkkinen *et al.*, 2011). The 1,5-lactone spontaneously rearranges to galactaro-1,4-lactone, which can be converted directly or indirectly to 2-keto-3-deoxy-L-threo-hexaric acid. In *Agrobacterium tumefaciens*, the enzyme galactarolactone cycloisomerase (EC 5.5.1.-) directly converts galactaro-lactone to 2-keto-3-deoxy-L-threo-hexaric acid (Andberg *et al.*, 2012). Alternatively, the metabolism proceeds indirectly, via *meso*-galactaric acid (mucic acid) (Chang and Feingold, 1969; Kilgore and Starr, 1959; Zajic, 1959) by spontaneous hydrolysis of the lactone or hydrolysis with the aid of a lactonase. *Meso*-galactaric acid is then dehydrated to 2-keto-3-deoxy-L-threo-hexaric acid (Chang and Feingold, 1969). A single enzyme (EC 4.2.1.41) catalyses the subsequent dehydratation and decarboxylation to form 2-ketoglutarate semialdehyde (Chang and Feingold, 1969; Jeffcoat *et al.*, 1969). Finally, 2-keto-glutarate semialdehyde is oxidised to 2-keto-glutarate, which can be further metabolized e.g. via the tricarboxylic acid cycle. For some enzymes of this pathway the corresponding gene has been identified (Richard and Hilditch, 2009).

The only known pathway for D-galacturonic acid metabolism in eukaryotes was described in *Hypocrea jecorina* (*Trichoderma reesei*) only relatively recently (Figure 3B). First D-galacturonic acid is reduced to L-galactonic acid (Kuorelahti *et al.*, 2005). This is then irreversibly dehydrated to L-threo-3-deoxy-hexulosonate (Kuorelahti *et al.*, 2006) and subsequently split into pyruvate and L-glyceraldehyde by L-threo-3-deoxy-hexulosonate aldolase (Hilditch *et al.*, 2007). Pyruvate is an intermediate of central metabolism, and L-glyceraldehyde is reduced to glycerol by a specific glycerol dehydrogenase (Gld1p) that is induced by D-galacturonic acid (Liepins *et al.*, 2006; Sealy-Lewis and Fairhurst, 1992). Since its discovery, this route has also been

identified in a number of other fungal species (Martens-Uzunova and Schaap, 2008; Zhang *et al.*, 2011) and (partly) in a psychrophilic yeast (Hamada *et al.*, 2011).

When evaluating these three pathways specifically for implementation in *S. cerevisiae* for ethanol production, the oxidative pathway found in bacteria is not suitable due to the large surplus of NADH that it generates and the fact that the pathway links to central metabolism via 2-keto-glutarate. Therefore, the bacterial isomerase pathway and the eukaryotic pathway (Figure 3) are the most qualified for expression in *S. cerevisiae* for ethanol production. Although the literature on heterologous expression of the genes for D-xylose and L-arabinose metabolism in yeast illustrates the relative ease with which fungal genes are functionally expressed in yeast, it also demonstrates the importance of taking into account redox metabolism. The same is true for D-galacturonic acid: when implemented in *S. cerevisiae* and linked to ethanol production (Figure 3), the two routes should have a similar overall reaction, but involve different intermediates and have a different cofactor preference. Unlike metabolism of D-glucose to ethanol, which is redox neutral, conversion of D-galacturonic acid to ethanol requires the input of redox equivalents because of the higher degree of oxidation of D-galacturonic acid compared to hexoses. Two molecules of NADH or NADPH are oxidised per molecule of D-galacturonic acid using the bacterial and the fungal route, respectively. The challenge of cofactor balancing is exemplified by the generally low ethanol yields obtained with galacturonic acid-metabolizing bacteria: a large part of the carbon is converted to products more oxidized than ethanol, e.g. acetate (Doran *et al.*, 2000; Grohmann *et al.*, 1998; Van Maris *et al.*, 2007). A similar situation is expected to occur when these routes are introduced in *S. cerevisiae* and unless an external electron donor is available, the formation of two molecules of ethanol from one molecule of D-galacturonic acid is not possible. As explained by Van Maris *et al.* (2007), the prokaryotic route is most promising for metabolic engineering of *S. cerevisiae* in the light of fermentation of hydrolysates: during anaerobic growth of *S. cerevisiae* on sugars, part of the sugar is converted to glycerol to reoxidise cytosolic NADH formed in biosynthetic reactions (Bakker *et al.*, 2001; Van Dijken and Scheffers, 1986). In fermentations on mixtures of sugars and D-galacturonic acid, metabolism of D-galacturonic acid could function as a redox sink for this excess NADH thereby preventing glycerol production, thus increasing the overall ethanol yield (Van Maris *et al.*, 2006).

Before metabolism is even possible, D-galacturonic acid has to enter the cell. In bacteria, galacturonic acid transporters have been identified: in *E.coli* (Jimeno Abendano and Kepes, 1973; Nemoz *et al.*, 1976), *Erwinia chrysanthemi* (San Francisco and Keenan, 1993) and *Erwinia caroteva* (Hugouvieux-Cotte-Pattat *et al.*, 1983) evidence indicated an active (energy-requiring) transport system, which transports

both D-glucuronate and D-galacturonate. The transporter, ExuT, is a member of the anion:cation symporter (ACS) family of the major facilitator superfamily (MFS) of transporters (Pao *et al.*, 1998). The gene encoding the galacturonate transporter was first identified in *E.coli* (Mata-Gilsinger and Ritzenthaler, 1983), and (putative) *exuT* genes have been identified in many prokaryotes now. However, there are hardly any examples of successful expression of prokaryotic membrane proteins in *S. cerevisiae*, which illustrates that functional expression of such transporters is not a trivial task.

Transporter genes originating from fungi may be more favourable. In fact, as a strategy to improve D-xylose metabolism in engineered *S. cerevisiae*, fungal xylose transporters were successfully expressed in this yeast in a number of studies (e.g. Leandro *et al.*, 2006; Saloheimo *et al.*, 2007; Young *et al.*, 2011). Fungal galacturonic acid transporters have not been characterized, but Martens-Uzunova and Schaap (2008) identified several putative transporter genes that were strongly upregulated in a transcriptome analysis of galacturonic acid-grown *Aspergillus niger*. A similar approach could identify alternative candidate genes for galacturonic acid transporters from other filamentous fungi or yeasts (Van Maris *et al.*, 2006).

Finally, it is conceivable that some level of native galacturonic acid transport occurs in *S. cerevisiae*. It is well known that some of the hexose transporters have a broad substrate range, not only accepting a range of hexose sugars, but even naturally non-metabolisable pentoses (Becker and Boles, 2003; Hamacher *et al.*, 2002; Kou *et al.*, 1970; Saloheimo *et al.*, 2007; Sedlak and Ho, 2004; Wisselink *et al.*, 2010; Young *et al.*, 2011). If some native transport of galacturonic acid would occur in *S. cerevisiae*, this would facilitate testing of a metabolic route as a heterologous transporter is initially not necessary.

Mixed substrates and Robustness

The three main monomers in pectin hydrolysates are D-glucose, L-arabinose and D-galacturonic acid (Micard *et al.*, 1996). Analogously to hydrolysates of lignocellulosic feedstocks, efficient (co-)consumption of pentoses and hexoses is therefore an important aspect. In addition, it is conceivable that the substantial amount of D-galacturonic acid in these hydrolysates may influence mixed substrate utilisation in two ways.

First, competitive inhibition of transport may occur: D-galacturonic acid in its non-dissociated form is structurally similar to D-galactose, and may therefore inhibit galactose (5-8% in hydrolysates of pectin-rich feedstocks; see Table 1) and arabinose uptake via the galactose transporter Gal2. Binding to the transporter can have a great effect when D-galacturonic acid is present in large excess, e.g. at the end of the fermentation. Second, D-galacturonic acid, which is inevitably formed during hydrolysis of pectin-rich feedstocks, is a weak acid with a pKa of 3.51 (Kohn and

Kovac, 1978). Weak acids are not only known fermentation inhibitors (Palmqvist and Hahn-Hägerdal, 2000; Piper *et al.*, 2001) but they may also affect mixed substrate utilization, as illustrated by the specific inhibition of the xylose consumption phase by acetic acid (Bellissimi *et al.*, 2009; Casey *et al.*, 2010).

Inhibition by compounds present in hydrolysates is not as extensively studied for pectin-rich feedstocks as for lignocellulosic feedstocks. However, substances present in pectin hydrolysates include compounds such as acetic acid, ferulic acid, and methanol, all of which are potential inhibitors of yeast fermentations (Klinke *et al.*, 2004; Larsson *et al.*, 2000; Micard *et al.*, 1996; Palmqvist and Hahn-Hägerdal, 2000; Taherzadeh *et al.*, 1997; Yasokawa *et al.*, 2010).

To achieve economically viable ethanol production from hydrolysates of pectin-rich feedstocks, both optimisation of mixed-substrate utilization and characterizing the potential effects of the significant fraction of D-galacturonic acid and other potential inhibitors present in these hydrolysates, is essential.

Scope and outline

Reducing our dependence on fossil transportation fuels has been a tremendous incentive for research and development of processes for production of biofuels such as ethanol. Currently, starch- and sucrose-based feedstocks are used to produce fuel ethanol, but ultimately second-generation feedstocks, such as waste-streams or dedicated crops should be utilized. *S. cerevisiae*, which is currently used for industrial ethanol production, is a very promising organism to realize this. This thesis explores the challenges related to the use of *S. cerevisiae* for production of ethanol from hydrolysates of pectin-rich feedstocks like sugar beet pulp or citrus peel. Compared to hydrolysates of other second-generation feedstocks, these hydrolysates are unique in the high concentration of D-galacturonic acid.

Chapter 1 gives an introduction on various aspects of ethanol production from second-generation feedstocks with emphasis on the expansion of the substrate range of *S. cerevisiae*, the utilization of substrate mixtures and the robustness of yeast in such hydrolysates. After describing the general challenges for these feedstocks, specific challenges related to pectin-rich feedstocks, such as metabolism and toxicity of D-galacturonic acid are introduced.

Since D-galacturonic acid is not metabolized by wild-type *S. cerevisiae*, it will remain present in high concentrations in hydrolysates of pectin-rich feedstocks. In **Chapter 2** the physiological effects of the presence of a significant amount of D-galacturonic acid in defined cultivation medium are studied. By monitoring the viability of *S. cerevisiae* in time, it was assessed whether the dissociated or

undissociated form of D-galacturonic acid is able to enter the cells of wild-type yeast, and whether this is dependent on the presence of hexose transporters. To further analyze the impact of the presence of D-galacturonic acid, both aerobic and anaerobic chemostat cultures were studied at low pH. This allowed accurate and reproducible determination of biomass yield, substrate uptake rate and product formation rates. Moreover, transcriptome analysis was used to evaluate the impact of D-galacturonic acid at the transcriptional level.

As ethanol production from second-generation feedstocks always involves mixtures of carbon substrates, it is vital to also study the effect of D-galacturonic acid on the fermentation of such sugar mixtures. Since the molecular structure of undissociated D-galacturonic acid resembles that of D-galactose, in **Chapter 3** the quantitative impact of the presence of D-galacturonic acid was first studied for anaerobic batch cultivations of the reference strain CEN.PK113-7D growing on a mixture of glucose and galactose. Subsequently, the physiology of the efficient pentose-fermenting *S. cerevisiae* strain IMS0010 was investigated on a mixture of D-glucose, D-xylose and L-arabinose in the presence of D-galacturonic acid.

Enabling D-galacturonic acid metabolism by *S. cerevisiae* through metabolic engineering would be the best scenario to avoid the inhibiting effect on alcoholic fermentation. Regrettably, multiple attempts in this project failed to achieve this ambitious goal. Therefore, in **Chapter 4**, a thorough investigation into the expression of the first two steps of the bacterial isomerase pathway for D-galacturonic acid metabolism (Figure 3A) in *S. cerevisiae* was performed. To this end, prokaryotic genes encoding D-galacturonate isomerase and D-tagaturonate reductase from phylogenetically diverse origin were expressed in yeast. Subsequently, qPCR and enzymatic assays (using D-tagaturonate reductase as a coupling enzyme to measure uronate isomerase activity) were used to evaluate expression levels.

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Chapter 2

Physiological and transcriptional response of *Saccharomyces cerevisiae* towards galacturonic acid inhibition.

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Manuscript in preparation

Abstract

Aim

Analyse inhibitory effects of galacturonic acid, an important constituent of plant biomass hydrolysates, on growing and starving cultures of *Saccharomyces cerevisiae*.

Methods & Results

Biomass yields in aerobic and anaerobic glucose-limited chemostat cultures (pH 3.5) were reduced by 25 and 10%, respectively, upon addition of 10 $g \cdot l^{-1}$ D-galacturonic acid. Genes previously reported to show a transcriptional response to other organic acids were overrepresented in a set of galacturonic-acid responsive genes identified by microarray analysis. These results suggested that galacturonic acid causes weak-acid uncoupling of the yeast plasma membrane pH gradient. Consistent with this hypothesis, galacturonic acid-accelerated loss of viability in starving cell suspensions was strongly pH dependent. Loss of viability was much slower in a strain in which all *HXT* (hexose transporter) genes were deleted. Moreover, deletion of *HXT* genes alleviated growth inhibition on ethanol observed at D-galacturonic acid concentrations of 10 $g \cdot l^{-1}$ and above.

Conclusions

At low pH, galacturonic acid negatively affects the physiology of *S. cerevisiae*. Reduced sensitivity of hexose-transporter mutants indicated that one or more hexose transporters are involved in transport of galacturonic acid.

Significance and Impact

This study shows that galacturonic acid toxicity should be taken into account in process development for yeast-based fermentative conversion of pectin-rich feedstocks such as sugar beet pulp and citrus peel. Involvement of hexose transporters in galacturonic acid toxicity provides leads for improving tolerance.

Introduction

To maximize the volume of plant biomass accessible to industrial biotechnology, abundant pectin-rich feedstocks such as citrus peel and sugar beet pulp are of considerable interest (Doran *et al.*, 2000; Edwards and Doran-Peterson, 2012; Grohmann and Bothast, 1994). Pectin is a complex polymer with a backbone of α-1,4-linked D-galacturonic acid which, depending on the type of pectin, is linked in different stoichiometries to the neutral sugars L-rhamnose, L-arabinose and D-galactose (Mohnen, 2008). While the relatively low lignin content of pectin-rich feedstocks (Doran *et al.*, 2000; Edwards and Doran-Peterson, 2012; Micard *et al.*, 1996) facilitates pretreatment and decreases inhibitor formation (Palmqvist and Hahn-Hägerdal, 2000), enzymatic hydrolysis is complicated by the complex structure of pectin (Grohmann and Bothast, 1994; Kashyap *et al.*, 2001). Hydrolysates of pectin-rich biomass contain D-glucose, L-arabinose, D-galactose and D-galacturonic acid as the main potentially fermentable carbon sources (Grohmann and Bothast, 1994; Micard *et al.*, 1996).

Technology development for microbial conversion of agricultural residues is currently mostly driven by the production of fuel ethanol, but once developed can be applied to a much wider range of biofuels and biochemicals. Although genetically engineered bacteria able to convert D-glucose, D-galactose, L-arabinose and D-galacturonic acid to ethanol have been described (Doran *et al.*, 2000; Edwards and Doran-Peterson, 2012; Sutton and Peterson, 2001), their low tolerance to acids, ethanol and other inhibitors, as well as their phage sensitivity, complicate the development of robust industrial processes. *Saccharomyces cerevisiae* is currently used for large-scale ethanol fermentation from corn starch and cane sugar, because of its high fermentation rates and robustness under process conditions. However, wild-type strains of this yeast cannot ferment L-arabinose or D-galacturonic acid (Barnett *et al.*, 1990; Huisjes *et al.*, 2012b; Van Maris *et al.*, 2006). Metabolic engineering has already yielded *S. cerevisiae* strains able to ferment L-arabinose and, although less relevant for pectin-rich feedstocks, D-xylose (Bera *et al.*, 2010; Kötter *et al.*, 1990; Kötter and Ciriacy, 1993; Kuyper *et al.*, 2003; Wisselink *et al.*, 2007). So far, however, no engineered *S. cerevisiae* strains capable of fermenting D-galacturonic acid have been reported (Huisjes *et al.*, 2012b; Van Maris *et al.*, 2006), which implies that the D-galacturonic acid in pectin hydrolysates will remain present throughout their fermentation by the yeast.

D-galacturonic acid is a weak acid (pKa = 3.51, (Kohn and Kovac, 1978)). Other weak organic acids occurring in plant biomass hydrolysates, such as acetic and ferulic acid, are known to negatively affect yeast fermentation performance (Abbott *et al.*, 2007; Larsson *et al.*, 2000; Piper *et al.*, 2001; Piper, 2011). In a previous study,

(see Chapter 3 of this thesis) we found that undissociated D-galacturonic acid inhibits fermentation of sugar mixtures by wild-type and engineered *S. cerevisiae* strains and, in particular, the conversion of D-galactose, D-xylose and L-arabinose (Huisjes *et al.*, 2012a).

Successful development of yeast-based fermentation processes that use sugar beet or citrus peel hydrolysates as feedstocks, clearly requires deeper insight in the impact of D-galacturonic acid on yeast physiology and sugar fermentation kinetics. Therefore, the goal of this study was to investigate the responses of *S. cerevisiae* to the presence of D-galacturonic acid in the fermentation medium, both in growing cultures and in starving cell suspensions. Chemostat-based microarray analysis (Daran-Lapujade *et al.*, 2008) was used to study genome-wide transcriptional responses to D-galacturonic acid. Since previous observations suggested a possible role of hexose transporters in growth inhibition by D-galacturonic acid (Huisjes *et al.*, 2012a; Souffriau *et al.*, 2012), the effect of D-galacturonic acid on viability and growth was also studied in *S. cerevisiae* strains in which genes encoding hexose transporters had been deleted (Wieczorke *et al.*, 1999).

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
CEN.PK 113-7D	*MATa MAL2-8^c SUC2*	(Entian and Kötter, 2007)
CEN.PK2-1C	*MATa leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2 hxt17Δ*	(Entian and Kötter, 2007)
EBY.VW4000	CEN.PK2-1C *hxt13Δ::loxP hxt15Δ::loxP hxt16Δ::loxP hxt14Δ::loxP hxt12Δ::loxP hxt9Δ::loxP hxt11Δ::loxP hxt10Δ::loxP hxt8Δ::loxP hxt514Δ::loxP hxt2Δ::loxP hxt367Δ::loxP gal2Δ stl1Δ::loxP agt1Δ::loxP ydl247wΔ::loxP yjr160cΔ::loxP*	(Wieczorke *et al.*, 1999)
IMK300	CEN.PK113-7D *dak2::loxP-KanMX::loxP*	This study
IMK317	CEN.PK113-7D *dak1::laxP-hphNT1::laxP dak2::loxP-KanMX::loxP*	This study
IMK319	CEN.PK113-7D *gal2::loxP-KanMX::loxP*	This study
IMK355	BY4741 *bar1::loxP-KanMX::loxP*	Euroscarf

Materials and methods

Strains and maintenance

Stock cultures of the *S. cerevisiae* strains used in this study (Table 1) were grown in shake flasks on 100 ml medium containing 10 g·l^{-1} Bacto yeast extract, 20 g·l^{-1} Bacto peptone and 20 g·l^{-1} D-glucose, maltose or ethanol. After addition of 30% (v/v) glycerol to stationary phase cultures, 1-ml aliquots were stored at -80°C.

Yeast strain construction

S. cerevisiae IMK300 was obtained by replacing the open reading frame of *GAL2* in strain CEN.PK113-7D with the loxP-KanMX-loxP cassette from pUG6 (Güldener *et al.*, 1996) using standard molecular biology techniques (Gietz and Woods, 2001). *S. cerevisiae* IMK317 was constructed with the pUG6 cassette to delete *DAK2*, and the *hphNT1* cassette from pUG-*hphNT1* (Zelle *et al.*, 2011) to delete *DAK1*. Deletion cassettes were prepared by PCR using Phusion™ Hot Start Polymerase (Finnzymes, Vantaa, Finland) and the primers specified in Table 2. Cultures transformed with deletion cassettes were plated on medium containing 10 g·l^{-1} Bacto yeast extract, 20 g·l^{-1} Bacto peptone, 20 g·l^{-1} Bacto agar and 20 g·l^{-1} D-glucose with G418 (200 mg·l^{-1}) and/or hygromycin B (200 mg·l^{-1}). Deletions were confirmed by diagnostic PCR .

Plate assay for galacturonic acid tolerance

The solid medium used for plate assays consisted of Yeast Nitrogen Base (YNB, 0.67% (w/v), Sigma-Aldrich) medium supplemented with agar (1.5% (w/v); Sigma-Aldrich) and ethanol (20 g·l^{-1}; Sigma-Aldrich). Uracil, L-leucine, L-tryptophan and L-histidine were added at the concentrations specified by Pronk (2002). The concentrations of D-galacturonic acid (>98%; Sigma-Aldrich) were 0, 10 and 20 g·l^{-1}. To neutralize the added D-galacturonic acid and to reach a final pH of 4.0, sterile

Table 2. Oligonucleotides used in this study

Oligo-nucleotide	Sequence
DAK1 KO FW	GAACACATCAAAGAATAAGATTACATTCTATATCTAAGACT CAGCTGAAGCTTCGTACGC
DAK1 KO RV	ATATATCATAAGTATCTTGGTATGTATTCGTGAGCCAATTA CTTAGCATAGGCCACTAGTGGATCTG
DAK2 KO FW	TCAAAATCTGACAAAACCCAACTACAATTGACTAAATAATC CAGCTGAAGCTTCGTACGC
DAK2 KO RV	CTTTAAAGCTGTTATGTTTGGCTTCTAGTGTGTACGAGCAA TTGCATAGGCCACTAGTGGATCTG

amounts of KOH were added after autoclaving the medium at 120°C. Stock solutions of D-galacturonic acid and auxotrophic requirements were filter-sterilized and added together with ethanol after autoclaving and cooling of the medium to ca. 50°C. Strains were pre-grown overnight on 10 $g \cdot l^{-1}$ yeast extract, 20 $g \cdot l^{-1}$ peptone and 20 $g \cdot l^{-1}$ ethanol (initial pH 6.0). Stationary-phase cells were washed, resuspended and diluted in synthetic medium without carbon source to optical densities (measured at 660 nm) of 1, 0.1, 0.01 and 0.001. Of these dilutions, 15 µl was spotted on the plates, which were incubated for 4 days at 30°C.

