Journal of Biotechnology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

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

Q1 Eline H. Huisjes[a], Marijke A.H. Luttik[a], Marinka J.H. Almering[a], Markus M.M. Bisschops[a], Dieu H.N. Dang[a], Michiel Kleerebezem[b], Roland Siezen[c,d,e], Antonius J.A. van Maris[a], Jack T. Pronk[a,*]

[a] Department of Biotechnology, Delft University of Technology and Kluyver Centre for Genomics of Industrial Fermentation, Julianalaan 67, 2628 BC Delft, The Netherlands
[b] NIZO Food Research, PO Box 20, 6710 BA Ede, The Netherlands
[c] Centre for Molecular and Biomolecular Informatics (CMBI), Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB, The Netherlands
[d] TI Food and Nutrition, PO Box 557, 6700 AN Wageningen, The Netherlands
[e] Netherlands Bioinformatics Centre (NBIC), PO Box 9101, The Netherlands

ARTICLE INFO

Article history:
Received 16 July 2012
Received in revised form 4 October 2012
Accepted 8 October 2012
Available online xxx

Keywords:
Pectin
D-Galacturonate
Ethanol
Metabolic engineering
D-Galacturonate isomerase
D-Tagaturonate reductase
Saccharomyces cerevisiae

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 *gpd1Δ gpd2Δ 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*.

1. 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 hydrolyzed 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, unpublished data).

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., 2005, 2006; Liepins et al., 2006). However, the involvement of both NADP(H)

* Corresponding author. Tel.: +31 152783214; fax: +31 152782355.
E-mail address: J.T.Pronk@TUDelft.NL (J.T. Pronk).

0168-1656/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jbiotec.2012.10.003

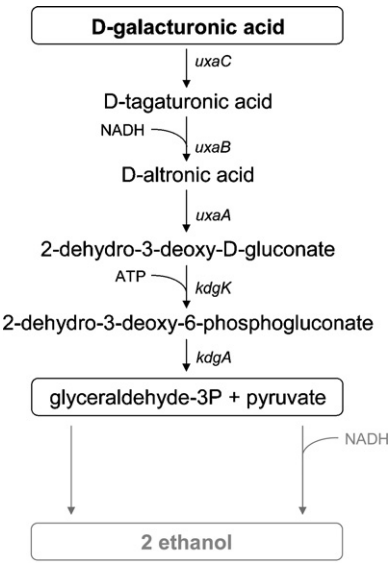

Fig. 1. Pathway for bacterial metabolism of D-galacturonate. Codes indicate the following genes and enzymes: *uxaC*, D-galacturonate isomerase (EC 5.3.1.12) *uxaB*, D-tagaturonate reductase (EC 1.1.1.58); *uxaA*, D-altronate dehydratase (EC 4.2.1.7); *kdgK*, 2-keto-3-deoxygluconate kinase (EC 2.7.1.45);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

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 (Fig. 1) involves five enzyme-catalyzed 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.

2. Materials and methods

2.1. 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, auxotrophic requirements were added at concentrations calculated according to Pronk (2002). After addition of 30% (v/v) glycerol to stationary phase cultures, 1-ml aliquots were stored at −80 °C.

2.2. 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-Prep^{TM} 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 l^{-1}) or kanamycin (50 mg 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.

Plasmids used in this study are listed in Table 1. 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

Please cite this article in press as: Huisjes, E.H., et al., Toward pectin fermentation by *Saccharomyces cerevisiae*: Expression of the first two steps of a bacterial pathway for D-galacturonate metabolism. J. Biotechnol. (2012), http://dx.doi.org/10.1016/j.jbiotec.2012.10.003

Table 1
S. cerevisiae strains used in this study.

Strain	Genotype	Source
CEN.PK 113-7D	*MATa MAL2-8c SUC2*	Entian and Kötter (2007)
CEN.PK 113-5A	*MATa MAL2-8c SUC2 URA3 trp1-289 leu2-3,112 his3-Δ1*	Entian and Kötter (2007)
CEN.PK 2-1C	*MATa MAL2-8c SUC2 ura3-52 trp1-289 leu2-3,112 his3Δ1*	Entian and Kötter (2007)
IME083	CEN.PK 113-5A pUDE049(*LEU2*)	This study
IME084	CEN.PK 113-5A pUDE058(*LEU2*)	This study
IME139	CEN.PK 113-5A 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.PK 2-1C pUDE23(*LEU2*) pUDi24(*URA3*)	This study
IME092	CEN.PK 113-5A 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)

P_{TPI1}-*kdgK*-T_{ADH1} and P_{TEF1}-*kdgA*-T_{CYC1} expression cassettes were subsequently cloned in the pBC P_{TDH3}-*uxaA*-T_{TEF} 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* 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 (GenBank 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 (NC_004663.1) and *L. lactis* subsp. *lactis* KF147 (CP001834.1) were codon optimized by Base Clear (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 2). 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.

2.3. 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 (OD_{660}), 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 2) were used as negative and positive controls, respectively.

2.4. 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.