Media and cultivation

Shake flask cultures were grown at 30°C in an orbital shaker (200 rpm) in synthetic medium (Verduyn *et al.*, 1990), set to pH 6.0 with 2M KOH prior to sterilization. Precultures were grown in 500-ml shake flasks containing 100 ml medium with 20 $g \cdot l^{-1}$ D-glucose as carbon and energy source, inoculated with a 1 ml glycerol stock. Chemostat cultivation was carried out at 30°C and at a dilution rate of 0.10 h^{-1} in 2-litre bioreactors (Applikon, Schiedam, the Netherlands) with a working volume of 1.0 litre and equipped with an electronic level sensor to maintain a constant volume. For aerobic cultures, synthetic medium (Verduyn *et al.*, 1990) with 7.5 $g \cdot l^{-1}$ D-glucose was used. Dissolved oxygen concentrations in the cultures were monitored with an autoclavable oxygen electrode (Applisens, Schiedam, the Netherlands). Anaerobic cultures were fed with synthetic medium supplemented with 25 $g \cdot l^{-1}$ D-glucose and the anaerobic growth factors ergosterol (0.01 $g \cdot l^{-1}$) and Tween 80 (0.42 $g \cdot l^{-1}$). Silicone antifoam was used to prevent foaming. To minimize oxygen diffusion, bioreactors were equipped with Norprene tubing (Cole-Parmer Instrument Company, Vernon Hills, IL) and Viton O-rings and the medium vessel was sparged with N_2. D-Galacturonic acid (Carbosynth, Compton (Berkshire), UK) fed to cultures was filter-sterilised separately and added to the autoclaved medium. The pH of the cultures was kept at 3.5 by the automatic addition of 2M KOH. Cultures were stirred at 800 rpm and sparged with 0.5 $l \cdot min^{-1}$ air or N_2 (<10 ppm oxygen). Chemostat cultures were assumed to be in steady state when, after at least 5 volume changes, the culture dry weight and specific carbon dioxide production rate changed by less than 3% over 2 consecutive volume changes. Steady-state samples were taken between 10 and 14 volume changes after inoculation to minimize evolutionary adaptation. Aerobic glucose-limited chemostat cultures at pH 3.5, supplemented with 0.23 $g \cdot l^{-1}$ D-galactose were run similarly.

Analytical methods

Culture dry weight was measured according to Postma *et al.* (1989). Supernatants were obtained by centrifugation of culture samples and analysed by high-performance liquid chromatograph (HPLC) analysis on a Waters Alliance 2690

HPLC (Waters, Milford, MA) containing a Bio-Rad HPX 87H column (Bio-Rad, Hercules, CA). The column was eluted at 60°C with 0.5 $g \cdot l^{-1}$ H_2SO_4 at a flow rate of 0.6 $ml \cdot min^{-1}$. Detection was by means of a Waters 2410 refractive-index detector and a Waters 2487 UV detector. When required, the galactose concentration was determined with the low-detection level EnzyPlus Lactose/Galactose determination kit (Biocontrol, Bellevue, WA). Spiking of known amounts of galactose was used to confirm that galacturonic acid did not interfere with the assay.

Gas analysis

Exhaust gas was cooled in a condenser (2°C) and dried with a Permapure type MD-110-48P-4 dryer (Permapure, Toms River, NJ). Oxygen and carbon dioxide concentrations were determined with an NGA 2000 analyser (Rosemount Analytical, Orrville, OH). Exhaust gas flow rate and carbon dioxide production rates were determined as described previously (Abbott *et al.*, 2009). In calculating these biomass-specific rates, a correction was made for volume changes caused by sampling.

Micro-arrays

Microarray analyses were performed on steady-state chemostat cultures. Sampling for transcriptome analysis was performed as previously described, using liquid nitrogen for rapid quenching of metabolism (Piper *et al.*, 2002). Samples were stored at -80°C in a mixture of phenol/chloroform and TEA buffer until further processing for total RNA extraction. Total RNA was extracted as previously described in De Nicola *et al.* (2007). Processing of total RNA was performed according to Affymetrix instructions. RNA target preparation for microarray expression analysis was carried out according to the GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA) using 200 ng of total RNA. The protocol was carried out with minor modifications, i.e., the Affymetrix polyA RNA controls were excluded from the aRNA amplification protocol and the IVT reactions were incubated for 16 h at 40°C. The quality of total RNA, cDNA, aRNA, and fragmented aRNA was checked using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Hybridization, washing and scanning of microarrays were performed following Affymetrix instructions. Results for each growth condition were derived from independent replicate cultures. Processing of expression data (normalization, expression cut-off, etc.) was performed according to Boer *et al.* (2003). Differentially expressed genes were identified using the Excel plug-in of the SAM software (Significance analysis of Microarrays (Storey and Tibshirani, 2003)) with a false positive discovery rate of 1%. The complete dataset is available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo)

under the accession number GSE43474. Hypergeometric distribution analysis to identify overrepresented MIPS categories (Ruepp *et al.*, 2004) was performed as previously described in Knijnenburg *et al.* (2007). Replicate probe sets for the same gene were removed to avoid falsely overrepresented categories.

Viability assays

Precultures were grown on synthetic medium (Verduyn *et al.*, 1990) (initial pH 6.0) containing 20 $g \cdot l^{-1}$ D-glucose or 20 $g \cdot l^{-1}$ maltose, harvested in the exponential growth phase, washed in synthetic medium (pH 6.0), and added to a 500 ml shake flask containing 100 ml starvation medium at a concentration of $1 \cdot 10^7$ $cells \cdot ml^{-1}$. Starvation medium consisted of synthetic medium without carbon source, set at pH 3.0 or pH 5.0 by the addition of 2M KOH or 2M H_2SO_4 prior to sterilization. Starvation medium containing 10 $g \cdot l^{-1}$ D-galacturonic acid was filter-sterilized. Cells were incubated at 30°C in an orbital shaker (200 rpm). Samples were centrifuged, and cells were resuspended in an equal volume of Isoton II® diluent (Beckman Coulter, Woerden, the Netherlands) to a concentration of $1 \cdot 10^7$ $cells \cdot ml^{-1}$. Viability measurement was performed using 1 ml of a suspension with $1 \cdot 10^7$ cells and a final concentration of 20 $nmol \cdot l^{-1}$ propidium iodide. 3000 Cells were counted on a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter, Woerden, the Netherlands), essentially as described by Boender *et al.* (2011). Viability was determined as the percentage of propidium iodide negative cells (PI^-), normalised to the average measured at the start of the experiment.

α-Factor halo assay

The halo assay, based on an α-factor-sensitive *S. cerevisiae* tester strain, was performed essentially as described by Sprague (1991). A *MATa bar1*$^-$ strain (IMK355) and its ancestor strain BY4741 were obtained from Euroscarf (Frankfurt, Germany). Plates containing 10 $g \cdot l^{-1}$ Bacto yeast extract, 20 $g \cdot l^{-1}$ Bacto peptone with 20 $g \cdot l^{-1}$ D-glucose and 20 $g \cdot l^{-1}$ Bacto agar, with or without 10 $g \cdot l^{-1}$ D-galacturonic acid, were covered with a lawn of either IMK355 or BY4741. Cell counts of overnight cultures, grown on medium containing 10 $g \cdot l^{-1}$ Bacto yeast extract, 20 $g \cdot l^{-1}$ Bacto peptone with 20 $g \cdot l^{-1}$ D-glucose, of strains CEN.PK113-7D and CEN.PK113-1A were determined with a Z2 Coulter Counter (Beckman Coulter, Woerden, the Netherlands) and spots were made containing various cell concentrations on the plates. Plates were incubated for 48 h at 30°C before the result was scored.

Results

Galacturonic acid reduces biomass yields in glucose-limited chemostat cultures

To investigate physiological responses of *S. cerevisiae* to galacturonic acid, glucose-limited chemostat cultures were grown at a dilution rate of 0.10 h^{-1} and at pH 3.5, in the presence and absence of 10 $g \cdot l^{-1}$ D-galacturonic acid. Under anaerobic conditions, galacturonic acid caused a ca. 10% decrease of the biomass yield on glucose and a corresponding increase of the specific rate of glucose consumption and of the ethanol and carbon dioxide production rates (Table 3). In aerobic cultures, addition of 10 $g \cdot l^{-1}$ D-galacturonic acid caused an even stronger decrease of the biomass yield on glucose, from 0.49 to 0.37 g biomass·(g glucose)$^{-1}$, corresponding to a 25% reduction. The biomass yield decrease in aerobic cultures was accompanied by increased specific rates of oxygen consumption (q_{O2}) and carbon dioxide production (q_{CO2}) (Table 3). No production of ethanol or other fermentation products was observed in these cultures. Aerobic, glucose-limited chemostat cultures grown at higher galacturonic acid concentrations (15 and 20 $g \cdot l^{-1}$) became respiro-fermentative. At these high galacturonic acid concentrations, biomass concentrations varied over time and between replicate cultures, thus precluding quantitative analysis.

Table 3. Biomass yields on D-glucose (g biomass·g glucose^{-1}) and metabolic fluxes in aerobic and anaerobic, glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D, grown on synthetic medium with or without 10 $g \cdot l^{-1}$ D-galacturonic acid and run at a dilution rate of 0.10 h^{-1} at pH 3.5.

		Galacturonic acid ($g \cdot l^{-1}$)	
		0	**10**
Anaerobic	Y_{SX} ($g \cdot g^{-1}$)	0.09 ± 0.00	0.08
	$q_{glucose}$(mmol·g dry weight$^{-1} \cdot h^{-1}$)	6.12 ± 0.14	6.79
	$q_{ethanol}$ (mmol·g dry weight$^{-1} \cdot h^{-1}$)	10.32 ± 0.35	11.03
	q_{CO2} (mmol·g dry weight$^{-1} \cdot h^{-1}$)	10.88 ± 0.37	11.63
	q_{O2} (mmol·g dry weight$^{-1} \cdot h^{-1}$)	-	-
Aerobic	Y_{SX} ($g \cdot g^{-1}$)	0.49 ± 0.02	0.37 ± 0.07
	$q_{glucose}$(mmol·g dry weight$^{-1} \cdot h^{-1}$)	1.19 ± 0.09	1.54 ± 0.24
	$q_{ethanol}$ (mmol·g dry weight$^{-1} \cdot h^{-1}$)	-	-
	q_{CO2} (mmol·g dry weight$^{-1} \cdot h^{-1}$)	3.00 ± 0.16	4.83 ± 1.02
	q_{O2} (mmol·g dry weight$^{-1} \cdot h^{-1}$)	2.92 ± 0.15	4.53 ± 0.90

Transcriptional responses to galacturonic acid

To further investigate the impact of galacturonic acid on *S. cerevisiae*, a DNA microarray-based transcriptome analysis was performed on aerobic, glucose-limited chemostat cultures grown in the presence and absence of 10 $g \cdot l^{-1}$ D-galacturonic acid. The average coefficients of variation for transcript levels in independent replicate cultures were below 23%, which is typical for chemostat-based transcriptome studies in *S. cerevisiae* (Piper *et al.*, 2002). With a fold-change threshold of 2 and a false-discovery rate of 1%, 218 genes (3.4% of the genome) showed a significantly changed transcript level in the presence of galacturonic acid. Of these genes, 127 showed higher transcript levels in the presence of galacturonic acid, while transcript levels of 91 genes were lower than in the reference cultures.

A first inspection showed that *GAL2*, *GAL7*, *GAL10* and *GAL1*, which are all involved in galactose metabolism, were strongly up-regulated in the presence of galacturonic acid (Table 4). Subsequent analysis indicated that the galacturonic acid used in the chemostat cultures contained 2.3% (w/w) galactose. To check whether this contamination could explain the up-regulation of the *GAL* regulon in galacturonic acid-fed cultures, glucose-limited chemostat cultures were grown with the addition of 0.23 $g \cdot l^{-1}$ D-galactose. Microarray analysis of cultures showed that, indeed, this low concentration of D-galactose strongly induced transcription of *GAL* genes (Table 4). Our data therefore do not support the conclusion that D-galacturonic acid induces the *GAL* regulon. Discarding galactose-responsive genes from the transcriptome analysis reduced the number of genes that were transcriptionally up- and down-regulated in the presence of galacturonic acid to 119 and 77, respectively.

Genes belonging to the MIPS category pheromone response, mating-type determination and sex-specific proteins (34.11.03.07) were strongly overrepresented (p-value $6.4 \cdot 10^{-8}$) among the set of galacturonic acid up-regulated genes. *STE2* and *STE3,* which encode G-protein coupled receptors for α- and a-factor, respectively (Burkholder and Hartwell, 1985; Hagen *et al.*, 1986) were both included in this set of genes, as well as *FAR1* and *CDC42*, which encode elements of the downstream signalling pathway (Chang and Herskowitz, 1990; Peter *et al.*, 1993; Simon *et al.*, 1995; Valtz *et al.*, 1995). Moreover, this gene set showed a clear overrepresentation (p-value $1.2 \cdot 10^{-10}$) of targets of Ste12p, a transcriptional activator located at the end of the MAP kinase cascade that binds to the pheromone response element (Dolan *et al.*, 1989; Kronstad *et al.*, 1987). Up-regulated Ste12p targets included genes encoding positive regulators of the mating pathway (*FAR1*, *STE2*), negative regulators involved in desensitizing to and recovery from pheromone signalling (*BAR1*, *SST2*), genes involved in cell fusion (*FUS1*, *FIG1*, *FIG2*), as well

Table 4. Transcript levels of GAL regulon genes, determined by microarray analysis in duplicate aerobic glucose-limited chemostat cultures at a dilution rate of 0.10 h^{-1}, to which either 10 $g \cdot l^{-1}$ D-galacturonic acid or 0.23 $g \cdot l^{-1}$ D-galactose was added. Data are presented as the average ± deviation of duplicate or triplicate (reference) measurements.

Gene Name	Reference	+ 10 $g \cdot l^{-1}$ galacturonate	+ 0.23 $g \cdot l^{-1}$ galactose	Description
Galactose metabolism				
GAL1	12 ± 0	89 ± 22	167 ± 17	Galactokinase
GAL2	12 ± 0	282 ± 25	1098 ± 40	Galactose permease
GAL7	12 ± 0	111 ± 18	314 ± 38	Galactose-1-phosphate uridyl transferase
GAL10	12 ± 0	121 ± 23	605 ± 290	UDP-glucose-4-epimerase
PGM2	659 ± 187	259 ± 20	389 ± 26	Phosphoglucomutase
Regulation of galactose metabolism				
GAL3	30 ± 13	68 ± 7	46 ± 1	Transcriptional regulator
GAL4	39 ± 11	46 ± 1	26 ± 11	Transcription factor
GAL80	50 ± 13	93 ± 24	149 ± 81	Transcriptional regulator
Average CV[1]	14%	16%	23%	

[1]Average coefficient of variation, calculated only over these genes

as α-factor (*MFalpha1*) and a-factor (*MFA1* and *MFA2*). The 27-fold up-regulation of *MFalpha1* in cultures of the heterothallic *MATa* CEN.PK113-7D strain used in this study was intriguing. To test whether this up-regulation of *MFalpha1* resulted in production of α-factor by this *MATa* strain, a *MATa bar1-* indicator strain hypersensitive to α-factor (Sprague, 1991) was used. On YPD plates supplemented with 10 $g \cdot l^{-1}$ D-galacturonic acid, spots of a MATα strain caused halos in a lawn of the *bar1-* tester strain. The diameter of these halos was not significantly different from those on YPD plates without galacturonic acid (data not shown). However, no halos were observed around spots of CEN.PK113-7D (*MAT*a), neither in the absence nor in the presence of galacturonic acid. The strong transcriptional up-regulation of *MFalpha1* by galacturonic acid did therefore not result in a significant net production of α-factor.

DAK2, which encodes a dihydroxyacetone kinase involved in dihydroxyacetone detoxification (Molin *et al.*, 2003), was among the most strongly up-regulated genes (42 fold) in chemostat cultures grown in the presence of galacturonic acid. The effect of galacturonic acid on strains carrying *dak2*Δ (IMK300) and *dak1*Δ *dak2*Δ (IMK317) mutations and on the reference strain CEN.PK113-7D was therefore tested in shake flasks grown on synthetic medium supplemented with either 20 $g \cdot l^{-1}$ D-galactose, 20 $g \cdot l^{-1}$ D-glucose or 10 $g \cdot l^{-1}$ D-galactose + 10 $g \cdot l^{-1}$ D-glucose, each in the presence and absence of 10 $g \cdot l^{-1}$ D-galacturonic acid. However, no differences in specific growth rate or final

biomass concentration were observed between these three strains (data not shown), indicating that, under the experimental conditions, Dak2p and Dak1p did not contribute to galacturonic acid tolerance.

In a chemostat-based transcriptome study on the effects of acetic, benzoic, propionic and sorbic acid on *S. cerevisiae*, Abbott *et al.* (2007) showed that transcriptional responses to these organic acids were different but overlapping and correlated to their hydrophobicity. Hypergeometric distribution analysis of the genes whose transcript levels were higher in the presence of galacturonic acid revealed a significant overrepresentation (Bonferroni-corrected p-value < 0.05) of genes that were previously shown to be upregulated in the presence of acetate, propionate and sorbate (p-values $2 \cdot 10^{-5}$, $9 \cdot 10^{-4}$ and $3 \cdot 10^{-4}$, respectively). In the set of 77 genes that were down regulated in the presence of galacturonic acid, an overrepresentation was found of genes whose transcription was also down regulated in the presence of acetic acid (p-value $4.6 \cdot 10^{-7}$) and sorbate (p-value $6.6 \cdot 10^{-4}$).

Galacturonic acid toxicity in starving cell suspensions is alleviated upon deletion of hexose-transporter genes

The overlapping transcriptional responses to galacturonic acid and other organic acids suggested that weak-acid uncoupling of the plasma membrane pH gradient is an important mechanism of galacturonic acid inhibition. The impact of weak-acid uncoupling is expected to be strongest when cells are energetically compromised,

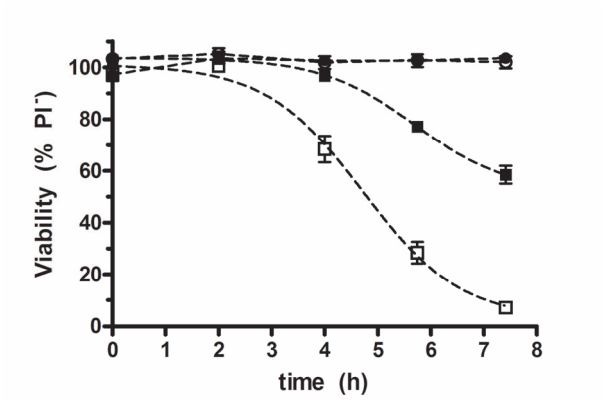

Figure 1. Viability of *S. cerevisiae* CEN.PK113-7D under starvation conditions (no carbon and energy source) in synthetic medium at pH 5 (●), pH 5 supplemented with 10 $g \cdot l^{-1}$ D-galacturonic acid (○), pH 3 (■) and pH 3 with 10 $g \cdot l^{-1}$ D-galacturonic acid (□). Viability (determined at least in triplicate cultures) is shown as the percentage of propidium iodide negative cells (PI^-), relative to the average measured at the start of the experiment.

thus precluding efficient proton pumping by the plasma membrane H^+-ATPase Pma1p (Abbott *et al.*, 2007; Larsson *et al.*, 2000; Piper *et al.*, 2001; Piper, 2011)). The impact of galacturonic acid was therefore further analysed in energy-starved cell suspensions. Exponentially growing cultures of *S. cerevisiae* CEN.PK113-7D were harvested and incubated in the presence and absence of galacturonic acid, at pH 5 and at pH 3. Viability was monitored by flow cytometry of cell suspensions stained with propidium iodide, which selectively enters cells with compromised cell membranes, and fluoresces red when intercalating with DNA. Cells incubated at pH 5 retained viability throughout these experiments, both in the presence or

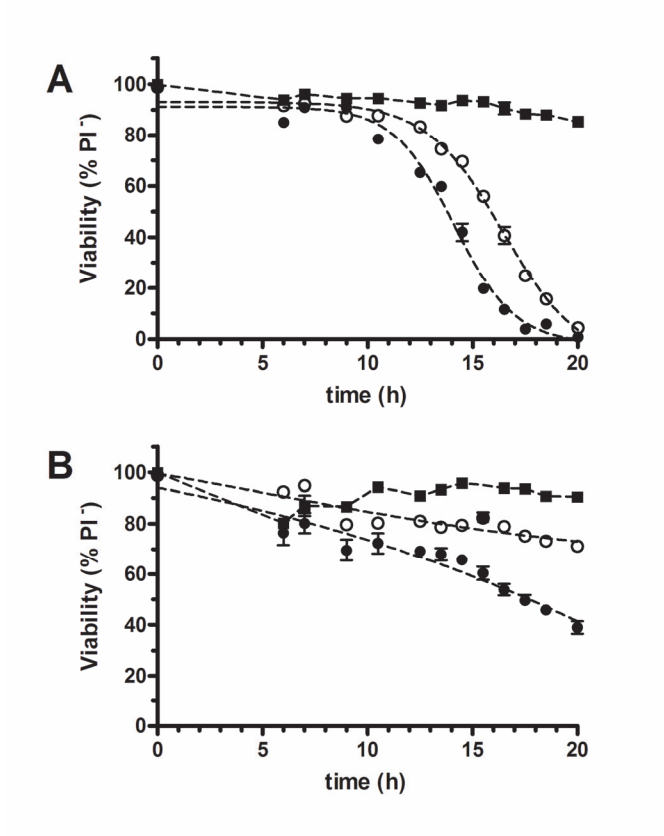

Figure 2. Viability of *S. cerevisiae* strains CEN.PK113-7D (●), IMK319 (*gal2*⊿) (○), and the hexose transporter-negative strain EBY.VW4000 (■) under starvation conditions (no carbon and energy source) incubated in synthetic medium at pH 3 in the presence of 10 $g \cdot l^{-1}$ D-galacturonic acid (panel A) and in the absence of D-galacturonic acid (panel B). Viability was measured from duplicate experiments as the percentage of propidium iodide negative cells (PI^-), relative to the average measured at the start of the experiment

absence of galacturonic acid (Figure 1), indicating that dissociated galacturonic acid did not affect viability. In the absence of galacturonic acid, starvation at pH 3 already led to a detectable loss of viability after 4 h of incubation, reaching 59% viability after 7.4 h. In the presence of 10 g·l^{-1} D-galacturonic acid, which at pH 3 is 76% undissociated, culture viability decreased almost from the start of the starvation experiment and reached 7% after 7.4 h (Figure 1).

Galacturonic acid is structurally related to the hexose-sugar galactose, and D-galacturonic acid was recently reported to inhibit galactose fermentation (Huisjes *et al.*, 2012a). To study whether galacturonic acid enters the cells via the galactose transporter Gal2p or one of the other hexose transporters, viability assays were performed under starvation conditions at pH 3 with the reference strain *S. cerevisiae* CEN.PK2-1C, strain EBY.VW4000, in which all hexose transporters have been deleted (Wieczorke *et al.*, 1999), and strain IMK319, which only lacks *GAL2*. Since EBY.VW4000 is unable to grow on glucose or galactose, all three strains were pre-grown on maltose. The presence of 10 g·l^{-1} D-galacturonic acid (Figure 2A) resulted in a much more pronounced decrease in the viability of reference strain CEN.PK113-7D and the *gal2*Δ strain IMK319 compared to incubations of these strains in the absence of galacturonic acid (Figure 2B). In contrast, the hexose-

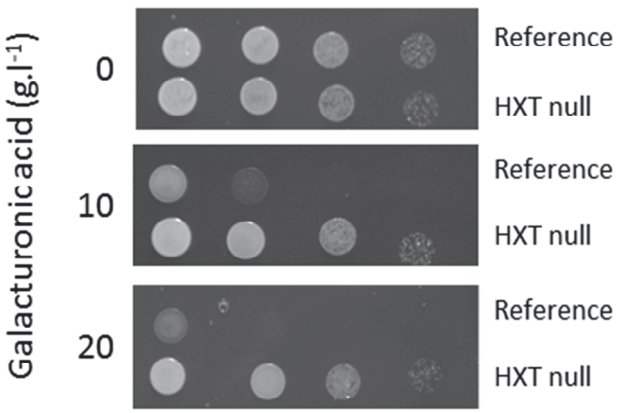

Figure 3. Involvement of HXT transporters in growth inhibition by D-galacturonic acid. *S. cerevisiae* CEN.PK2-1C (reference strain) and EBY.VW4000 (*hxt13*Δ*::loxP hxt15*Δ*::loxP hxt16*Δ*::loxP hxt14*Δ*::loxP hxt12*Δ*::loxP hxt9*Δ*::loxP hxt11*Δ*::loxP hxt10*Δ*::loxP hxt8*Δ*::loxP hxt514*Δ*::loxP hxt2*Δ*::loxP hxt367*Δ*::loxP gal2*Δ *stl1*Δ*::loxP agt1*Δ*::loxP ydl247w*Δ*::loxP yjr160c*Δ*::loxP*; (Wieczorke *et al.*, 1999) were grown on YNB-ethanol agar plates (pH 4) containing 0, 10 or 20 g·l^{-1} D-galacturonic acid. Spots were made with 15 µl aliquots of cell suspensions with, from left to right, optical densities at 660 nm of 1, 0.1, 0.01 and 0.001.

transporter-negative strain EBY.VW4000 showed identical viability loss curves in the presence and absence of galacturonic acid, indicating that absence of all hexose transporters renders *S. cerevisiae* more resistant to galacturonic acid.

Growth inhibition by galacturonic acid is relieved in a hexose-transporter-negative strain

To test whether absence of hexose transporters also increased tolerance of *S. cerevisiae* to galacturonic acid in growing cultures, the hexose-transporter-negative strain EBY.VW4000 (Wieczorke *et al.*, 1999) and the isogenic reference strain CEN.PK2-1C were plated on ethanol medium with D-galacturonic acid concentrations ranging from 0-20 $g \cdot l^{-1}$ (Figure 3). Ethanol was used in these experiments since glucose does not support growth of HXT null strains (Wieczorke *et al.*, 1999). In line with the decreased viability under starvation conditions, galacturonic acid strongly inhibited growth of the reference strain CEN.PK2-1C on ethanol at a concentration of 10 $g \cdot l^{-1}$. In contrast, growth of the hexose-transporter-negative strain was not inhibited at this concentration of galacturonic acid (Figure 3). These results support the hypothesis that hexose transporters are involved in galacturonic acid uptake by *S. cerevisiae*.

Discussion

The negative impact of galacturonic acid on biomass yields in glucose-limited chemostat cultures and its pH-dependent stimulation of viability loss in starving cell suspensions are consistent with the hypothesis that galacturonic acid primarily affects the physiology of *S. cerevisiae* via 'classical' weak acid uncoupling. This mechanism encompasses an increased ATP-expenditure for proton expulsion, required to counter acidification of the cytosol caused by weak organic acid influx (Abbott *et al.*, 2007; Larsson *et al.*, 2000; Piper *et al.*, 2001; Piper, 2011). Its involvement in galacturonic acid inhibition was further supported by the chemostat-based transcriptome analysis, in which a set of galacturonic-acid-responsive genes was found to show a strong overrepresentation of genes previously found to be transcriptionally responsive to other organic acids. Two other strong transcriptional responses to galacturonic acid, an activation of the mating factor pathway and a strong up-regulation of the *DAK2* gene, could not be functionally linked to galacturonic acid tolerance or sensitivity.

Weak-acid uncoupling as the main mechanism of galacturonic acid inhibition is also consistent with the previous observation that growth and alcoholic fermentation of D-galactose, D-xylose and L-arabinose by *S. cerevisiae* are much more

strongly affected by galacturonic acid than D-glucose fermentation (Huisjes *et al.*, 2012a). Faster fermentation of glucose enables a faster generation of ATP, which is crucial for countering the dissipation of the plasma membrane pH gradient by the plasma membrane H^+-ATPase. A similar difference in sensitivity of xylose and glucose fermentation by *S. cerevisiae* was previously demonstrated for acetic acid (Bellissimi *et al.*, 2009; Casey *et al.*, 2010).

In a chemostat-based study on responses of *S. cerevisiae* to benzoic, sorbic, acetic and propionic acid, Abbott *et al.* (2007) demonstrated a clear correlation between the hydrophobicity of these acids and their uncoupling effect, expressed as the concentration that reduced the biomass yield in anaerobic, glucose-limited cultures by 50%. Our results show that this correlation does not hold for galacturonic acid which, with an octanol/water partition coefficient of -2.5 (calculated with EPI Suite, (EPA, 2011)), is quite hydrophilic. Nevertheless, at a relatively low concentration of 26 mM undissociated acid, it decreased the biomass yield in aerobic, glucose-limited cultures by 25%. This strongly suggested involvement of (a) transport protein(s) in the translocation of galacturonic acid across the yeast plasma membrane. Based on a kinetic study on the uptake of radiolabelled substrate, Souffriau *et al.* (2012) recently concluded that, indeed, *S. cerevisiae* harbours a constitutively expressed, low-affinity transporter for undissociated galacturonic acid. While the observed transport kinetics supported this hypothesis, calculated intracellular galacturonic acid concentrations did, even at low extracellular pH, not exceed extracellular concentrations (Souffriau *et al.*, 2012). This observation is not consistent with facilitated diffusion of undissociated galacturonic acid which, in the absence of an energy driven export system, would be expected to cause intracellular accumulation of galacturonate.

The galacturonate-induced loss of viability in energy-starved *S. cerevisiae* cultures and the growth inhibition by galacturonic acid on ethanol medium observed in our study were both alleviated in a hexose-transporter-negative mutant. This indicates that galacturonate transport is mediated by one or more members of the HXT family of hexose transporters. Several hexose transporters in *S. cerevisiae* are able to transport sugars that cannot be metabolized by wild type *S. cerevisiae* strains, such as arabinose by Gal2p (Becker and Boles, 2003; Kou *et al.*, 1970; Wisselink *et al.*, 2010; Young *et al.*, 2011) or xylose by Hxt4p, Hxt5p, Hxt7p and Gal2p (Hamacher *et al.*, 2002; Saloheimo *et al.*, 2007; Sedlak and Ho, 2004; Young *et al.*, 2011). Involvement of hexose transporters in galacturonic acid transport is in line with the induction of genes resembling *S. cerevisiae* HXT transporters during growth of filamentous fungi on galacturonic acid (Martens-Uzunova and Schaap, 2008). Involvement of specific Hxt transporters in galacturonic acid transport, combined with the differential regulation of the members of the HXT gene family (Diderich *et*

al., 1999), might explain the different degrees of uncoupling caused by galacturonic acid in aerobic and anaerobic chemostat cultures grown under otherwise identical conditions. Based on analysis of radiolabelled galacturonic acid uptake in a hexose-transporter negative strain, Souffriau *et al.* (2012) concluded that hexose transporters are not responsible for galacturonic acid uptake by *S. cerevisiae*. Resolving this apparent discrepancy will require systematic analysis of galacturonic acid inhibition and transport in engineered yeast strains expressing individual hexose transporters.

Identification of the transporter(s) involved in galacturonic acid uptake should ultimately enable the rational design and construction of strains with increased galacturonic acid tolerance. Alternatively, it may be possible to increase galacturonic acid tolerance by evolutionary engineering, which has previously been used to select for tolerance to acetic acid (Wright *et al.*, 2011). Along with the design and construction of galacturonic acid fermenting *S. cerevisiae* strains (Huisjes *et al.*, 2012b; Van Maris *et al.*, 2006), development of galacturonic acid tolerant strains remains a key challenge in development of efficient yeast-based process for bioethanol production from pectin-rich feedstocks.

Acknowledgements

The PhD research of EHH was financed by the Dutch Ministry of Economic Affairs via an EOS-LT grant (http://www.agentschapnl.nl/programmas-regelingen/energie-onderzoek-subsidie-eos). The Kluyver Centre for Genomics of Industrial Fermentation is supported by the Netherlands Genomics Initiative. We thank Erik de Hulster and Marinka Almering for experimental assistance, Eckhard Boles for permission to use the 'HXT null' strain and Rintze Zelle for critical reading of the manuscript.

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Chapter 3

Galacturonic acid inhibits growth of *Saccharomyces cerevisiae* on galactose, xylose and arabinose.

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Applied and Environmental Microbiology 2012, 78:5052-5059.

Abstract

The efficient fermentation of mixed substrates is essential for the microbial conversion of second-generation feedstocks, including pectin-rich waste streams such as citrus peel and sugar beet pulp. Galacturonic acid is a major constituent of hydrolysates of these pectin-rich materials. The yeast *Saccharomyces cerevisiae*, the main producer of bioethanol, cannot use this sugar acid. The impact of galacturonic acid on alcoholic fermentation by *S. cerevisiae* was investigated with anaerobic batch cultures grown on mixtures of glucose and galactose at various galacturonic acid concentrations and on a mixture of glucose, xylose, and arabinose. In cultures grown at pH 5.0, which is well above the pKa value of galacturonic acid (3.51), the addition of 10 $g \cdot l^{-1}$ galacturonic acid did not affect galactose fermentation kinetics and growth. In cultures grown at pH 3.5, the addition of 10 $g \cdot l^{-1}$ galacturonic acid did not significantly affect glucose consumption. However, at this lower pH, galacturonic acid completely inhibited growth on galactose and reduced galactose consumption rates by 87%. Additionally, it was shown that galacturonic acid strongly inhibits the fermentation of xylose and arabinose by the engineered pentose-fermenting *S. cerevisiae* strain IMS0010. The data indicate that inhibition occurs when non-dissociated galacturonic acid is present extracellularly and corroborate the hypothesis that a combination of a decreased substrate uptake rate due to competitive inhibition on Gal2p, an increased energy requirement to maintain cellular homeostasis, and/or an accumulation of galacturonic acid 1-phosphate contributes to the inhibition. The role of galacturonic acid as an inhibitor of sugar fermentation should be considered in the design of yeast fermentation processes based on pectin-rich feedstocks.

Background

The shift of industrial biotechnology from highly refined sugar syrups to more sustainable and cheaper carbon and energy sources, such as lignocellulosic hydrolysates, also represents a shift from single-sugar to mixed-substrate utilization (Jojima *et al.*, 2010; Van Maris *et al.*, 2006). Many traditional applications of the yeast *Saccharomyces cerevisiae* are already based on substrate mixtures, such as mixtures of glucose and fructose in grape must and mixtures of maltose, sucrose, glucose, and fructose in beer wort. Typically, the yeast will first consume its preferred substrate, glucose or fructose, by the glucose repression of genes involved in uptake and the consumption of other substrates. This results in the sequential consumption of multiple substrates, also known as diauxic growth (Monod, 1945).

Lignocellulosic hydrolysates are desirable feedstocks for bioethanol production by *S. cerevisiae*. These hydrolysates contain not only fermentable sugars but also inhibitors and sugars that are nonfermentable by this yeast (Hahn-Hägerdal *et al.*, 2006; Palmqvist and Hahn-Hägerdal, 2000; Van Maris *et al.*, 2006). Examples of feedstocks that contain multiple substrates are sugar beet pulp and citrus peel hydrolysates (Table 1), which are currently used mostly as animal feed. Alternatively, they could be hydrolysed and used as a substrate for fermentation (Doran *et al.*, 2000; Grohmann and Bothast, 1994). In contrast to commonly investigated sources of lignocellulose, such as corn stover, wheat straw, and switch grass, sugar beet pulp and citrus peel contain less lignin (Grohmann and Bothast, 1994) but instead contain a significant amount of pectin (Grohmann and Bothast, 1994; Micard *et al.*, 1996). Pectin is a complex polysaccharide that consists of a backbone of galacturonic acid residues and can have side chains containing various neutral sugars (Mohnen, 2008). In addition, the polymer can be methylesterified and acetylated (Mohnen, 2008).

Hydrolysates of sugar beet pulp and citrus peel consist of predominantly glucose, galactose, arabinose, xylose, and galacturonic acid (Micard *et al.*, 1996) (Table 1). Glucose and galactose are consumed sequentially by wild-type *S. cerevisiae* strains (see, e.g. Klein *et al.*, 1999). Galactose is metabolized via the Leloir pathway (Frey, 1996), which is repressed by glucose and induced by galactose (Gancedo, 1998; Lohr *et al.*, 1995; Mechler, 1997). The inability of some *S. cerevisiae* strains to

Table 1. Composition of the cell-wall fraction of pectin-rich feedstocks[a]

	Weight fraction of feedstock (%)	
Substrate	Sugar beet pulp	Orange peel
Carbohydrate (%)		
Glucose	24.1	23.7
Galactose	4.6	8.2
Mannose	0.9	-
Arabinose	18.2	14.2
Xylose	1.5	<5
Rhamnose	1.6	<2
Galacturonic acid	20.7	26.0
Non-carbohydrate (%)		
Lignin	1.5	3.0
Protein	5.8	6.3
Ash	8.2	4.0

[a] Data adapted from Grohmann and Bothast (1994)

switch from anaerobic glucose-limited growth to galactose consumption illustrates the energetic costs associated with the expression of the Leloir pathway enzymes (Van den Brink *et al.*, 2009). Although *S. cerevisiae* cannot naturally ferment the pentose sugars xylose and arabinose, this limitation has been alleviated through various metabolic engineering strategies (Bera *et al.*, 2010; Kötter *et al.*, 1990; Kötter and Ciriacy, 1993; Kuyper *et al.*, 2003; Wisselink *et al.*, 2007). A major difference between pectin-rich hydrolysates and other lignocellulosic hydrolysates is the high concentration of galacturonic acid (Table 1). Wild-type *S. cerevisiae* cannot ferment the galacturonic acid fraction present in sugar beet pulp hydrolysates, and so far, this has also not been achieved through metabolic engineering. As a consequence, when sugar beet pulp or citrus peel is used as a feedstock for alcoholic fermentation, high concentrations of galacturonic acid will be present in the fermentation broth.

Galacturonic acid is the uronic acid of galactose and has a dissociation constant (pKa) of 3.51 (Kohn and Kovac, 1978). Therefore, at pH values relevant for lignocellulosic fermentation, both the dissociated and undissociated forms of galacturonic acid will be present. Several weak organic acids are known to negatively affect yeast growth and alcoholic fermentation when they are present in their non-dissociated forms (Abbott *et al.*, 2007; Bellissimi *et al.*, 2009; Palmqvist and Hahn-Hägerdal, 2000; Piper *et al.*, 2001). To our knowledge, possible inhibitory effects of galacturonic acid on yeast performance have not been previously investigated. The goal of the present study was to study the impact of galacturonic acid on sugar fermentation by *S. cerevisiae*. To this end, anaerobic batch cultures on mixtures of glucose and galactose were run at various galacturonic acid concentrations, both at an optimal pH (pH 5.0) and at a low pH (pH 3.5). Additionally, the impact of galacturonic acid on the fermentation of a mixture of glucose, xylose, and arabinose in anaerobic cultivations of the engineered pentose-fermenting *S. cerevisiae* strain IMS0010 was investigated (Wisselink *et al.*, 2009).

Materials and Methods

Strains and maintenance

Stock cultures of *S. cerevisiae* laboratory reference strain CEN.PK113-7D were grown in shake flasks in 100 ml medium containing 1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone, and 2% (w/v) glucose. Pentose-fermenting *S. cerevisiae* strain IMS0010 (Wisselink *et al.*, 2009) was cultivated in synthetic medium (Verduyn *et al.*, 1990) containing 2% (w/v) arabinose. After the addition of 30% (vol/vol) glycerol to stationary-phase cultures, 1-ml aliquots were stored at -80°C.

Media and cultivation

Shake flask cultivation in synthetic medium (Verduyn *et al.*, 1990) was performed at 30°C in an orbital shaker (200 rpm). The pH of the medium was set to 6.0 with 2M KOH prior to sterilization. Precultures were prepared by the inoculation of 100 ml medium with a 1-ml glycerol stock. Galactose (2%, w/v) was used as a carbon and energy source for *S. cerevisiae* CEN.PK 113-7D, and 2% (w/v) arabinose was used for *S. cerevisiae* IMS0010, unless mentioned otherwise. Batch cultivation was carried out at 30°C in 2-liter laboratory bioreactors (Applikon, Schiedam, Netherlands) with a working volume of 1 liter. Synthetic medium with either 10 $g \cdot l^{-1}$ glucose and 10 $g \cdot l^{-1}$ galactose or 20 $g \cdot l^{-1}$ glucose, 10 $g \cdot l^{-1}$ arabinose, and 10 $g \cdot l^{-1}$ xylose was supplemented with 0.3 $g \cdot l^{-1}$ silicone antifoam (antifoam C; Sigma-Aldrich, St. Louis, MO) as well as with the anaerobic growth factors ergosterol (0.01 $g \cdot l^{-1}$) and Tween 80 (0.42 $g \cdot l^{-1}$) dissolved in ethanol. If galacturonic acid was added to cultures, this was filter sterilized separately. Cultures with 10 $g \cdot l^{-1}$ glucose and 10 $g \cdot l^{-1}$ glucuronic acid were prepared similarly; glucuronic acid was also sterilized by filter sterilization. The pH of the cultures was kept at 3.5 or 5.0 by the automatic addition of 2M KOH. Cultures were stirred at 800 rpm and sparged with 0.5 $l \cdot min^{-1}$ nitrogen (<10 ppm oxygen). To minimize the diffusion of oxygen, bioreactors were equipped with Norprene tubing (Saint-Gobain Performance Plastics, Courbevoie, France) and Viton O-rings (Eriks, Alkmaar, Netherlands).

Determination of substrates, metabolites, culture dry weights, and rates

The culture dry weight was measured according to methods described previously by Postma *et al.* (1989). Additionally, culture growth was monitored via readings of the optical density at a wavelength of 660 nm (OD_{660}) on a Novaspec II spectrophotometer (GE Life Sciences, Diegem, Belgium). Supernatants were obtained by the centrifugation of culture samples and analysed by high-performance liquid chromatography (HPLC) analysis on a Waters Alliance 2690 HPLC instrument (Waters, Milford, MA) containing a BioRad HPX 87H column (BioRad, Hercules, CA). The column was eluted at 60°C with 0.5 $g \cdot l^{-1}$ H_2SO_4 at a flow rate of 0.6 $ml \cdot min^{-1}$. Detection was performed by means of a Waters 2410 refractive-index detector and a Waters 2487 UV detector. In calculations of the ethanol production rate and yield, a correction was made for the evaporation of ethanol through the off gas, as described previously by Guadalupe Medina *et al.* (2010).

Growth rates and specific rates were based on constant stoichiometry during exponential growth phases. In the galactose consumption phase of the batch fermentations with 10 $g \cdot l^{-1}$ galacturonic acid, growth was absent, and the above-mentioned approach could not be used. Instead, average specific rates were determined during this phase.

Gas analysis

The exhaust gas was cooled in a condenser (2°C) and dried with a Permapure type MD-110-48P-4 dryer (Permapure, Toms River, NJ). Carbon dioxide concentrations were determined with an NGA 2000 analyser (Rosemount Analytical, Orrville, OH). The exhaust gas flow rates and carbon dioxide production rates were determined as described previously (Van Urk *et al.*, 1988). In calculating these biomass-specific rates, a correction was made for volume changes caused by the withdrawal of culture samples.

Culture viability

Viability measurements were performed by using the Fungalight CFDA (5-carboxyfluorescein diacetate) AM (acetoxymethyl ester)-propidium iodide yeast vitality kit (Invitrogen, Carlsbad, CA), by counting 3,000 cells on a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter, Woerden, Netherlands) in duplicate, as described previously by Boender *et al.* (2011).

Enzyme activity assays

Cell extracts for galactokinase activity assays were prepared from exponentially growing shake flask cultures with galactose as the carbon source and analysed for protein content as described previously (Postma *et al.*, 1989). Galactokinase was assayed with freshly prepared cell extracts according to methods described previously (Van den Brink *et al.*, 2009), with the following minor modifications: 13 units of pyruvate kinase and 14.3 units of lactate dehydrogenase (both from Sigma-Aldrich, St. Louis, MO) were added. A 0.5-mol·l^{-1} solution of galacturonic acid in 1M potassium phosphate buffer (pH 7.5) was used to prevent pH changes in the assay mixture and was added to a final concentration of 5 mmol·l^{-1} galacturonic acid.

Measurement of galacturonic acid derivatives

Two independent batch cultures with 10 g·l^{-1} glucose, 10 g·l^{-1} galactose, and 10 g·l^{-1} galacturonic acid were sampled for intracellular metabolite measurements at the point where the CO_2 peak of the galactose consumption phase was just past its maximum. Samples were taken and processed according to methods described previously (Canelas *et al.*, 2008). The concentrations of the metabolites galacturonic acid 1-phosphate and UDP-galacturonic acid were determined by electrospray ionization-liquid chromatography-tandem mass spectrometry (ESI-LC-MS/MS) (Van Dam *et al.*, 2002). Calibration was performed with standard mixes of galacturonic acid 1-phosphate (Sigma-Aldrich, St. Louis, MO) and UDP-

galacturonic acid (CarboSource Services, Athens, GA), and the fragments at m/z 97 and m/z 403, respectively, were used for determinations of concentrations.

Results

Galacturonic acid inhibits galactose consumption in anaerobic fermentations at low pH

As a reference, anaerobic batch cultures on a mixture of glucose and galactose were performed in the absence of galacturonic acid (Figure 1A and B). Under anaerobic conditions, glucose and galactose are fermented to equimolar amounts of ethanol and CO_2. The production of CO_2, which was continuously monitored via the CO_2 concentration in the exhaust gas, is therefore a measure of the rate of fermentation. No significant differences in the fermentation kinetics were observed between cultures grown at pH 5.0 (Figure 1A) and those grown at pH 3.5 (Figure 1B). The first peak in the CO_2 profile, which represents the glucose consumption phase (Figure 1), was completed in 11 h. Subsequently, the induction of the Leloir pathway enabled the complete consumption of galactose in the next 10 h, as indicated by the second peak in the CO_2 profile.

To examine the effects of galacturonic acid on the fermentation of sugar mixtures, anaerobic batch cultures were grown on a mixture of glucose and galactose (10 $g \cdot l^{-1}$ each) with galacturonic acid concentrations up to 10 $g \cdot l^{-1}$. At pH 5.0, where 97% of the galacturonic acid is present as the anion, the addition of either 5 or 10 $g \cdot l^{-1}$ galacturonate did not influence the glucose consumption phase and had only a minor influence on the CO_2 profiles during the galactose consumption phase (Figure 1A).

At pH 3.5, half of the galacturonic acid (pKa = 3.51) will be present as the undissociated acid, and half will be present as the anion. Therefore, comparisons of data from growth experiments at this pH with data from experiments performed at pH 5 enable a differentiation between the effects of these two species. The addition of galacturonic acid at concentrations of 2.5, 5.0, 7.5, or 10 $g \cdot l^{-1}$ did not affect the glucose consumption phase at pH 3.5, as indicated by the nearly identical first peaks in the CO_2 profile (Figure 1B). However, in sharp contrast to the experiments at pH 5.0, a large impact of the addition of galacturonic acid on the galactose consumption phase at pH 3.5 was observed. Already, at a concentration of 2.5 $g \cdot l^{-1}$, galacturonic acid increased the duration of the galactose consumption phase by 27% (from 11 h to 14 h). At 5.0 $g \cdot l^{-1}$ galacturonic acid, the length of the galactose consumption phase was almost doubled in comparison to that of the reference culture. An even stronger inhibition of galactose fermentation was observed for cultures grown with

7.5 $g \cdot l^{-1}$ and 10 $g \cdot l^{-1}$ galacturonic acid, in which the galactose consumption phases lasted 58 h and 81 h, respectively. In the reference cultures, the rate of CO_2 production during the galactose phase rapidly dropped after reaching its maximum value. In cultures grown at pH 3.5 in the presence of galacturonic acid, this decrease of the CO_2 production rates was much more gradual. Such a decreasing fermentation rate with decreasing galactose fermentation rates suggests that galacturonic acid causes a decreased affinity of the yeast cells for galactose.

When, after sugar depletion, the galacturonic acid concentrations in the anaerobic batch cultures were measured, no significant consumption was observed for either cultures grown at pH 5.0 or cultures grown at pH 3.5. This is consistent with the previously reported inability of *S. cerevisiae* to grow on galacturonic acid (Barnett *et al.*, 1990).

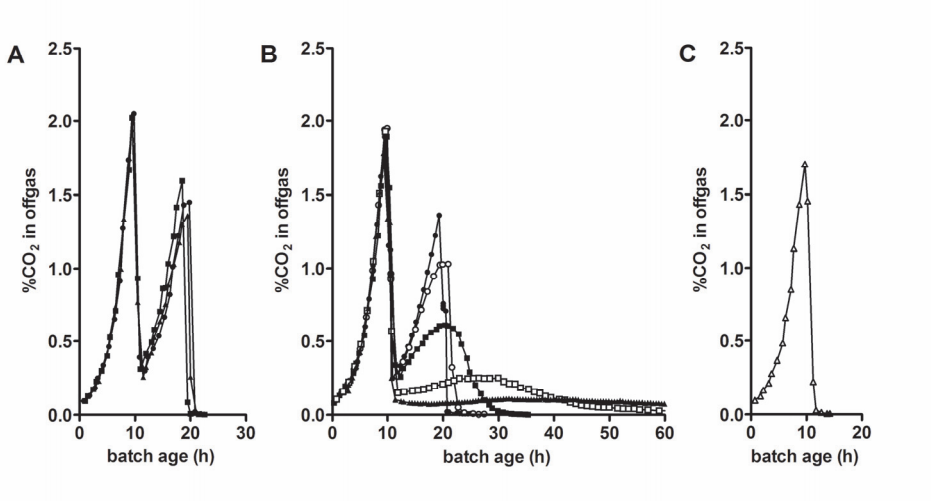

Figure 1. Impact of galacturonic acid on performance of *S. cerevisiae* CEN.PK113-7D during growth on glucose-galactose mixtures in batch fermentations. The result of one representative batch experiment is shown for each condition. Replicate experiments yielded essentially the same results. Fermentation performance is indicated by the CO_2 (percent) in the exhaust gas (offgas) of anaerobic batch cultures of *S. cerevisiae* CEN.PK113-7D, which were flushed with nitrogen gas at a constant rate of 0.5 $l \cdot l^{-1} \cdot h^{-1}$. (A) Cultures grown at pH 5.0 on a mixture of 10 $g \cdot l^{-1}$ glucose and 10 $g \cdot l^{-1}$ galactose with either 0 $g \cdot l^{-1}$ (●), 5 $g \cdot l^{-1}$ (■), or 10 $g \cdot l^{-1}$ (▲) galacturonic acid. (B) Cultures grown at pH 3.5 on a mixture of 10 $g \cdot l^{-1}$ glucose, 10 $g \cdot l^{-1}$ galactose, and either 0 $g \cdot l^{-1}$ (●), 2.5 $g \cdot l^{-1}$ (○), 5 $g \cdot l^{-1}$ (■), 7.5 $g \cdot l^{-1}$ (□), or 10 $g \cdot l^{-1}$ (▲) galacturonic acid. (C) Cultures grown at pH 3.5 on a mixture of 10 $g \cdot l^{-1}$ glucose and 10 $g \cdot l^{-1}$ glucuronic acid (Δ).

Physiological analysis of galacturonic acid inhibition of galactose metabolism

To gain further insight into the observed inhibition of galactose metabolism by galacturonic acid, sugar consumption, ethanol production, and growth were determined in independent duplicate fermentation experiments with a mixture of 10 $g \cdot l^{-1}$ glucose and 10 $g \cdot l^{-1}$ galactose with and without 10 $g \cdot l^{-1}$ galacturonic acid at pH 3.5. The finding from the CO_2 profiles that galacturonic acid has a minor effect on the glucose phase (Figure 1 and 2A and B) was confirmed. Galacturonic acid even had a slight stimulatory effect on the specific growth rate (μ increased from 0.28 ± 0.00 to 0.32 ± 0.01 h^{-1}; $p < 0.06$) and the glucose consumption rate (q_s increased from 14.4 ± 0.4 to 15.8 ± 0.2 mmol·g[dry weight]$^{-1} \cdot h^{-1}$; $p < 0.09$).

Consistent with the strongly reduced rates of CO_2 production (Figure 1), the addition of 10 $g \cdot l^{-1}$ galacturonic acid decreased ($p < 0.01$) the galactose consumption rate from 4.6 ± 0.4 to an average rate of 0.6 ± 0.0 mmol·g[dry weight]$^{-1} \cdot h^{-1}$ (Table 2). In the presence of 10 $g \cdot l^{-1}$ galacturonic acid, the biomass concentration remained constant after glucose was completely consumed, indicating that galactose fermentation was not coupled to growth. The final glycerol concentration increased ($p < 0.002$) from 14.4 ± 0.1 mM in the reference cultures to 16.9 ± 0.0 mM in the cultures with galacturonic acid (Figure 2). Glycerol formation (at a low osmotic pressure) is coupled to the reoxidation of excess NADH (Van Dijken and Scheffers, 1986), which can be formed either from biosynthesis or from the formation of oxidized products, such as acetic acid. Indeed, the increased glycerol concentration was balanced by an increase ($p < 0.04$) of the final

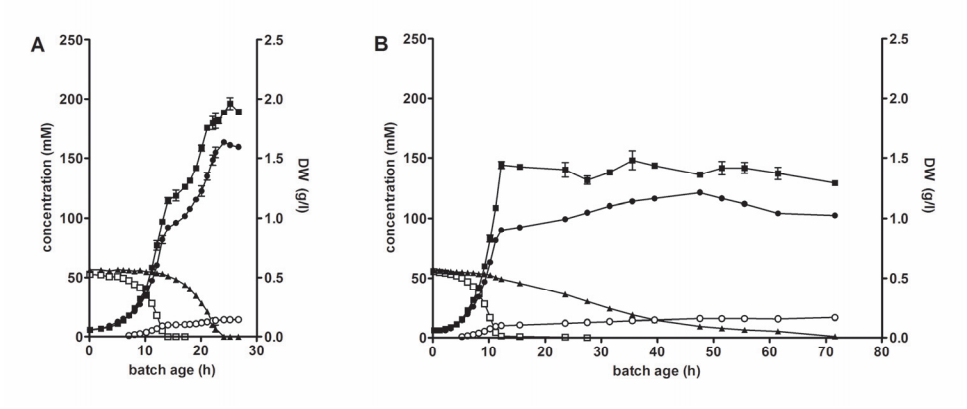

Figure 2. Growth and metabolite production in anaerobic batch cultures of *S. cerevisiae* CEN. PK113-7D cultivated in duplicate at pH 3.5 on a mixture of 10 $g \cdot l^{-1}$ glucose (□) and 10 $g \cdot l^{-1}$ galactose (▲) in the absence of galacturonic acid (A) and in the presence of 10 $g \cdot l^{-1}$ galacturonic acid (B). Ethanol (●), glycerol (○), and biomass dry weight (DW) (■) were formed during these fermentations.

Table 2. Physiological parameters of anaerobic batch cultures (pH 3.5) of *S. cerevisiae* CEN.PK113-7D grown on a mixture of 10 g·l^{-1} glucose and 10 g·l^{-1} galactose in the presence and absence of 10 g·l^{-1} galacturonic acid (GalUA). Data are represented as the average ± devation of duplicate batch experiments.

		μ (h^{-1})	q_s ($mmol \cdot g^{-1} \cdot h^{-1}$)	$q_{ethanol}$ ($mmol \cdot g^{-1} \cdot h^{-1}$)	Y_{sx} ($g \cdot g^{-1}$)
Culture	Phase	Avg ± SD	Avg ± SD	Avg ± SD	Avg ± SD
Reference	glucose	0.28 ± 0.00	-14.4 ± 0.4	23.2 ± 0.4	0.11 ± 0.00
	galactose	0.10 ± 0.00	-4.6 ± 0.4	9.4 ± 0.1	0.12 ± 0.01
10 g·l^{-1} GalUA	glucose	0.32 ± 0.01	-15.8 ± 0.2	24.8 ± 0.4	0.11 ± 0.00
	galactose[a]	0.00 ± 0.00	-0.6 ± 0.0	0.8 ± 0.0	0.01 ± 0.00

[a] In the absence of growth, time-averaged specific rates were determined during this phase.

concentration of acetic acid from 0.5 ± 0.1 mM to 3.6 ± 0.0 mM, despite the decreased biomass formation. In addition, small amounts (<1 mM) of pyruvate and lactate were produced, but no significant effects of galacturonic acid on their concentrations were observed (data not shown).

To investigate whether the low metabolic activity during the galactose consumption phase in the presence of 10 g·l^{-1} galacturonic acid was caused by a decreased viability of the culture, this parameter was measured by viability staining and flow cytometry. Culture viability was still at 81% ± 8% 20 h into the galactose consumption phase (batch age, 31.5 h).

Galacturonic acid inhibits pentose fermentation by engineered *S. cerevisiae*

In batch cultivations of IMS0010, an engineered *S. cerevisiae* strain able to efficiently consume a mixture of glucose, xylose, and arabinose (Wisselink *et al.*, 2009), we investigated whether galacturonic acid also inhibits growth on xylose and/or arabinose. For this, IMS0010 was precultured on arabinose and characterized by using mixtures of 20 g·l^{-1} glucose, 10 g·l^{-1} xylose, and 10 g·l^{-1} arabinose in anaerobic bioreactors in the presence and absence of 10 g·l^{-1} galacturonic acid at pH 3.5 (Figure 3). In the absence of galacturonic acid, growth and metabolism were essentially the same as those described previously for growth at pH 5.0 (Wisselink *et al.*, 2009): first, glucose was consumed, followed by the simultaneous and complete consumption of xylose and arabinose within 40 h (Figure 3A). The addition of 10 g·l^{-1} galacturonic acid to an otherwise identical experimental setup had a drastic impact on the fermentation performance of IMS0010. Whereas the glucose consumption rate did not differ significantly, the consumption of the pentose sugars was drastically affected. As was observed in the absence of galacturonic acid, at glucose concentrations below 10 g·l^{-1}, part of the xylose (28%) and part of the

arabinose (14%) were co-consumed, while the remaining glucose was depleted. Strikingly, in the presence of galacturonic acid at pH 3.5, the consumption of xylose and arabinose stopped immediately after glucose was depleted (Figure 3B), clearly indicating that galacturonic acid also inhibits pentose fermentation in *S. cerevisiae* IMS0010.

Glucuronic acid does not inhibit glucose fermentation

Glucuronic acid (pKa of 3.28) is the uronic acid derived from glucose (Kohn and Kovac, 1978). To investigate whether the observed inhibition of galactose

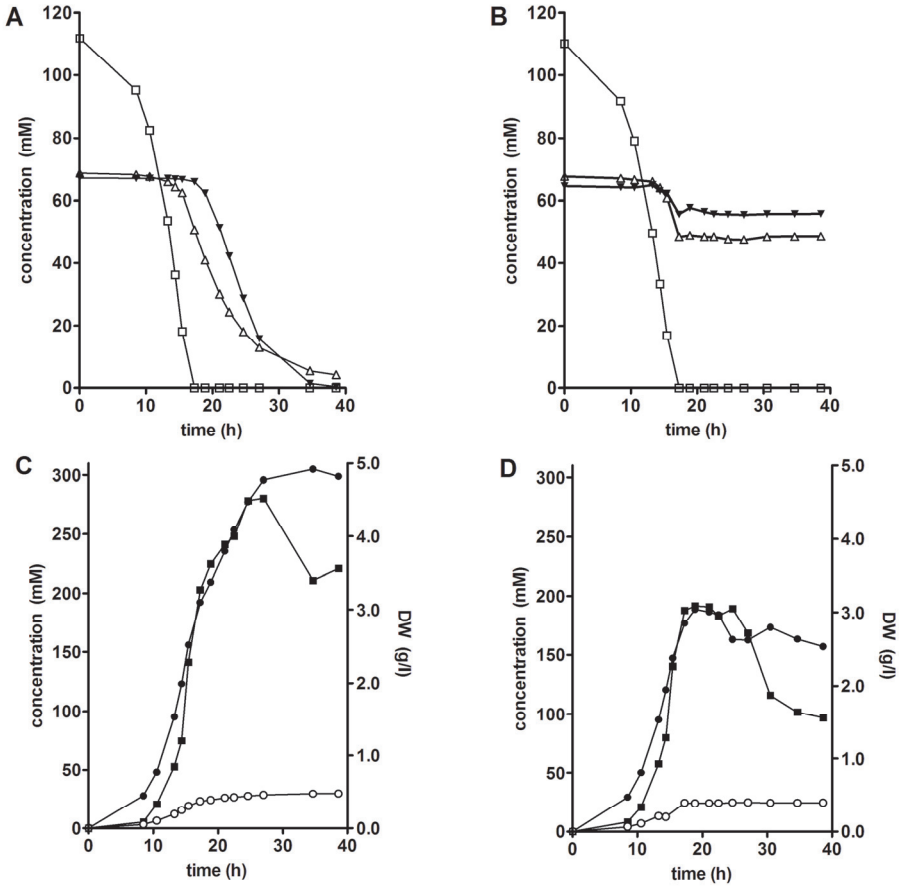

Figure 3. Growth and metabolite production in anaerobic batch cultures of *S. cerevisiae* IMS0010 grown at pH 3.5 on a mixture of 20 g·l^{-1} glucose (□), 10 g·l^{-1} xylose (Δ), and 10 g·l^{-1} arabinose (▼) in the absence of galacturonic acid (A and C) and in the presence of 10 g·l^{-1} galacturonic acid (B and D). Ethanol (●), glycerol (○), and biomass dry weight (■) were formed during these fermentations. The data are from single-batch cultivations and are representative of duplicate experiments.

metabolism by galacturonic acid reflects a more general impact of uronic acids on sugar metabolism by yeasts, anaerobic batch cultures were grown at pH 3.5 on 10 $g \cdot l^{-1}$ glucose and 10 $g \cdot l^{-1}$ glucuronic acid. In these experiments, cells were precultured on glucose, since the induction of the Leloir pathway was not necessary. At pH 3.5, 38% of the acid is in its undissociated form. Glucuronic acid did not significantly influence the fermentation characteristics on glucose, as shown by the virtually identical CO_2 profiles in the presence and in the absence of glucuronic acid (Figure 1C).

Possible interactions of galacturonic acid with the galactokinase

When glucose concentrations are low, the *GAL* genes are induced both in wild-type *S. cerevisiae* cells growing on galactose (Gancedo, 1998; Lohr *et al.*, 1995; Mechler, 1997) and in IMS0010 cells growing on arabinose (Wisselink *et al.*, 2010). Since galactose and galacturonic acid are structurally related compounds, the (competitive) inhibition of galactokinase (Gal1p) might explain the strong effect of galacturonic acid on galactose consumption. To test this hypothesis, the activities of galactokinase, the first enzyme in the Leloir pathway, in cell extracts of galactose-grown shake flask cultures were assayed. In both the presence and the absence of 5 $mmol \cdot l^{-1}$ galacturonic acid in the assay mixtures, which is the same as the galactose concentration, the specific galactokinase activity in the cell extracts was 1.1 ± 0.2 $U \cdot [mg\ protein]^{-1}$. This demonstrated that this concentration of galacturonic acid did not inhibit galactokinase activity *in vitro*. *In vitro* galacturonic acid phosphorylation activities, assayed with a modified galactokinase assay, remained below the detection limit of 0.028 $U \cdot [mg\ protein]^{-1}$.

To investigate the possible *in vivo* phosphorylation of galacturonic acid, which might lead to the intracellular accumulation of galacturonic acid 1-phosphate, samples were taken during the galactose consumption phase of cultures grown on a mixture of 10 $g \cdot l^{-1}$ glucose and 10 $g \cdot l^{-1}$ galactose in the presence of 10 $g \cdot l^{-1}$ galacturonic acid at pH 3.5. In the presence of galacturonic acid, an intracellular concentration of galacturonic acid 1-phosphate of 1.02 ± 0.15 $\mu mol \cdot [g\ dry\ weight]^{-1}$ was detected, which was >50-fold higher than control measurements in the absence of galacturonic acid. The concentration of UDP-galacturonic acid, which might conceivably be formed in a reaction analogous to the Leloir pathway reactions catalysed by galactose-1-phosphate uridyltransferase (Gal7p), did not differ from that of the control and remained below 13 $nmol \cdot [g\ dry\ weight]^{-1}$.

Discussion

Mechanisms of inhibition by galacturonic acid

The different degrees of inhibition at pH 3.5 and pH 5 indicate that the observed effect on galactose metabolism occurs when undissociated galacturonic acid is present extracellularly. Furthermore, the observation of intracellular galacturonic acid 1-phosphate indicates that at least some galacturonic acid can enter the yeast cell at pH 3.5. Since free diffusion over the cell membrane of a highly polar molecule such as galacturonic acid is unlikely, it seems plausible that a permease is involved in galacturonic acid transport. Especially in their protonated, noncharged forms, uronic acids bear a strong structural resemblance to the corresponding aldose sugars. Therefore, the competitive inhibition of the galactose transporter Gal2p, which in IMS0010 is also responsible for arabinose transport (Wisselink *et al.*, 2010), offers a plausible mechanism for galacturonic acid inhibition. The competitive inhibition by a constant concentration of galacturonic acid should become more pronounced as the concentration of the other (transported) species decreases due to its consumption by the yeast cells. This was indeed observed during anaerobic growth on glucose-galactose mixtures in the presence of galacturonic acid at pH 3.5 (Figure 1). In line with this, a *gal2*Δ strain, which cannot grow on galactose or on arabinose and which for that reason was excluded from this study, was shown to be less sensitive to galacturonic acid (Huisjes *et al.*, unpublished data, see Chapter 2 of this thesis). However, the fact that galacturonic acid also inhibits the fermentation of xylose, which is not (solely) transported by Gal2p (Hamacher *et al.*, 2002; Young *et al.*, 2011), indicates that galacturonic acid must have additional inhibitory effects.

Once galacturonic acid enters the cytoplasm of *Saccharomyces cerevisiae* (possibly via Gal2p), multiple additional mechanisms of inhibition are possible. In the cytosol, galacturonic acid will dissociate due to the near-neutral intracellular pH, potentially resulting in classical weak organic acid toxicity (Piper *et al.*, 2001). Since galacturonic acid cannot be metabolized by *S. cerevisiae*, the anion either accumulates, which may inhibit cellular processes, or has to be excreted at the expense of ATP. Additionally, to maintain pH homeostasis, the proton has to be exported via a plasma membrane H^+-ATPase, which in *S. cerevisiae* requires 1 ATP molecule. The maintenance energy requirement for anaerobic growth on glucose was reported previously to be 1 mmol ATP·[g dry weight]$^{-1}$·h^{-1} (Boender *et al.*, 2009). The ATP production associated with the observed galactose consumption rate at pH 3.5 in the presence of 10 g·l^{-1} galacturonic acid would be 1.2 mmol ATP·g [dry weight]$^{-1}$·h^{-1} and was only just above this value. In this situation, the higher energy requirement to maintain cellular homeostasis combined with a low substrate uptake rate due to the competitive inhibition of Gal2p would

result in a situation where there is not enough ATP available to sustain growth. This is consistent with the observation that galactose was consumed, and the majority of the population remained viable, but growth did not take place at pH 3.5 in the presence of 10 $g \cdot l^{-1}$ galacturonic acid. Since even in the absence of galacturonic acid, the consumption rates of xylose and arabinose are already lower than that of galactose, the impact of competitive inhibition and weak organic acid toxicity will be even more drastic. One additional possible mechanism of inhibition is linked to the measured intracellular concentrations of galacturonic acid 1-phosphate (ca. 1 $\mu mol \cdot g\ [dry\ weight]^{-1}$), which were in the same range as the concentrations of the hexose-phosphates that are intermediates of central yeast metabolism, such as glucose-6-phosphate or fructose-6-phosphate (Canelas *et al.*, 2008). Although not previously described for galacturonic acid 1-phosphate, the inhibitory effects of other phosphorylated compounds have been well documented (Ciriacy and Breitenbach, 1979; Gancedo and Gancedo, 1985; Heredia *et al.*, 1964; Maitra, 1971; Platt, 1984). High levels of UDP-sugars can also have toxic effects (Daran *et al.*, 1995), but the intracellular UDP-galacturonic acid concentration remained below the detection limit in this study. Taken together, the experiments with both the mixture of glucose and galactose and the mixture of glucose, xylose, and arabinose corroborate the hypothesis that a combination of mechanisms is responsible for the observed inhibition by galacturonic acid.

In this study, no evidence was found for an inhibition of glucose fermentation by glucuronic acid. In *S. cerevisiae*, hexose transport can be facilitated by at least 20 different members of the hexose and maltose transporter family (Wieczorke *et al.*, 1999). Given the strong diversity in the affinities of different hexose transporters for glucose and other monosaccharides, it seems plausible that at least some of them have a low inhibition constant for glucuronic acid. In addition, the higher rate of ATP formation from the rapid conversion of glucose to ethanol would still allow both growth and cellular maintenance.

Impact of galacturonic acid on fermentation characteristics of pectin-rich feedstocks

The efficient alcoholic fermentation of sugar mixtures, especially when dealing with nonnatural substrates of *S. cerevisiae* such as xylose and arabinose, is already challenging without inhibitory compounds (Kuyper *et al.*, 2005; Wisselink *et al.*, 2009). The strong and negative effect of relatively low concentrations of galacturonic acid on the fermentation of galactose, xylose, and arabinose by *S. cerevisiae* represents a previously unknown challenge. Possible process design solutions include the operation of fermentation processes at pH values that permit the growth of *S. cerevisiae* at the required galacturonic acid concentrations or a

reduction of total sugar concentrations. However, the former approach might increase the risk of contamination, while the second approach leads to decreased product concentrations and, therefore, increased costs of ethanol distillation. Previous observations of the impact of acetic acid at low pHs on the xylose consumption rates in glucose-xylose mixtures demonstrated that this could be alleviated by a continuous glucose-limited feed to provide the ATP necessary to overcome the toxic effect (Bellissimi *et al.*, 2009). Such a relatively simple operational strategy might also relieve the weak-acid toxicity of galacturonic acid but does not impact the competitive inhibition of galacturonic acid on transport.

Alternatively, galacturonic acid inhibition may be alleviated by evolutionary or metabolic engineering. In previous experiments, evolutionary engineering was shown to be able to improve both the fermentation of mixtures of glucose, xylose, and arabinose (Wisselink *et al.*, 2009) and the fermentation kinetics of engineered strains for the nonnative substrate xylose in the presence of acetic acid (Bellissimi *et al.*, 2009; Wright *et al.*, 2011). Such an approach could potentially also yield yeast strains capable of mixed-substrate utilization in the presence of galacturonic acid. Obviously, it would be even more beneficial to metabolize galacturonic acid, preferably by its conversion into ethanol. Although wild-type *S. cerevisiae* cannot ferment galacturonic acid (Grohmann and Bothast, 1994; Van Maris *et al.*, 2006), there are many advantages that make yeast the desired organism for bioethanol production from pectin-rich second-generation feedstocks (Van Maris *et al.*, 2006). The implications of the implementation of metabolic routes from other microorganisms that can naturally consume galacturonic acid in *S. cerevisiae* were described previously (Van Maris *et al.*, 2006). As long as these strategies have not been implemented in practice, galacturonic acid inhibition will represent a key issue in the yeast-based production of bioethanol and other products from pectin-rich feedstocks.

Acknowledgements

The Ph.D. research of E.H.H. is financed by the Dutch Ministry of Economic Affairs via an EOS-LT grant (http://www.agentschapnl.nl/programmas-regelingen/energie-onderzoek-subsidie-eos). The Kluyver Centre for Industrial Fermentation is supported by the Netherlands Genomics Initiative.

We thank Victor Guadalupe Medina, Marijke Luttik, Bart Oud, and Zhen Zeng for experimental assistance.

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Chapter 4

Toward pectin fermentation by *Saccharomyces cerevisiae:* expression of the first two steps of a bacterial pathway for D-galacturonate metabolism.

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Journal of Biotechnology 2012, 162:303-310

Abstract

Saccharomyces cerevisiae cannot metabolize D-galacturonate, an important monomer of pectin. Use of *S. cerevisiae* for production of ethanol or other compounds of interest from pectin-rich feedstocks therefore requires introduction of a heterologous pathway for D-galacturonate metabolism. Bacterial D-galacturonate pathways involve D-galacturonate isomerase, D-tagaturonate reductase and three additional enzymes. This study focuses on functional expression of bacterial D-galacturonate isomerases in *S. cerevisiae*. After demonstrating high-level functional expression of a D-tagaturonate reductase gene (*uxaB* from *Lactococcus lactis*), the resulting yeast strain was used to screen for functional expression of six codon-optimized bacterial D-galacturonate isomerase (*uxaC*) genes. The *L. lactis uxaC* gene stood out, yielding a tenfold higher enzyme activity than the other *uxaC* genes. Efficient expression of D-galacturonate isomerase and D-tagaturonate reductase represents an important step toward metabolic engineering of *S. cerevisiae* for bioethanol production from D-galacturonate. To investigate *in vivo* activity of the first steps of the D-galacturonate pathway, the *L. lactis uxaB* and *uxaC* genes were expressed in a *gpd1*Δ *gpd2*Δ *S. cerevisiae* strain. Although D-tagaturonate reductase could, in principle, provide an alternative means for re-oxidizing cytosolic NADH, addition of D-galacturonate did not restore anaerobic growth, possibly due to absence of a functional D-altronate exporter in *S. cerevisiae*.

Introduction

Saccharomyces cerevisiae is currently the microorganism of choice for production of bioethanol, presently the largest-volume product in industrial biotechnology. Moreover, *S. cerevisiae* is a popular metabolic engineering platform for production of a wide and increasing range of other products (Hong and Nielsen, 2012; Nevoigt, 2008). The limited range of carbon substrates for growth of wild-type *S. cerevisiae* strains is a disadvantage for such applications. Expanding the substrate range of *S. cerevisiae* is particularly important for its application in the conversion of second generation feedstocks, including agricultural residues and energy crops (Van Maris *et al.*, 2006). Hitherto, research on this subject has focused on the hemicellulose fraction of lignocellulosic plant biomass hydrolysates. Significant progress has been made in metabolic engineering of *S. cerevisiae* for the utilization of D-xylose and L-arabinose, two key monomers of hemicellulose that cannot be fermented by wild-type *S. cerevisiae* strains (Hahn-Hägerdal *et al.*, 2007; Weber *et al.*, 2010; Wisselink *et al.*, 2009).

In addition to cellulose and hemicellulose, plant biomass contains pectin as a third major carbohydrate polymer, with glucose, L-arabinose and D-galacturonate as its main monomers. Wild-type *S. cerevisiae* strains cannot ferment D-galacturonate (Barnett *et al.*, 1990), which makes up ca. 20% of polymeric pectin (Micard *et al.*, 1996). Engineering *S. cerevisiae* for D-galacturonate metabolism is essential to achieve economically viable conversion of hydrolysed pectin-rich agricultural residues such as sugar beet pulp and citrus peel to bioethanol or other products. Additionally, consumption of D-galacturonate from pectin-containing feedstocks could prevent its negative effects on growth and sugar fermentation kinetics (Huisjes *et al.*, 2012).

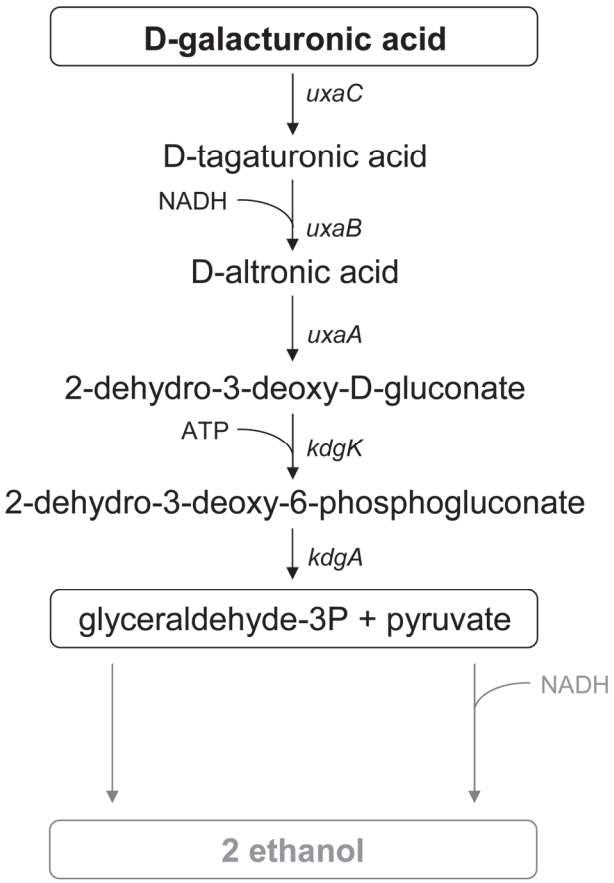

Figure 1. Pathway for bacterial metabolism of D-galacturonate. Codes indicate the following genes and enzymes: *uxaC*, D-galacturonate isomerase (EC 5.3.1.12) *uxaB*, D-tagaturonate reductase (EC 1.1.1.58); *uxaA*, D-altronate dehydratase (EC 4.2.1.7); *kdgK*, 2-keto-3-deoxygluconate kinase (EC 2.7.1.45); *kdgA*, 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14). Reactions in grey indicate conversion of the products pyruvate and 3-phosphoglyceraldehyde to ethanol via yeast glycolysis and alcoholic fermentation.

The pathway for fungal D-galacturonate metabolism has been extensively studied in *Hypocrea jecorina* (*Trichoderma reesei*) and the individual enzymes have been heterologously expressed in *S. cerevisiae* (Hilditch *et al.*, 2007; Kuorelahti *et al.*, 2006; Kuorelahti *et al.*, 2005; Liepins *et al.*, 2006). However, the involvement of both NADP(H) and NAD(H) as redox cofactors in the fungal D-galacturonate pathway, as well as the involvement of glycerol as an intermediate, may present problems in yeast-based anaerobic processes such as the production of bioethanol. For such applications, expression of a bacterial pathway for D-galacturonate metabolism may be preferable (Van Maris *et al.*, 2006).

The predominant pathway for D-galacturonate metabolism in bacteria (Figure 1) involves five enzyme-catalysed reactions and is initiated by isomerization of D-galacturonate to D-tagaturonate (Ashwell *et al.*, 1960). After NADH-dependent reduction of D-tagaturonate to D-altronate (Hickman and Ashwell, 1960), D-altronate is dehydrated to 2-dehydro-3-deoxy-D-gluconate (Smiley and Ashwell, 1960), whose phosphorylation yields 2-keto-3-deoxy-6-phosphogluconate (Cynkin and Ashwell, 1960). This compound is subsequently split by an aldolase to yield pyruvate and glyceraldehyde-3-phosphate (Kovachevich and Wood, 1955), thereby linking D-galacturonate metabolism to glycolysis.

Since D-galacturonate is more oxidized than sugars, its conversion via either the fungal or bacterial pathway does not enable the formation of 2 mol of ethanol/mol of D-galacturonate, unless an external electron donor is available. During anaerobic growth of *S. cerevisiae* on sugars, part of the sugar is converted to glycerol to reoxidize cytosolic NADH formed in biosynthetic reactions (Bakker *et al.*, 2001; Van Dijken and Scheffers, 1986). It has been proposed that, in mixed-substrate cultures of an *S. cerevisiae* strain expressing a bacterial D-galacturonate pathway, metabolism of D-galacturonate could obviate the need for glycerol production, thereby increasing the ethanol yield on sugar as well as on D-galacturonate (Van Maris *et al.*, 2006).

An initial isomerization of the substrate is also a key element in several metabolic engineering strategies for fermentation of D-xylose and L-arabinose by *S. cerevisiae*. Despite a clear lack of structural similarities between bacterial D-xylose and L-arabinose isomerases (Becker and Boles, 2003), functional expression of both these isomerases in *S. cerevisiae* proved to be a significant challenge (Amore *et al.*, 1989; Becker and Boles, 2003; Gárdonyi and Hahn-Hägerdal, 2003; Moes *et al.*, 1996; Sarthy *et al.*, 1987; Sedlak and Ho, 2001; Walfridsson *et al.*, 1996).

The goal of the present study is to investigate the functional expression of the initial reactions of the bacterial pathway for D-galacturonate metabolism in *S. cerevisiae*. After expression of a bacterial D-tagaturonate reductase, the resulting yeast strain was used to screen for functional expression of several bacterial

D-galacturonate isomerase genes, using the reductase as a coupling enzyme in *in vitro* enzyme assays. Subsequently, the *in vivo* activity of bacterial D-galacturonate pathway genes was explored in growth experiments with engineered yeast strains.

Materials and Methods

Strains and maintenance

Stock cultures of *S. cerevisiae* strains used in this study (Table 1) were grown in shake flasks on 100 ml YPD medium (Bacto yeast extract, 1% (w/v); Bacto peptone, 2% (w/v) and glucose, 2% (w/v)) or synthetic medium (Verduyn *et al.*, 1990) with 2% (w/v) glucose as carbon source. Where relevant, histidine, leucine, tryptophan and uracil were added at concentrations calculated according to Pronk (2002) to complement for auxotrophic requirements. After addition of 30% (v/v) glycerol to stationary phase cultures, 1-ml aliquots were stored at −80 °C.

Strain construction

A BAC clone containing the genes for the D-galacturonate pathway of the *Lactococcus lactis* subsp. *lactis* strain KF147 (Van Hylckama Vlieg *et al.*, 2006) was used to PCR-amplify coding sequences for *uxaC*, *uxaB*, *uxaA*, *kdgK* and *kdgA*, using Phusion Hot Start high- fidelity DNA polymerase (Finnzymes Oy, Espoo, Finland) according to the manufacturer's specifications in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany). PCR products were purified using the DNA Clean and Concentrator kit from Zymo Research Corp. (Irvine, CA, USA). Plasmids were isolated with the Genelute Plasmid Mini-PrepTM Kit (Sigma–Aldrich, St. Louis, MO, USA). Restriction endonucleases were purchased from New England Biolabs (Ipswich, MA, USA) and T4 DNA ligase from Roche (Roche Applied Science, Indianapolis, IN, USA). Linear DNA fragments were isolated from 1% (w/v) agarose gel in 1× TAE buffer using the Zymoclean gel DNA Recovery kit from Zymo Research Corp. (Irvine, CA, USA). DNA was stained with SYBR safe from Invitrogen (Carlsbad, CA, USA). Correct plasmid construction was confirmed by restriction analysis. Transformations of reaction products into competent *Escherichia coli* K-12 were performed with the Z-Competent *E. coli* transformation kit Zymo Research Corp. (Irvine, CA, USA) and plated on LB medium (Bacto tryptone 1% (w/v), Bacto yeast extract 0.5% (w/v), NaCl 1% (w/v)) containing ampicillin (100 $mg \cdot l^{-1}$) or kanamycin (50 $mg \cdot l^{-1}$). Yeast transformations were performed by the method of Burke *et al.* (2000). After transformations, cells were plated on selective synthetic media and plasmid identity was confirmed by diagnostic PCR.

Table 1. *S. cerevisiae* strains used in this study.

Strain	Genotype	Source
CEN.PK113-7D	*MATa MAL2-8^c SUC2*	(Entian and Kötter, 2007)
CEN.PK113-5A	*MATa MAL2-8^c SUC2 URA3 trp1-289 leu2-3,112 his3Δ1*	(Entian and Kötter, 2007)
CEN.PK2-1C	*MATa MAL2-8^c SUC2 ura3-52 trp1-289 leu2-3,112 his3Δ1*	(Entian and Kötter, 2007)
IME083	CEN.PK113-5A pUDE049(*LEU2*)	This study
IME084	CEN.PK113-5A pUDE058(*LEU2*)	This study
IME139	CEN.PK113-5A pRS425(*LEU2*) pRS424(*TRP1*)	This study
IME094	IME084 pUDE092(*TRP1*)	This study
IME097	IME084 pUDE093(*TRP1*)	This study
IME098	IME084 pUDE094(*TRP1*)	This study
IME099	IME084 pUDE095(*TRP1*)	This study
IME136	IME084 pUDE147(*TRP1*)	This study
IME137	IME084 pUDE148(*TRP1*)	This study
IME090	IME084 pUDE090(*TRP1*)	This study
IMI005	CEN.PK2-1C pUDE23(*LEU2*) pUDI24(*URA3*)	This study
IME092	CEN.PK113-5A pUDE049(*LEU2*) pUDE096(*HIS3*)	This study
IMX031	*MATa MAL2-8c SUC2 ura3 HIS3 leu2::LEU2*[pRS405] *TRP1 gpd1::loxP gpd2::loxP-hygmx-loxP*	This study
IMZ243	IMX031 pUDE100 (*URA3*)	This study
IMZ244	IMX031 pRS426(*URA3*)	This study
IME076	*MATa ura3 GPD1 GPD2* p426_GPD(*URA3*)	(Guadalupe Medina *et al.*, 2010)

Plasmids used in this study are listed in Table 2. A fragment containing P_{TDH3}-*uxaC*-T_{CYC1} and P_{TEF2}-*uxaB*-T_{ADH1} expression cassettes was synthesized and cloned in pRS405 by BaseClear (Leiden, the Netherlands) to yield pUDE23. The P_{TDH3}-*uxaA*-T_{TEF} expression cassette was synthesized and supplied in vector pBC (BaseClear, Leiden, the Netherlands). The *L. lactis kdgK* and *kdgA* genes were PCR amplified from the BAC clone mentioned above. The *TPI1* promoter, *ADH1* terminator, *TEF1* promoter and *CYC1* terminator fragments needed for the *kdgK* and *kdgA* expression cassettes were amplified from pYX222 (R&D Systems Europe Ltd., Abingdon, UK), pRW231 (Wisselink *et al.*, 2007), pYM-N18 (Janke *et al.*, 2004) and p426-GPD (Mumberg *et al.*, 1995), respectively. Expression cassettes were assembled using a combination of fusion PCR and restriction/ligation and were verified by sequencing. The resulting P_{TPI1}-*kdgK*-T_{ADH1} and P_{TEF1}-*kdgA*-T_{CYC1} expression cassettes were subsequently cloned in the pBC P_{TDH3}-*uxaA*-T_{TEF}

Table 2. Plasmids used in this study. Unless mentioned otherwise, the *uxaC*, *uxaB*, *uxaA*, *kdgK* and *kdgA* genes originate from *L. lactis* subsp. *lactis* KF147, and are not codon-optimized.

Plasmid	Characteristics	Reference
pSH47	*cre* recombinase expression vector, *URA3*	(Güldener *et al.*, 1996)
pRS405	Integration, *LEU2*	(Sikorski and Hieter, 1989)
pRS406	Integration, *URA3*	(Sikorski and Hieter, 1989)
pRS423	2µm, *HIS3*	(Christianson *et al.*, 1992)
pRS424	2µm, *TRP1*	(Christianson *et al.*, 1992)
pRS425	2µm, *LEU2*	(Christianson *et al.*, 1992)
pRS426	2µm, *URA3*	(Christianson *et al.*, 1992)
pUDE23	pRS405 P_{TDH3}-*uxaC*-T_{CYC1}, P_{TEF2}-*uxaB*-T_{ADH1}	
pUDi24	pRS406 P_{TDH3}-*uxaA*-T_{TEF}, P_{TPI1}-*kdgK*-T_{ADH1} P_{TEF1}-*kdgA*-T_{CYC1}	
pUDE049	pRS425 P_{TDH3}-*uxaC*-T_{CYC1}, P_{TEF2}-*uxaB*-T_{ADH1}	
pUDE058	pRS425 P_{TEF2}-*uxaB*-T_{ADH1}	
pUDE090	pRS424 P_{TDH3}-*uxaC*-T_{CYC1}	
pUDE092	pRS424 P_{TDH3}-Ec_*uxaC*- T_{CYC1} (*uxaC* from *E.coli* K12[a])	
pUDE093	pRS424 P_{TDH3}-Ca_*uxaC*- T_{CYC1} (*uxaC* from *Clostridium acetobutylicum* ATCC 824[a])	
pUDE094	pRS424 P_{TDH3}-Rs_*uxaC*- T_{CYC1} (*uxaC* from *Rhodobacter sphaeroides* ATCC BAA-808[a])	
pUDE095	pRS424 P_{TDH3}-Ba_*uxaC*- T_{CYC1} (*uxaC* from *Bacillus amyloliquefaciens* FZB42[a])	
pUDE096	pRS423 P_{TDH3}-*uxaA*-T_{TEF}, P_{TPI1}-*kdgK*-T_{ADH1} P_{TEF1}-*kdgA*-T_{CYC1}	
pUDE100	pRS426 P_{TDH3}-*uxaC*-T_{CYC1}, P_{TEF2}-*uxaB*-T_{ADH1}	
pUDE147	pRS424 P_{TDH3}-Bt_*uxaC*- T_{CYC1} (*uxaC* from *Bacteroides thetaiotaomicron*[a])	
pUDE148	pRS424 P_{TDH3}-*uxaC*-T_{CYC1}[a]	

[a]codon-optimized

intermediate vector using the HindIII and NotI restriction sites. The resulting plasmid was then cut with XmaI and SpeI, and the three expression cassettes were ligated into pRS406 digested with SpeI and NgoMIV to yield pUDI24. pUDE049 was obtained by cloning the *uxaC* and *uxaB* expression cassettes from pUDE23 in pRS425 using the PstI and SpeI restriction sites. The *uxaA*, *kdgK* and *kdgA*

Table 3. Oligonucleotide primers used in this study

Primers	Target	Sequences[a]
uxaAkdgAkdgK (FW)	*L. lactis uxaAkdgAkdgK* cassette	5'-CCCGGGGCCGGGAGTTTATCATTATC-3'
uxaAkdgAkdgK (RV)	*L. lactis uxaAkdgAkdgK* cassette	5'-ACTAGTGCCGCAAATTAAAGCCTTC-3'
Q-uxaC-RV	*L. lactis uxaC*	5'-TTCTACCAAGCGGCCAAAG-3'
Q-uxaC-FW	*L. lactis uxaC*	5'-TGACCGATTCACGGAGTTTC-3'
Q-uxaB-RV	*L. lactis uxaB*	5'-TCAACACCAGCAAGTAGAG-3'
Q-uxaB-FW	*L. lactis uxaB*	5'-CCATACCGTGAACGTAAAG-3'
Q-uxaA-RV	*L. lactis uxaA*	5'-AAACAGAGCTGCCACCTTG-3'
Q-uxaA-FW	*L. lactis uxaA*	5'-AGACGGTTTCTCAGGTATCAC-3'
Q-kdgK-RV	*L. lactis kdgK*	5'-GCACAAGCTCGACTCACTG-3'
Q-kdgK-FW	*L. lactis kdgK*	5'-TGGTTTCGCAGTCGGATTAG-3'
Q-kdgA-FW2	*L. lactis kdgA*	5'-AGTGGCTCAATATGCTGGAAC -3'
Q-kdgA-RV2	*L. lactis kdgA*	5'-ACTATAGCCAAACGAGCTGAG -3'
q_Ec_uxaC (FW)	Codon-optimized (CO) *E. coli uxaC*	5'-GTCGGTATGTTGACTGACTC-3'
q_Ec_uxaC (RV)	(CO) *E. coli uxaC*	5'-ACCCATCTACCGATCATTTG-3'
q_Ca_uxaC (FW)	(CO) *C. acetobutylicum uxaC*	5'-ATTGGTATGCTGACGGATTC-3'
q_Ca_uxaC (RV)	(CO) *C. acetobutylicum uxaC*	5'-CCCATTTCCCGATCAAGTC-3'
q_Rs_uxaC (FW)	(CO) *R. sphaeroides uxaC*	5'-AGCTAGACGTGTGGATTG-3'
q_Rs_uxaC (RV)	(CO) *R. sphaeroides uxaC*	5'-AACTTCAGGTGCCTCTTC-3'
q_Ba_uxaC (FW)	(CO) *B. amyloliquefaciens uxaC*	5'-CAGCAAATGAGAGCGTTATC-3'
q_Ba_uxaC (RV)	(CO) *B. amyloliquefaciens uxaC*	5'-AAGTATTCATGCCTGGGATAG-3'
q BT uxaC co FW	(CO) *B. thetaiotaomicron uxaC*	5'-CGTTGGTATGTTGACGGATAG-3'
q BT uxaC co Rv	(CO) *B. thetaiotaomicron uxaC*	5'-TTCTCCGTTCTCCACATCC-3'
q LL uxaC co Fw	(CO) *L. lactis uxaC*	5'-GGTATGCTGACCGATTCAAG-3'
q LL uxaC co Rv	(CO) *L. lactis uxaC*	5'-TTCGACCAATCTGCCAAAG-3'

[a]restriction sites underlined

expression cassettes were amplified from pUD124 with primers uxaAkdgAkdgK (FW) and uxaAkdgAkdgK (RV) (Table 3), thereby introducing SpeI and XmaI restriction sites. The amplified fragment and vector pRS423 were then cut with SpeI and XmaI and ligated to yield pUDE096. pUDE58 was constructed by restriction of pUDE49 with XhoI and ligating the resulting fragment. pUDE90 was obtained by cloning the *uxaC* expression cassette from pUDE49 in pRS424 using the NotI and XmaI restriction sites.

The native *uxaC* genes from *E. coli* K12 DH10B (Gen-Bank accession no. CP000948.1), *Clostridium acetobutylicum* ATCC 824 (AE001437.1), *Rhodobacter sphaeroides* ATCC BAA-808 (CP000143.1), *Bacillus amyloliquefaciens* FZB42 (CP000560.1), *Bacteroides thetaiotaomicron* VPI-5482 (NC004663.1) and *L. lactis* subsp. *lactis* KF147 (CP001834.1) were codon optimized by BaseClear (Leiden, the Netherlands) by avoiding codons that are rare (<10%) in *S. cerevisiae*. The *E. coli* gene

was followed by a synthetic *CYC1* terminator and a SacII restriction site. Each synthetic gene was flanked by BamHI and SpeI restriction sites to enable cloning to the destination vector. pUDE092 was constructed by cloning the *E. coli uxaC* gene and the *CYC1* terminator in pUDE090 using the SacII and BamHI restriction sites, thus yielding an *uxaC* expression cassette including the TDH3 promoter. pUDE093, pUDE094, pUDE095, pUDE147 and pUDE148 were all constructed similarly: the SpeI and BamHI restriction sites were used to clone the various *uxaC* genes in the pUDE092 backbone.

Plasmids were transformed to CEN.PK strains of *S. cerevisiae*, thereby complementing their auxotrophic markers (Table 1). The genetic background of IMX031 is strain RWB094 (Guadalupe Medina *et al.*, 2010). First, the loxP-*KanMX*-loxP marker in strain RWB094 was removed using pSH47, which contains the Cre recombinase (Güldener *et al.*, 1996). Next, the *LEU2* marker was restored by transformation with BstEII-digested pRS405. pUDE100 was constructed by interchanging the pRS425 backbone for that of pRS426 using the SpeI and SalI restriction sites. Strains IMZ243 and IMZ2044 were constructed by transformation of IMX031 with pUDE100 and pRS426, respectively.

Media and cultivation

Shake-flask cultivation of *S. cerevisiae* strains was performed at 30°C in an orbital shaker (200 rpm) in synthetic medium (Verduyn *et al.*, 1990) supplemented with the appropriate auxotrophic requirements (Pronk, 2002). Media containing D-galacturonate were filter-sterilized. The pH of the medium was set to the desired value with 2 M KOH or 2 M H_2SO_4 prior to sterilization. 100-ml cultures were inoculated with a 1-ml glycerol stock.

Analysis of the ability of *S. cerevisiae* strains to grow on or co-consume D-galacturonate was done in shake-flask cultures on synthetic medium (Verduyn *et al.*, 1992). Cultures were incubated at pH 3 or at pH 5, using 0.2% or 1% (w/v) D-galacturonate as a carbon source, either alone or in combination with 1% (w/v) glucose or ethanol or 0.2% (w/v) D-galactose. To prevent culture acidification, ammonium sulfate was replaced by an equimolar amount (based on nitrogen content) of urea (Hensing *et al.*, 1995). To compensate for the reduced sulfate content of these media, 6.6 g of K_2SO_4 l^{-1} was added as well. Cultures were monitored by measuring the optical density at 660 nm (OD660), HPLC analysis of supernatants, pH measurements and microscopic evaluation of culture purity.

Anaerobic growth of *S. cerevisiae* IMZ243 was tested on agar plates with synthetic medium at pH 4 with 2% (w/v) glucose, with or without 0.5% (w/v) D-galacturonate, supplemented with the anaerobic growth factors Tween 80 and ergosterol (Verduyn *et al.*, 1990). Cultures were incubated at 30 °C in an anaerobic

jar using an Oxoid Anaerobic Gas Generating Kit (Oxoid Ltd., Basingstoke, UK) to remove oxygen. *S. cerevisiae* strains IMZ244 and IME076 (Table 1) were used as negative and positive controls, respectively.

Tagaturonic acid

Tagaturonic acid, a gift of J. van der Toorn (Department of Biocatalysis and Organic Chemistry, Delft University of Technology, The Netherlands) was produced according to Ehrlich and Guttmann (1934), with the following adaptations: the calcium salt was neutralized with Dowex H^+, the solvent was evaporated and the product was crystallized via an anti-crystallization from water and ethanol.

Enzyme assays

Cell extracts were prepared by sonication from exponentially growing *S. cerevisiae* cultures and analyzed for protein content as described by Postma *et al.* (1989). Enzyme activities were assayed with freshly prepared cell extracts in spectrophotometric assays, using a Hitachi model U-3010 spectrophotometer. All determinations were performed at 30°C and 340 nm ($\varepsilon_{NAD(P)H}$ at 340 nm = 6.33 mM^{-1}). D-Galacturonate isomerase assays were performed according to Linster and Van Schaftingen (2004), with one modification: the $MnCl_2$ concentration was increased to 0.5 mM as this led to higher activities. The reaction was started with 5 mM sodium D-galacturonate. The assay for D-tagaturonate reductase was based on the uronate isomerase assay described by Linster and Van Schaftingen (2004). The assay mixture contained 50 mM HEPES pH 7, 0.1 mM $MnCl_2$, 0.01 mM EDTA and 0.15 mM NADH. The reaction was started with 5 mM calcium D-tagaturonate (pH 7). Glucose-6-phosphate dehydrogenase activity was measured according to Postma *et al.* (1989).

qPCR

The sampling procedure was based on the method used by Piper *et al.* (2002): exponentially growing *S. cerevisiae* shake-flask cultures on glucose were cooled, and 72 mg of cells was harvested by centrifugation. The cell pellet was resuspended in 1.08 ml ice-cold AE buffer and immediately 1.08 ml acid phenol-chloroform (5:1, pH 4.5) and 108 µl 10% (w/v) SDS were added. After vortexing vigorously, the tubes were placed in a water bath for 5 min at 65°C. The content was homogenized by vortexing, and divided over 3 RNase-free tubes and stored at −80°C. RNA extraction was performed by the method of Schmitt *et al.* (1990) and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Dusseldorf, Germany). qPCR was performed in triplicate on three dilutions of the sample using

the QuantiTect SYBR Green PCR Kit (Qiagen, Dusseldorf, Germany) with a primer concentration of 0.5 µM in a total volume of 20 µl in the Rotor-Gene Q (Qiagen, Dusseldorf, Germany). All qPCR primers are listed in Table 3. The PCR-efficiencies (E) were calculated from standard curves. The average efficiency over all product-yielding qPCR reactions was 1.8 ± 0.1. C_T values were determined with the Rotor-Gene Q Series Software by automatic scanning for the threshold setting which delivers optimal estimates of the given concentrations. IME083 *ACT1* was always included as an internal standard. For each individual strain, the relative expression of *uxaC*, *uxaB*, *uxaA*, *kdgK* and *kdgA* was quantified by the method of Pfaffl (2001) using the following formula, with *ACT1* as the reference gene: $E^{\Delta C_T (\text{reference-target})}$.

Results

S. cerevisiae is unable to grow on D-galacturonate

Wild-type *S. cerevisiae* strains are unable to grow on D-galacturonate (Barnett *et al.*, 1990). Prolonged incubation of microbial cultures sometimes leads to enrichment of spontaneous mutants with the ability to utilize compounds that did not initially support growth. For example, such experiments have yielded non-recombinant *S. cerevisiae* strains capable of growth on D-xylose (Attfield and Bell, 2006). Prolonged incubation (over 8 weeks) of *S. cerevisiae* CEN.PK113-7D in synthetic media with D-galacturonate, either as sole carbon source or in combination with ethanol, did not result in either growth or D-galacturonate conversion (data not shown). This result is consistent with the absence, in the genome of *S. cerevisiae* CEN.PK113-7D, of genes with sequence homology to fungal or bacterial genes involved in D-galacturonate metabolism (Nijkamp *et al.*, 2012).

Expression of a bacterial D-tagaturonate reductase gene in *S. cerevisiae*

In cell extracts of glucose-grown shake-flask cultures of the empty-vector reference strain *S. cerevisiae* IME139, the background activity of the NADH-dependent D-tagaturonate reductase assay was below the detection limit of 0.005 µmol·min^{-1}·[mg protein]$^{-1}$. This observation corroborates the predicted lack of a galacturonate isomerase encoding gene in the *S. cerevisiae* genome, and also indicates that, under these conditions, *S. cerevisiae* does not express non-specific NADH-dependent reductases capable of reducing D-tagaturonate to D-altronate. However, when the *uxaB* gene from *L. lactis* KF147, encoding D-tagaturonate reductase, was expressed on a multi-copy vector in *S. cerevisiae* CEN.PK113-5A,

under control of the constitutive *TEF1* promoter, D-tagaturonate reductase activities in cell extracts were 6.3 ± 0.5 μmol·min^{-1}·[mg protein]$^{-1}$.

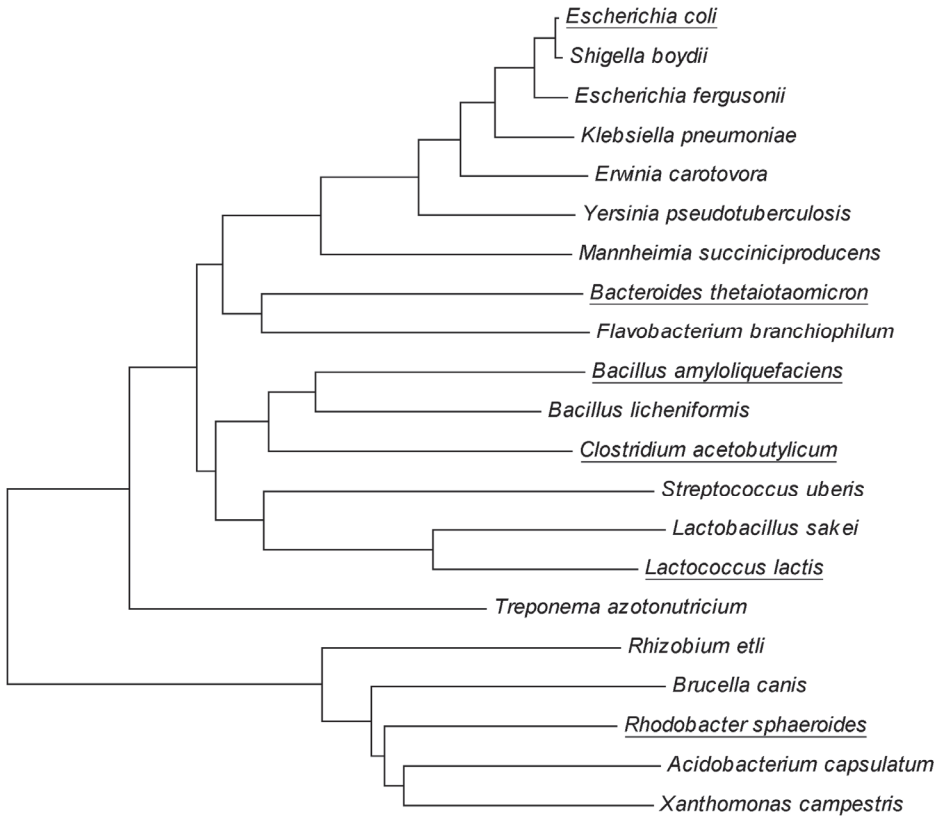

Figure 2. Phylogenetic tree of bacterial genes encoding D-galacturonate isomerase (*uxaC*). The underlined species names indicate *uxaC* genes that were included in the present study. The nucleotide sequences were obtained from GenBank (accession numbers, from top to bottom in the figure, CP000948.1, CP001063.1, CU928158.2, CP000647.1, BX950851.1, CP000720.1, NC_006300.1, NC_004663.1, NC_016001.1, CP000560.1, CP000002.3, AE001437.1, AM946015.1, CR936503.1, CP001834.1, CP001841.1, CP000133.1, CP000873.1, CP000143.1, NC_012483.1, AM039952.1). The sequences were aligned using ClustalW (Thompson *et al.*, 1994), phylogeny was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004). All positions containing gaps and missing data were eliminated. There were a total of 1250 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

Expression of codon-optimized bacterial *uxaC* genes leads to low D-galacturonate isomerase activities

Based on a phylogenetic tree of bacterial D-galacturonate isomerase genes, six candidate genes from different branches were selected (Figure 2). Optimization of codon usage can strongly improve expression of prokaryotic genes in *S. cerevisiae*, as illustrated by a study on the introduction of a bacterial L-arabinose pathway (Wiedemann and Boles, 2008). Therefore, synthetic variants of the codon regions of the six bacterial D-galacturonate isomerase genes, codon optimized for *S. cerevisiae*, were custom synthesized. The codon-optimized open reading frames were then cloned behind the constitutive *TDH3* promoter on a multicopy vector. D-galacturonate isomerase can be measured by coupling its activity to that of NADH-dependent D-tagaturonate reductase (Linster and Van Schaftingen, 2004). Therefore, the expression vectors were transformed to a strain already expressing the *L. lactis uxaB* gene.

Activities of D-galacturonate isomerase in cell extracts of the transformed strains were low and varied from 0.005 to 0.46 μmol·min^{-1}·[mg protein]$^{-1}$ (Table 4). Enzyme activity in cell extracts of strain IME137, which expresses the codon-optimized *L. lactis uxaC* gene, was ten-fold higher than that in the next best strain,

Table 4. *In vitro* activities of D-galacturonate isomerase and *uxaC* mRNA levels of different *S. cerevisiae* strains, grown in shake-flask cultures on synthetic medium with glucose. Data are presented as the average ± deviation of independent duplicate analyses. The average activity of the reference enzyme glucose-6-phosphate dehydrogenase in cell extracts of the different strains was 0.50 μmol·min^{-1}·[mg protein]$^{-1}$ and differed by less than 10% between the different strains. With the exception of strain IME139, activities of D-tagaturonate reductase in all strains were above 3 μmol·min^{-1}·[mg protein]$^{-1}$.

Strain	Description	D-galacturonate isomerase μmol·min^{-1}·[mg protein]$^{-1}$	*uxaC* mRNA expression relative to *ACT1*
IME139	Empty-vector control reference strain	< 0.005	*uxaC* mRNA not detected
IME094	*E. coli uxaC*, codon optimized	0.014 ± 0.003	*uxaC* mRNA not detected
IME097	*C. acetobutylicum uxaC,* codon optimized	0.041 ± 0.009	3.1 ± 0.2
IME098	*R. sphaeroides uxaC,* codon optimized	0.011 ± 0.011	0.1 ± 0.1
IME099	*B. amyloliquefaciens uxaC*, codon optimized	0.005 ± 0.001	5.3 ± 1.1
IME136	*B. thetaiotaomicron uxaC*, codon optimized	0.045 ± 0.006	5.3 ± 0.5
IME137	*L. lactis uxaC*, codon optimized	0.46 ± 0.05	4.3 ± 0.4
IME090	*L. lactis uxaC*, not codon optimized	0.36 ± 0.02	4.9 ± 0.6

IME136, which expresses the codon-optimized *B. thetaiotaomicron uxaC* gene. To assess the impact of codon optimization on the expression of the *L. lactis uxaC* gene,its native coding sequence was expressed using the same multicopy vector. The resulting D-galacturonate isomerase activity was only about 20% lower than that observed with the codon-optimized sequence (Table 4).

qPCR experiments yielded very low and undetectable mRNA levels for the strains expressing codon-optimized *R. sphaeroides* and *E. coli uxaC* genes, respectively (Table 4). This indicated that the low D-galacturonate isomerase activities found in these strains are at least partially caused by suboptimal transcription and/or mRNA stability. In contrast, high *uxaC* mRNA levels were observed for strains expressing codon-optimized *uxaC* genes of *C. acetobutylicum*, *B. amyloliquefaciens* and *B. thetaiotaomicron*, although these strains exhibited low enzyme activities (Table 4). This observation implies that more effective post-transcriptional processes or superior enzyme characteristics contribute to the higher enzyme activity observed for the expression of the *L. lactis uxaC* gene in *S. cerevisiae*.

In vivo analysis of yeast strains expressing heterologous D-galacturonate genes

After optimization of the expression of *uxaB* and *uxaC* genes, we attempted to express the entire *L. lactis* D-galacturonate pathway in *S. cerevisiae*. To this end, the structural genes of *uxaC*, *uxaB*, *uxaA*, *kdgK* and *kdgA* were cloned behind strong constitutive yeast promoters (Table 2) on two episomal multicopy vectors and on two single-copy integration vectors (Table 1) that were subsequently integrated at the *LEU2* and *URA3* loci. Single-copy integration of the *L. lactis uxaB* and *uxaC* genes led to enzyme activities in cell extracts that were approximately two-fold lower than those in a yeast strain that expressed all D-galacturonate pathway genes from a multicopy vector (Table 5). Since substrates for the other enzymes of the pathway are not commercially available, we were unable to establish their activity in cell extracts. However, qPCR experiments confirmed that all five genes were transcribed (Table 5). Nevertheless, prolonged incubation (over 8 weeks) in aerobic shake-flask cultures on synthetic medium with D-galacturonate as the sole carbon source did not result in growth. These experiments were not only performed at an initial pH of 6, but also at an initial pH of 3.5 in order to facilitate D-galacturonate import (Huisjes *et al.*, 2012; Souffriau *et al.*, 2012). Moreover, in experiments with mixed carbon sources (10 $g \cdot l^{-1}$ glucose, D-galactose or ethanol combined with 2 or 10 $g \cdot l^{-1}$ D-galacturonate), no significant co-consumption of D-galacturonate was observed (data not shown).

Table 5. *In vitro* enzyme activities and mRNA levels of the bacterial galacturonate pathway in *S. cerevisiae* strains expressing the *L. lactis uxaC*, *uxaB*, *uxaA*, *kdgK* and *kdgA* genes from single- and multicopy vectors. Strains were grown in shake-flask cultures on synthetic medium with glucose. Data are presented as the average ± deviation of duplicate analyses. Activities of the reference enzyme glucose-6-phosphate dehydrogenase in cell extracts of strains IMI005 and IME092 were both 0.53 ± 0.03 μmol·min^{-1}·[mg protein]$^{-1}$. n.d. = not determined due to unavailability of substrate.

Gene	*S. cerevisiae* IMI005 single copy, integrated		*S. cerevisiae* IME092 multicopy	
	mRNA expression relative to *ACT1*	Enzyme activity μmol·min^{-1}·[mg protein]$^{-1}$	mRNA expression relative to *ACT1*	Enzyme activity μmol·min^{-1}·[mg protein]$^{-1}$
uxaC	3.1 ± 0.5	0.08 ± 0.01	2.8 ± 0.3	0.18 ± 0.02
uxaB	0.6 ± 0.1	0.68 ± 0.07	0.7 ± 0.1	1.55 ± 0.24
uxaA	0.1 ± 0.0	n.d.	0.5 ± 0.1	n.d.
kdgK	1.3 ± 0.3	n.d.	2.8 ± 0.6	n.d.
kdgA	0.2 ± 0.1	n.d.	1.0 ± 0.1	n.d.

To specifically investigate the *in vivo* activity of the *uxaB* and *uxaC* genes, they were expressed in an *S. cerevisiae gpd1*Δ *gpd2*Δ (Gpd$^-$) mutant, yielding strain IMZ243. Since Gpd$^-$ mutants cannot reoxidize cytosolic NADH by glycerol production, they cannot grow under anaerobic conditions in the absence of an external electron acceptor (Björkqvist *et al.*, 1997; Van Dijken and Scheffers, 1986). Conversion of D-galacturonate to D-altronate via D-galacturonate isomerase and D-tagaturonate reductase should, in theory, provide an alternative mechanism for reoxidation of NADH (Figure 1). However, addition of either 2 or 10 g·l^{-1} D-galacturonate to anaerobic, glucose-grown shake-flask and plate cultures of strain IMZ243 did not result in anaerobic growth, not even after prolonged incubation or when the pH of the cultures was decreased to 3.5 to facilitate import of D-galacturonate (Huisjes *et al.*, 2012; Souffriau *et al.*, 2012). These results may indicate the absence of a functional D-altronate exporter in *S. cerevisiae*.

Discussion

Previous research on bioethanol production from D-galacturonate focused on the use of bacteria such as *E. coli* that have a native D-galacturonate pathway (Doran *et al.*, 2000; Edwards and Doran-Peterson, 2012). Although unable to ferment D-galacturonate (Barnett *et al.*, 1990; Grohmann and Bothast, 1994; Van Maris *et al.*, 2006), wild-type and engineered *S. cerevisiae* strains have many characteristics such as

high ethanol and inhibitor tolerance, insensitivity to bacteriophages and fast fermentation kinetics that are attractive to large-scale bioethanol production from second-generation feedstocks (Van Maris *et al.*, 2006). Unlocking the potential of pectin-rich feedstocks for anaerobic yeast-based processes therefore remains an important challenge in microbial biotechnology. This study represents the first successful expression of enzymes from a bacterial isomerase-based D-galacturonate pathway in a eukaryotic host.

In vitro activities of heterologously expressed *L. lactis* D-tagaturonate reductase were high enough to sustain high rates of D-galacturonate fermentation. As observed for the expression of bacterial D-xylose and L-arabinose isomerases (Becker and Boles, 2003; Brat *et al.*, 2009; Sedlak and Ho, 2001; Van Maris *et al.*, 2007; Wiedemann and Boles, 2008), introduction of codon-optimized *uxaC* genes from different bacteria in *S. cerevisiae* yielded substantially different enzyme activities in cell extracts (Table 4). The *L. lactis uxaC* gene stood out in this comparison, yielding a ten-fold higher enzyme activity than the other *uxaC* genes. The D-galacturonate isomerase activity observed with the *L. lactis uxaC* gene is comparable to that of the glycolytic enzyme phosphofructokinase in anaerobic, glucose-grown cultures of *S. cerevisiae* (Van Hoek *et al.*, 2000). Based on the assumption that dry yeast biomass contains 33% soluble protein (Postma *et al.*, 1989), the D-galacturonate isomerase activity of 0.46 $\mu mol \cdot min^{-1} \cdot [mg\ protein]^{-1}$ measured in an *L. lactis uxaC*-expressing yeast strain (Table 4) would correspond to an *in vivo* D-galacturonate conversion rate of 9 $mmol \cdot g^{-1} \cdot h^{-1}$. If the enzyme activities assayed in cell extracts accurately reflect *in vivo* capacity, they should therefore be sufficient to sustain fast alcoholic fermentation of D-galacturonate.

The successful expression of the first two enzymes of the bacterial D-galacturonate pathway in *S. cerevisiae* provides a useful platform for studying the expression of the other enzymes in the pathway. Our initial attempts to express the entire 5-enzyme *L. lactis* D-galacturonate pathway in *S. cerevisiae* did not enable aerobic or anaerobic growth on D-galacturonate. This negative result is very unlikely to be due to an inability of *S. cerevisiae* to transport D-galacturonic acid across its plasma membrane because, in a recent study, Souffriau *et al.* (2012) showed low affinity uptake of ^{3}H-labeled D-galacturonate by *S. cerevisiae* at low pH (ca. 3 $mmol \cdot g\ biomass^{-1} \cdot h^{-1}$ at 20 $g \cdot l^{-1}$ D-galacturonate and pH 3). Presence of such a D-galacturonic acid transport activity is in line with the observation that, at low pH, D-galacturonic acid strongly inhibits growth of engineered *S. cerevisiae* strains on L-arabinose, D-xylose and D-galactose (Huisjes *et al.*, 2012). Optimization of D-galacturonate transport kinetics is likely to be required for engineering of efficient D-galacturonate utilization by *S. cerevisiae*. Prokaryotic genes encoding D-galacturonate transporters are known (Mata-Gilsinger and Ritzenthaler, 1983), but

it is notoriously difficult to express prokaryotic transporters in a eukaryotic plasma membrane. Martens-Uzunova and Schaap (2008), in a transcriptome analysis of *Aspergillus niger*, identified several putative transporter genes that were strongly upregulated during growth on D-galacturonate. These, and putative transporter genes obtained in a similar way from other filamentous fungi or yeasts offer interesting candidates for engineering D-galacturonate transport in *S. cerevisiae* (Van Maris *et al.*, 2006).

In the case of *S. cerevisiae* strains engineered for L-arabinose fermentation, prolonged incubation finally resulted in growth, primarily due to the derepression of the *GAL2*-encoded D-galactose transporter, which also transports L-arabinose (Becker and Boles, 2003; Wisselink *et al.*, 2010). The absence of growth after long-term incubation of engineered strains carrying expression cassettes for the *L. lactis* D-galacturonate pathway suggests that more extensive mutations, either in the heterologous genes or in native yeast genes, are needed for *in vivo* activity of the bacterial D-galacturonate pathway.

Biochemical studies on the enzymes involved in the final three steps of the bacterial D-galacturonate pathway are complicated by the lack of commercial availability of their substrates. The biochemical literature on these enzymes does not indicate the use of cofactors that are absent from *S. cerevisiae*, nor a need for accessory proteins. With only three genes involved, it is conceivable to perform a combinatorial screen with structural genes from different prokaryotes, by selecting for aerobic growth on D-galacturonate in a strain background that already expresses *uxaC* and *uxaB*. A similar approach, based on *in vivo* recombination of DNA fragments, has been successfully applied to the optimization of plant metabolic pathways (Naesby *et al.*, 2009).

Conclusions

The first two enzymes of the bacterial pathway for D-galacturonate metabolism, D-galacturonate isomerase and D-tagaturonate reductase, have been functionally expressed in *S. cerevisiae*. Heterologous expression of D-galacturonate isomerase depended strongly on the origin of the bacterial *uxaC* gene sequence. The *uxaC* gene from *L. lactis* was shown to yield high expression levels and can be used in research on the expression of a fully functional pathway for D-galacturonate metabolism in *S. cerevisiae*.

Acknowledgements

The PhD research of EHH is financed by the Dutch Ministry of Economic Affairs via an EOS-LT grant (http://www.agentschapnl.nl/programmas-regelingen/energie-onderzoek-subsidie-eos). The Kluyver Centre for Industrial Fermentation is supported by the Netherlands Genomics Initiative. We thank Johan van Hylckama Vlieg for the gift of the *L. lactis* BAC clone and Victor Guadalupe Medina and Erwin Suir for their help in strain construction.

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Chapter 5

Summary
Samenvatting

Summary

In the beginning of the 20th century, bioethanol was already viewed as a promising transportation fuel. The ambition to reduce our current dependence on fossil transportation fuels has driven renewed interest in bioethanol. Today, most bioethanol is still produced from starch- and sucrose-based feedstocks. However, the availability of these relatively expensive feedstocks is limited and their increased use potentially results in undesirable competition with food production. Therefore, bioethanol production from second-generation feedstocks, such as agricultural residues or dedicated crops, is eventually desired. Often-studied examples of such agricultural residues are the lignocellulosic feedstocks corn stover, wheat straw or bagasse. To expand the available quantity of biomass, pectin-rich feedstocks like sugar beet pulp and citrus peel, which are currently sold as cattle feed, could be used for the production of bioethanol.

Efficient fermentation is essential for production of bioethanol from second generation feedstocks, including pectin-rich waste streams. The yeast *Saccharomyces cerevisiae* is the microorganism used in present-day processes for manufacturing bioethanol, which is currently the largest product in industrial biotechnology in terms of both volume and market value. Its robustness under process conditions also makes this yeast a very suitable microorganism for industrial ethanol production from second-generation feedstocks. Enzymatic hydrolysis of sugar beet pulp results in a hydrolysate rich in glucose (21% dry matter basis), arabinose (21%), galacturonic acid (21%) and galactose (5%). Glucose is a preferred carbon source of *S. cerevisiae* and is readily converted to ethanol. Also galactose can naturally be converted to ethanol by this yeast. However, arabinose and galacturonic acid are not naturally consumed or converted by this yeast. In previous research, *S. cerevisiae* was successfully engineered to ferment arabinose to ethanol. This leaves galacturonic acid as a major challenge in the production of bioethanol from pectin-rich feedstocks. The goal in this project was to improve fermentation of pectin-rich hydrolysates, such as sugar beet pulp, by developing *S. cerevisiae* strains capable of converting galacturonic acid to ethanol, and by addressing fermentation characteristics of yeast in these hydrolysates.

In the absence of *S. cerevisiae* strains able to consume galacturonic acid, high concentrations of galacturonic acid will remain in the fermentation broth. Galacturonic acid is a weak acid with a pKa of 3.51. For other weak organic acids (e.g. acetic acid) it is known that they have a large impact on yeast fermentations. Therefore it is vital to know the physiological effects of galacturonic acid on *S. cerevisiae* and its impact on fermentation. In **Chapter 2**, a combination of chemostat cultivation and transcriptome analysis was used to investigate the effect

of galacturonic acid on yeast physiology. The inhibitory effect of galacturonic acid was stronger under aerobic than under anaerobic conditions in glucose-limited chemostat cultures at pH 3.5, resulting in a 25% lower biomass yield on glucose in aerobic cultures with 10 $g \cdot l^{-1}$ galacturonic acid. Subsequent transcriptome analysis revealed a strong upregulation of genes involved in mating response, a very strong upregulation of *DAK2*, encoding a dihydroxyacetone kinase, and a strong upregulation of the *GAL* regulon, involved in metabolism of galactose. However, notwithstanding significant efforts, follow-up work did not provide additional leads on the mechanism of inhibition of galacturonic acid.

Viability studies using a combination of fluorescent staining with propidium iodide and flow cytometry, showed that the presence of galacturonic acid resulted in a pH-dependent decrease in viability under starvation conditions. This pH-dependency indicated that the undissociated acid was the inhibiting species. To study whether undissociated galacturonic acid enters the cells via non-specific transport through the *S. cerevisiae* hexose transporters (*HXT*), similar viability studies were carried out using a *gal2*Δ knockout strain, and a strain where all hexose transporters were knocked out (Hxt$^-$). These viability assays showed that yeast cells in which all genes known to encode hexose transporters had been deleted, displayed a much slower loss of viability in the presence of galacturonic acid than cells of a reference strain. In an independent experiment, deletion of all *HXT* transporters was shown to alleviate the inhibitory effect of 10 or 20 $g \cdot l^{-1}$ galacturonic acid on *S. cerevisiae* strains growing on ethanol. This study revealed the impact of galacturonic acid on the physiology of *S. cerevisiae* and indicated that modified expression of hexose transporters may improve robustness under process conditions with high galacturonic acid concentrations.

The impact of galacturonic acid on alcoholic fermentation of sugar mixtures by *S. cerevisiae* was investigated in **Chapter 3**. Anaerobic batch cultures were performed on mixtures of glucose and galactose at various galacturonic acid concentrations. In cultures grown at pH 5.0, which is well above the pKa value of galacturonic acid (3.51), addition of 10 $g \cdot l^{-1}$ galacturonic acid did not affect fermentation kinetics and growth. In cultures grown at pH 3.5, addition of 10 $g \cdot l^{-1}$ galacturonic acid did not significantly affect glucose consumption. However, at this lower pH, adding 10 $g \cdot l^{-1}$ galacturonic acid increased the duration of the galactose consumption phase from 11 h for the reference culture (no galacturonic acid added) to 81 h. Physiological characterization showed that galacturonic acid completely inhibited growth on galactose and reduced the galactose consumption rate by 87% relative to reference cultures without galacturonic acid. A combination of viability staining and flow cytometry showed that the low metabolic activity during the galactose consumption phase in the presence of 10 $g \cdot l^{-1}$ galacturonic acid was not

caused by a decreased viability of the culture. Additionally, *in vitro* assays showed that galacturonic acid did not inhibit galactokinase. Although no decrease in the concentration of galacturonic acid was observed in the cultures, galacturonic acid 1-phosphate was detected intracellularly. To investigate whether the observed inhibition of galactose metabolism by galacturonic acid reflects a more general impact of uronic acids on sugar metabolism by yeasts, similar experiments were carried out with glucuronic acid and glucose. However, identical CO_2 profiles of these experiments compared to the control demonstrated that glucuronic acid did not inhibit glucose consumption. Subsequently, it was shown in anaerobic batch fermentations of the engineered pentose-fermenting strain *S.cerevisiae* IMS0010 on a mixture of glucose, xylose and arabinose, that galacturonic acid strongly inhibited the fermentation of xylose and arabinose. Taken together, these data show that inhibition occurs when the undissociated acid is present, and suggest that a combination of a decreased substrate uptake rate due to competitive inhibition on Gal2p, an increased energy requirement to maintain cellular homeostasis, and/or an accumulation of galacturonic acid 1-phosphate contributes to the inhibition. As an alternative approach, it might very well be possible to overcome this toxicity of galacturonic acid through evolutionary engineering, as has previously been demonstrated for other organic acids, such as acetic acid. However, until resolved, the role of galacturonic acid as an inhibitor of sugar fermentation should be considered in the design of yeast fermentation processes based on pectin-rich feedstocks. One crucial parameter in that design is the operational pH of the fermentation, with higher pH values decreasing the galacturonic acid toxicity and lower pH values being beneficial for process economy and to avoid contaminations. Another process adaptation that could be beneficial, is the supply of a continuous glucose feed to provide the ATP necessary to overcome toxic effects.

The best solution would of course be to maintain low levels of galacturonic acid through conversion of this substrate to ethanol, which in addition increases the overall ethanol yield. This ultimately desired scenario was explored in **Chapter 4**. Prolonged cultivation confirmed that *S. cerevisiae* cannot utilize galacturonic acid. Therefore, achieving efficient conversion of this compound through metabolic engineering requires introduction of a heterologous pathway for galacturonate metabolism. One of the bacterial galacturonate pathways involves galacturonate isomerase, tagaturonate reductase and three additional enzymes. This chapter focused on functional expression of bacterial galacturonate isomerases in *S. cerevisiae*. After demonstrating high-level functional expression of a tagaturonate reductase gene (*uxaB* from *Lactococcus lactis*), the resulting yeast strain was used to screen for functional expression of six codon-optimized bacterial galacturonate isomerase (*uxaC*) genes. The *L. lactis uxaC* gene stood out, yielding a ten-fold higher enzyme

activity than the other *uxaC* genes. Efficient expression of galacturonate isomerase and tagaturonate reductase represents an important step towards metabolic engineering of *S. cerevisiae* for bioethanol production from galacturonate. To investigate *in vivo* activity of the first steps of the galacturonate pathway, the *L. lactis uxaB* and *uxaC* genes were expressed in a *gpd1Δ gpd2Δ S. cerevisiae* strain. Although tagaturonate reductase could, in principle, provide an alternative means for re-oxidizing cytosolic NADH, addition of galacturonate did not restore anaerobic growth, possibly due to the absence of a functional altronate exporter in *S. cerevisiae*. In addition, all five enzymes of the pathway were expressed in yeast, but this did not lead to growth on, or consumption of, galacturonic acid.

The implementation of a complete functional catabolic route for galacturonic acid in *S. cerevisiae* proved much more difficult than envisioned based on previous results for other non-natural substrates and on the desk-top studies that were performed at the start of the project. The very recent developments in yeast synthetic biology, such as transformation associated recombination (TAR) cloning, would allow for the extension of the phylogenetic screening to the other enzymes of the bacterial pathway. However, as clearly shown in this study, this either requires activity of the complete pathway or the ability to measure the individual activities of the enzymes. For these measurements the substrates of these reactions, which are not commercially available, will have to be synthesized or purified. In the past, evolutionary engineering has been proven as an effective tool in strain improvement aiming at expanding the substrate range of *S. cerevisiae*. As a starting point for such an evolution strategy, strains with low levels of activity of enzymes are needed. However, in this study, activities of the three last enzymes of the pathway could not be measured. The amount of mutations required to overcome these limitations might have been too much to overcome by evolutionary engineering and/or classical strain improvement. In this study, the difference in co-factor use between the fungal and the bacterial isomerase pathway, was the motivation to choose for the bacterial route. In light of the negative results with the bacterial route, an alternative would be to adapt the co-factor preference of the enzymes in the fungal route to become NAD(H)-dependent. Even though results in this study indicate entry of galacturonic acid into the cell, the exact mechanism and kinetics of 'transport' are unknown. Future functional expression of a catabolic pathway might therefore require additional expression of a transporter. In this light, the currently ongoing world-wide screening initiatives for (fungal) transporters for the compounds that can be found in lignocellulosic hydrolysates might provide interesting leads.

Despite many attempts at metabolic and evolutionary engineering, the project did not yet result in a *S. cerevisiae* strain capable of galacturonic acid

consumption. However, a rigorous study did lead to the functional expression of uronate isomerase and tagaturonate dehydrogenase. In a parallel research line, it was demonstrated that, especially at low pH, galacturonic acid has a drastic impact on cellular viability and galactose, arabinose and xylose consumption. As long as galacturonic acid is not consumed by *S. cerevisiae*, these inhibitory effects of galacturonic acid will remain a key issue in the yeast-based production of bioethanol and other products from pectin-rich feedstocks.

Samenvatting

Aan het begin van de 20ste eeuw werd bioethanol al beschouwd als een veelbelovende motorbrandstof. De wereldwijde ambitie om onze huidige maatschappij minder afhankelijk te maken van fossiele brandstoffen heeft de interesse in bioethanol sterk doen opleven. Momenteel wordt de meeste bioethanol geproduceerd uit grondstoffen die gebaseerd zijn op sacharose of zetmeel. Deze grondstoffen zijn echter beperkt beschikbaar en relatief duur. Bovendien zijn er zorgen dat het toegenomen gebruik van deze grondstoffen mogelijk leidt tot ongewenste competitie met voedselproductie. Om die redenen is bioethanol gewenst die wordt geproduceerd uit tweede-generatie grondstoffen, zoals afvalstromen uit de landbouw of speciaal gekweekte gewassen. Zo is er bijvoorbeeld veel onderzoek gedaan naar het gebruik van de resten van tarwe, suikerriet en maïsplanten. Om de hoeveelheid en diversiteit van tweede-generatie grondstoffen voor bioethanol productie te vergroten, zou ook pectine-rijke biomassa, zoals suikerbietenpulp en citrusschillen, gebruikt kunnen worden. Economisch gezien is dit aantrekkelijk omdat deze grondstoffen momenteel vooral als veevoer worden verkocht.

Voor de productie van bioethanol uit tweede-generatie grondstoffen, inclusief pectine-rijke grondstoffen, is het essentieel dat de fermentatie efficiënt is. In huidige processen wordt bakkersgist (*Saccharomyces cerevisiae*) gebruikt om op industriële schaal ethanol te maken. Omdat *S. cerevisiae* heel robuust is onder industriële procescondities is deze gist ook zeer geschikt voor productie van bioethanol uit tweede-generatie grondstoffen. Enzymatische hydrolyse van suikerbietenpulp levert een hydrolysaat op dat veel glucose (21% op basis van drooggewicht), arabinose (21%), galacturonzuur (21%) en galactose (5%) bevat. *S. cerevisiae* heeft een sterke voorkeur voor glucose als koolstofbron en vergist deze suiker snel en efficiënt tot ethanol. Tevens kan bakkersgist van nature galactose omzetten naar ethanol. Arabinose en galacturonzuur worden echter niet van nature geconsumeerd of omgezet door *S. cerevisiae*. Inmiddels zijn bakkersgiststammen ontwikkeld die wel in staat zijn arabinose naar ethanol te fermenteren. Galacturonzuur blijft echter over en vormt een grote uitdaging in de productie van bioethanol uit pectine-rijke grondstoffen. Het doel van dit project was het verbeteren van de fermentatie van pectine-rijke hydrolysaten door nieuwe *S. cerevisiae* stammen te ontwikkelen die in staat zijn om galacturonzuur om te zetten naar ethanol en door de fermentatie van deze hydrolysaten in gist te onderzoeken.

Zolang er geen *S. cerevisiae* stammen zijn die galacturonzuur kunnen consumeren, zal er in het fermentatiebeslag een hoge concentratie galacturonzuur aanwezig zijn. Galacturonzuur is een zwak zuur met een pKa van 3,51. Omdat

bekend is dat andere zwakke zuren (zoals azijnzuur) fermentaties met gist sterk kunnen beïnvloeden, is het belangrijk om de effecten van galacturonzuur op zowel de fysiologie van gist als op de suikervergisting in kaart te brengen. In **hoofdstuk 2** is onderzoek aan groei en productvorming in chemostaatcultures gecombineerd met transcriptoom-analyse om dit te bewerkstelligen.

Het remmende effect van galacturonzuur was sterker onder aërobe dan onder anaërobe condities in glucose-gelimiteerde chemostaatcultures gekweekt bij pH 3,5. Onder aërobe condities met 10 $g \cdot l^{-1}$ galacturonzuur bleek de biomassa-opbrengt op glucose 25% lager dan in afwezigheid van galacturonzuur. In de daaropvolgende transcriptoom-analyse van deze cultures viel een aantal veranderingen in de transcriptniveaus van gistgenen op. Ten eerste was er een sterke toename in het transcriptniveau van genen die betrokken zijn bij de geslachtelijke voortplanting van gist. Ten tweede was het transcriptniveau van het *DAK2*-gen, dat codeert voor het enzym dihydroxyacetonkinase, sterk verhoogd. Ten derde waren de transcriptniveaus van een aantal genen van het *GAL* regulon, dat betrokken is bij de afbraak van galactose, ook sterk verhoogd. Ondanks aanzienlijke inspanningen, leverde het najagen van deze aanwijzingen geen verklaring voor het mechanisme van galacturonzuurinhibitie.

Door de gistcellen met propidiumjodide fluorescent te kleuren, is via een flow-cytometer gemeten welk deel van de gistpopulatie nog leefde als deze geïncubeerd werd in een medium zonder energiebron (zoals glucose) in aanwezigheid van galacturonzuur. Dit experiment liet zien dat de gistpopulatie geleidelijk afsterft met een snelheid die afhankelijk is van de pH waarop de incubatie plaatsvond. Dit wees erop dat het ongedissocieerde zuur verantwoordelijk is voor de inhibitie door galacturonzuur. Vervolgens is gekeken of ongedissocieerd galacturonzuur de cellen binnenkomt via niet-specifiek transport of door hexose (*HXT*) transporters. Hiervoor zijn vergelijkbare experimenten uitgevoerd met een *gal2*Δ stam, en een stam waar alle *HXT* transporters verwijderd zijn (Hxt$^-$). Dit liet zien dat in een stam waar alle *HXT* transporters verwijderd zijn, de gistpopulatie veel minder snel afstierf in aanwezigheid van galacturonzuur dan in de originele stam die deze transporters nog bevatte. In een afzonderlijk experiment waar *S. cerevisiae* stammen werden gekweekt op ethanol, bleek het verwijderen van alle *HXT* genen het remmende effect van 10 of 20 $g \cdot l^{-1}$ galacturonzuur te verminderen. Deze studie laat de invloed van galacturonzuur op de fysiologie van *S. cerevisiae* zien en suggereert dat het aanpassen van de expressie van individuele hexosetransporter-genen zou kunnen leiden tot verbeterde robuustheid van gist in aanwezigheid van een hoge concentratie galacturonzuur.

De invloed van galacturonzuur op de fermentatie van suikermengsels naar ethanol door *S. cerevisiae* is onderzocht in **hoofdstuk 3**. Anaërobe batch cultures op

mengsels van glucose en galactose bij verschillende concentraties galacturonzuur lieten zien dat de toevoeging van 10 $g \cdot l^{-1}$ galacturonzuur bij pH 5,0 – wat ruim boven de pKa van galacturonzuur (3,51) is – geen effect had op de fermentatiekinetiek en op de groei van gist. In cultures met een pH van 3,5 had de toevoeging van een zelfde hoeveelheid galacturonzuur geen effect op de fase waarin de glucose geconsumeerd werd door de gist. Echter, de fase waarin de galactose werd geconsumeerd duurde veel langer dan wanneer geen galacturonzuur was toegevoegd: 81 uur in plaats van 11 uur. Een fysiologische analyse liet zien dat galacturonzuur de groei op galactose volledig remde en dat de snelheid waarmee galactose geconsumeerd werd met 87% omlaag ging vergeleken met de situatie zonder galacturonzuur. Door de gistcellen wederom fluorescent te kleuren (propidiumjodide) en via een flow-cytometer te meten welk deel van de gistpopulatie nog leeft, werd duidelijk dat de lage stofwisselingsactiviteit gedurende de fase waarin de galactose werd geconsumeerd in aanwezigheid van 10 $g \cdot l^{-1}$ galacturonzuur, niet werd veroorzaakt door het afsterven van de gistpopulatie. Daarnaast is met behulp van *in vitro* metingen van de enzymactiviteit van galactokinase aangetoond dat galacturonzuur dit enzym niet remt. Hoewel in de cultures geen afname van de hoeveelheid galacturonzuur te meten was, is wel intracellulair galacturonzuur-1-fosfaat gedetecteerd. Om vast te stellen of de inhibitie van het galactosemetabolisme door galacturonzuur een voorbeeld is van een algemeen effect van uronzuren op suikermetabolisme in gist, is een vergelijkbaar experiment uitgevoerd met glucuronzuur en glucose. Uit het CO_2-profiel van de fermentatie bleek echter dat glucuronzuur de glucosestofwisseling niet inhibeert. Vervolgens is met behulp van *S. cerevisiae* IMS0010, een giststam die geschikt is gemaakt voor het fermenteren van pentose-suikers, in anaërobe batchcultures op een mengsel van glucose, xylose en arabinose vastgesteld dat galacturonzuur tevens de fermentatie van xylose en arabinose sterk remt. Uit de resultaten blijkt dat de ongedissocieerde vorm van galacturonzuur verantwoordelijk is voor remming van de vergisting van verschillende suikers in gist. Tevens lijkt een combinatie van het afnemen van de opnamesnelheid van het substraat door competitieve remming van de galactosetransporter Gal2p, gecombineerd met een toegenomen energiebehoefte om de gistcellen in homeostase te houden en/of een ophoping van galacturonzuur-1-fosfaat, bij te dragen aan deze remming. Wellicht is het mogelijk deze remming door galacturonzuur te overwinnen door evolutie-experimenten, zoals eerder is gedaan voor andere organische zuren zoals azijnzuur. Zolang deze remming door galacturonzuur echter niet opgelost is, zal het noodzakelijk zijn hier rekening mee te houden bij het ontwerpen van industriële fermentatieprocessen voor omzetting van pectine-rijke hydrolysaten met bakkersgist. De pH van de fermentatie zal een cruciale parameter in dit ontwerp vormen, waarbij hogere pH waarden zullen leiden

tot minder remming door galacturonzuur, terwijl lagere pH waarden gunstig zijn voor de proceseconomie en helpen om besmetting van de cultures met andere micro-organismen te voorkomen. Verder zou wellicht een continue toevoer van glucose aan het proces gunstig kunnen zijn omdat op deze manier ATP beschikbaar is om de remming door galacturonzuur te kunnen overwinnen.

Uiteindelijk is de beste oplossing om ervoor te zorgen dat de hoeveelheid galacturonzuur tijdens de fermentatie laag blijft door het om te zetten naar ethanol. Dit zou dan tevens de opbrengst van ethanol verhogen. Deze optie is in **hoofdstuk 4** onderzocht. Langdurige incubatie van *S. cerevisiae* met galacturonzuur bevestigde dat deze gist niet in staat is om galacturonzuur te gebruiken. Om dit te bereiken, is het daarom nodig om de gist genetisch aan te passen en te voorzien van een stofwisselingsroute voor het omzetten van galacturonzuur. Een van de routes voor galacturonzuurafbraak in bacteriën bestaat uit het enzym galacturonaat-isomerase, tagaturonaatreductase, en drie andere enzymen. In dit hoofdstuk is specifiek gekeken naar de expressie van bacteriële galacturonaat-isomerases in *S. cerevisiae*. Nadat een tagaturonaatreductase-gen (*uxaB* van *Lactococcus lactis*) succesvol tot expressie was gebracht in *S. cerevisiae*, is deze giststam gebruikt om zes verschillende codon-geoptimaliseerde galacturonaat-isomerase-(*uxaC*)-genen te testen. Het *uxaC* gen uit *L. lactis* vertoonde een tien maal hogere enzymactiviteit dan de andere *uxaC* genen. De efficiënte expressie van galacturonaat-isomerase en tagaturonaatreductase vormt een belangrijke stap op weg naar een *S. cerevisiae* stam die in staat zal zijn om bioethanol uit galacturonzuur te maken. Om te kijken of deze eerste twee stappen van de route voor omzetting van galacturonzuur ook binnen de cel functioneren, zijn de *L. lactis uxaB* en *uxaC* genen tot expressie gebracht in een *gpd1*⊿ *gpd2*⊿ *S. cerevisiae* stam. Hoewel tagaturonaat reductase in theorie een alternatieve route zou kunnen vormen om het cytosolische NADH te her-oxideren, was deze stam niet in staat onder anaërobe condities te groeien in aanwezigheid van galacturonzuur. Dit kan wellicht verklaard worden door de afwezigheid van een functionele altronaattransporter in *S. cerevisiae*. De gehele bacteriële route voor galacturonzuur-afbraak, bestaande uit 5 genen, is tevens in gist gekloneerd. Dit leidde echter niet tot groei op, of consumptie van galacturonzuur.

Het bleek veel moeilijker dan van tevoren ingeschat om een complete werkende route voor de afbraak van galacturonzuur in *S. cerevisiae* in te brengen. De recente ontwikkelingen op het gebied van de synthetische biologie in gist, zoals het kloneren door middel van transformatie-geassocieerde recombinatie, maken het eenvoudiger om veel meer genen en enzymen te testen, inclusief de drie overige enzymen van de bacteriële route. Hiervoor zal het nodig zijn om de activiteit van deze enzymen individueel te bepalen. Echter, omdat de substraten voor deze enzymen niet commercieel beschikbaar zijn, zullen deze dan gemaakt of

opgezuiverd moeten worden. In het verleden zijn goede resultaten behaald met evolutie-experimenten om giststammen te verkrijgen die in staat zijn suikers af te breken die van nature niet omgezet worden. Als startpunt van een dergelijk experiment is het echter wel vereist dat een lage activiteit van de betreffende enzymen aanwezig is. In deze studie konden de activiteiten van de laatste drie enzymen van de route niet gemeten worden. Wellicht was het aantal mutaties dat nodig was om de afbraak van galacturonzuur op gang te krijgen, te groot om via spontane evolutie te overwinnen. De reden om aan het begin van dit project voor de bacteriële route te kiezen in plaats van de route die voorkomt in schimmels, was het verschil in cofactorgebruik bij galacturonzuurafbraak. Gezien de behaalde resultaten met bacteriële route, zou het interessant zijn te proberen de cofactorvoorkeur van de enzymen in de schimmelroute te veranderen zodat ook deze route NAD(H)-afhankelijk wordt. Hoewel de resultaten van dit onderzoek er sterk op wijzen dat galacturonzuur de gistcel binnen kan komen, is het precieze mechanisme niet bekend. Het is daarom wellicht nodig om een galacturonzuur-specifieke transporter in te brengen in gist wanneer een functionele route voor de afbraak van galacturonzuur tot expressie wordt gebracht. De wereldwijde zoektocht naar (schimmel)transporters voor de verschillende verbindingen die aanwezig zijn in hydrolysaten zou hier aan kunnen bijdragen.

Ondanks vele pogingen tot het genetisch aanpassen en evolueren van *S. cerevisiae* heeft dit project niet geleid tot een giststam die in staat is om galacturonzuur daadwerkelijk te consumeren. Het heeft echter wel geleid tot de functionele expressie van galacturonaatisomerase en tagaturonaatreductase. In parallel is aangetoond dat – met name bij lage pH – galacturonzuur een grote invloed heeft op de levensvatbaarheid van gist en op de consumptie van galactose, arabinose en xylose. Zolang galacturonzuur niet wordt omgezet door *S. cerevisiae*, zullen de remmende effecten van galacturonzuur een belangrijk aandachtspunt blijven in de productie van ethanol en andere producten uit pectine-rijke grondstoffen met behulp van bakkersgist.

Chapter 6

Curriculum Vitae
List of Publications
Acknowledgements

Curriculum Vitae

Eline Huisjes was born on November 23rd, 1981 in Zaltbommel, the Netherlands. She finished her pre-university education (VWO) at Scholengroep Cambium in 2000. Subsequently she started her study in Life Science and Technology, a joint education programme of Delft University of Technology and Leiden University. She dedicated one year to work in the board of the Life Science and Technology student's association LIFE. Her BSc thesis on 'engineering of redox metabolism in yeast for improved glycerol production' in the Industrial Microbiology section of Delft University of Technology, was supervised by Jan-Maarten Geertman and Jack Pronk. This triggered her fascination for rational improvement of microorganisms as sustainable production systems. During her MSc studies, Eline performed a 6-month internship on membrane bioreactor (MBR) technology for wastewater treatment at the Berlin Centre of Competence for Water (Kompetenzzentrum Wasser Berlin), supervised by Boris Lesjean. Under the guidance of Guus Roeselers and Gerard Muyzer, Eline then completed her MSc thesis on the diversity and activity of uptake hydrogenase genes in phototrophic biofilms in the Environmental Biotechnology section of Delft University of Technology. In 2007, she returned to Industrial Microbiology to deepen her knowledge in this field during her PhD research, entitled "Towards fermentation of galacturonic acid-containing feedstocks with *Saccharomyces cerevisiae*", which was supervised by Ton van Maris and Jack Pronk. Currently, Eline works as program director at the Institute of Biotechnology Studies Delft Leiden (BSDL), an organisation for postdoctoral education of scientists from industry and academia in the field of biotechnology.

List of Publications

Huisjes, E.H., M.A.H. Luttik, P. P. de Waal, A. Teunissen, J.T. Pronk and A.J.A. van Maris. 2012. Physiological and transcriptional response of *Saccharomyces cerevisiae* towards galacturonic acid inhibition. Manuscript in preparation.

Huisjes, E.H., M.A.H. Luttik, M.J.H. Almering, M.M.M. Bisschops, D.H.N. Dang, M. Kleerebezem, R. Siezen, A.J.A. Van Maris and J.T. Pronk. 2012. Toward pectin fermentation by *Saccharomyces cerevisiae*: expression of the first two steps of a bacterial pathway for galacturonate metabolism. Journal of Biotechnology **162**:303-310.

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Acknowledgements

Nu het leukste stuk van dit proefschrift om te schrijven, al was het maar omdat het waarschijnlijk het meest gelezen stuk wordt. Er zijn zo onnoemelijk veel mensen die in enige mate hebben bijgedragen aan de totstandkoming van dit proefschrift, dat het vrijwel onmogelijk is hen allen te noemen. Met het risico mensen te vergeten, ga ik toch een poging wagen in dit dankwoord.

Allereerst wil ik mijn promotor, Jack Pronk, bedanken voor het vertrouwen, alle raad en advies, en de niet-aflatende stroom ideeën. Jouw enthousiasme werkt aanstekelijk, en dat had ik ook af en toe nodig. Ik vond (en vind) het fijn dat ik altijd bij je kon binnenwandelen om even snel, of niet zo snel, iets te bespreken. Mijn begeleider, Ton van Maris, wil ik bedanken voor zijn altijd kritische blik. Ton, ook jij bent erg toegankelijk, en dat werkt heel prettig. Naast de goede momenten en shop-dagen hebben we ook wat minder goede momenten uit deze jaren gedeeld, en ik wil je bedanken voor je steun. Gelukkig hoef ik nog geen afscheid te nemen van jullie, want we zullen nog veel samenwerken met de advanced courses.

Mijn promotieproject stond niet op zichzelf. Ik heb veel plezier gehad in de EOS-bijeenkomsten; dit heeft mij geholpen mijn horizon te verbreden, en mijn onderzoek in perspectief te plaatsen. De vaste groep: Henk Schols, Mirjam Kabel, Laurice Pouvreau en Stefan Kühnel van de Universiteit Wageningen, Jan Wery, Jaap Visser en Sandra Hinz van Dyadic, Jan de Bont, Aloys Teunissen, Inge Minneboo, Paul de Waal en Christiaan Koolloos van Nedalco, Hans Reith, Raimo van der Linden en Claudia Daza van ECN: hartelijk dank voor de samenwerking.

Hoewel de afgelopen jaren niet altijd even makkelijk waren, hebben alle geweldige collega's voor een goede werksfeer gezorgd. De stafanalisten Erik, Marinka, en Marijke zijn allen co-auteurs op minstens een van mijn artikelen, en niet voor niets. Zonder jullie jarenlange ervaring, advies, en experimentele hulp was dit boekje er niet geweest. Ik heb ontzettend veel van jullie geleerd, en met veel plezier samengewerkt. Erik, ik zal onze discussies over zoölogische melkproductie niet snel vergeten. Marinka, jouw nuchterheid, en lijfspreuk ("komt goed"), kon ik goed gebruiken. Marijke, met jou kan je het over alles hebben, en daar heb ik dankbaar gebruik van gemaakt!

I would like to thank my initial roommates Wouter, Derek and Léonie: as the youngest in the room, I learned a lot from you, and I enjoyed our scientific and non-scientific discussions. Also my later roomies Marit, Harmen, Yulia and Jules: thanks for the "gezelligheid". The long list of (former) colleagues illustrates that

IMB has changed a lot over the years, but the good atmosphere always remained. Many of you have contributed to this thesis in some way: Alexey, Ali, Ana, Andreas, Barbara C., Barbara K., Bart, Benjamin, Dani, Daniel, Diana, Dick, Duygu, Eleonora, Erwin, Filipa, Frank, Frieda, Gabriele, Guoliang, Han, Huong, Irina, Ishtar, Ishwar, Jean-Marc, Jeremiah, Joost, Jurgen, Lizanne, Lucie, Marcel, Maurice, Nick, Niels, Nuria, Pascale, Rintze, Rogier, Ruben, Stefan, Tânia, Theo, Thiago, Tim, Tracey, Victor, Zita, and all the students who did their internship with IMB: you were great, thanks!

Het begeleiden van studenten in hun bachelor- of master-eindstage vond ik een erg leuk onderdeel van het promotietraject. Inês, as my first student, you were the "guinea pig". I learned a lot from supervising you; thank you! Hoewel van het werk van zowel Inês als mijn latere studenten Bianca, Maaike, Mark en Hien slechts een fractie, of zelfs helemaal niets, in dit proefschrift is beland -wat overigens niet aan hen lag!-, wil ik jullie bedanken voor al het werk dat jullie hebben verzet! Mark, ik vind het heel leuk dat ook jij bent gaan promoveren bij IMB, en dat je nu zelfs letterlijk mijn plek hebt ingenomen!

De afdeling Biotechnologie is door de goede sfeer een leuke plek om te werken. Hoewel alle medewerkers hieraan bijdragen, wil ik een aantal specifiek bedanken, in willekeurige volgorde: Astrid, Apilena, Jannie, Sjaak, Jos, Arno, Marcel, Carlo, Elly, Mo, Kees, Elena, Astrid, Ginie, Herman, Hans, Robert en Nayyar, zonder jullie ondersteuning zou het lab niet zo vloeiend draaien. Esengül, bedankt dat ik als oud-EBT'er nog altijd in jouw lab mocht werken. Birdies, hoewel jullie al meerdere keren zijn verhuisd sinds ik begon, is het altijd gezellig om bij te kletsen onder het genot van thee / lunch / taart-van-Zita.

In het bijzonder wil ik Rintze en Stefan bedanken voor het kritisch lezen van mijn werk, Regine voor haar creativiteit, en tenslotte mijn paranimfen Marit en Tim: bedankt voor jullie hulp bij de voorbereidingen. Ik ben blij jullie tijdens de verdediging aan mijn zijde te hebben!

Tijdens een promotie is de indirecte bijdrage van vrienden en familie onmisbaar. Voor de broodnodige afleiding wil ik mijn vrienden (o.a. Bagijnhoffers, LST/LIFE'ers, jaarclubgenoten, GeNeYouS vrienden) bedanken. Mijn familie, en in het bijzonder mijn ouders, wil ik bedanken voor de kansen die ze mij geboden hebben, en hun steun en vertrouwen.

Tenslotte de belangrijkste: Bert, het is ook voor jou niet altijd makkelijk geweest. Bedankt voor je steun, geduld, en vertrouwen. We hebben het toch maar mooi voor elkaar gekregen met onze Siem en Tijn!