2.5. Enzyme assays

Cell extracts were prepared by sonication from exponentially growing *S. cerevisiae* cultures and analyzed for protein content

Please cite this article in press as: Huisjes, E.H., et al., Toward pectin fermentation by *Saccharomyces cerevisiae*: Expression of the first two steps of a bacterial pathway for D-galacturonate metabolism. J. Biotechnol. (2012), http://dx.doi.org/10.1016/j.jbiotec.2012.10.003

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	2 μm, *HIS3*	Christianson et al. (1992)
pRS424	2 μm, *TRP1*	Christianson et al. (1992)
pRS425	2 μm, *LEU2*	Christianson et al. (1992)
pRS426	2 μm, *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.

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).

2.6. 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: $E^{\Delta C_T(\text{reference-target})}$.

3. Results

3.1. 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).

3.2. 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 galaturonate 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]$^{-1}$.

3.3. 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 (Fig. 2). Optimization of codon usage can strongly improve expression of prokaryotic genes in *S. cerevisiae*, as illustrated by

Please cite this article in press as: Huisjes, E.H., et al., Toward pectin fermentation by *Saccharomyces cerevisiae*: Expression of the first two steps of a bacterial pathway for D-galacturonate metabolism. J. Biotechnol. (2012), http://dx.doi.org/10.1016/j.jbiotec.2012.10.003

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.

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.

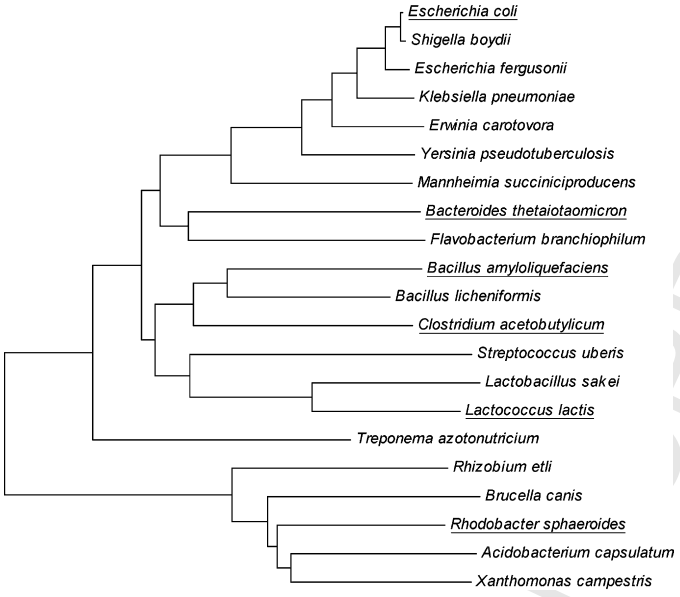

Fig. 2. Phylogenetic tree of bacterial genes encoding D-galacturonate isomerase (*uxaC*). The underlined species names indicate *uxaC* genes that were included in the present study. The nucleotide sequences were obtained from GenBank (accession numbers, from top to bottom in the figure, CP000948.1, CP001063.1, 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).

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 tenfold higher than that in the next best strain, 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*.

3.4. *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

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

that were approximately twofold 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 l^{-1} glucose, D-galactose or ethanol combined with 2 or 10 g l^{-1} D-galacturonate), no significant co-consumption of D-galacturonate was observed (data not shown).

To specifically investigate the *in vivo* activity of the *uxaB* and *uxaC* genes, they were expressed in an *S. cerevisiae gpd1Δ gpd2Δ* (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 (Fig. 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*.

4. 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 tenfold 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 μmol min^{-1} [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 g^{-1} 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

Table 5
In vitro enzyme activities mRNA levels of the bacterial D-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.

Please cite this article in press as: Huisjes, E.H., et al., Toward pectin fermentation by *Saccharomyces cerevisiae*: Expression of the first two steps of a bacterial pathway for D-galacturonate metabolism. J. Biotechnol. (2012), http://dx.doi.org/10.1016/j.jbiotec.2012.10.003

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 g biomass^{-1} h^{-1} at 20 g 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).

5. 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 yielded 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.

References

Amore, R., Wilhelm, M., Hollenberg, C.P., 1989. The fermentation of xylose – an analysis of the expression of *Bacillus* and *Actinoplanes* xylose isomerase genes in yeast. Applied Microbiology and Biotechnology 30, 351–357.
Ashwell, G., Wahba, A.J., Hickman, J., 1960. Uronic acid metabolism in bacteria. 1. Purification and properties of uronic acid isomerase in *Escherichia coli*. Journal of Biological Chemistry 235, 1559–1565.
Attfield, P.V., Bell, P.J.L., 2006. Use of population genetics to derive nonrecombinant *Saccharomyces cerevisiae* strains that grow using xylose as a sole carbon source. FEMS Yeast Research 6, 862–868.
Bakker, B.M., Overkamp, K.M., Van Maris, A.J.A., Kötter, P., Luttik, M.A.H., Van Dijken, J.P., Pronk, J.T., 2001. Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiology Review 25, 15–37.
Barnett, J.A., Payne, R.W., Yarrow, D., 1990. A Guide to Identifying and Classifying Yeasts. Cambridge University Press
Becker, J., Boles, E., 2003. A modified *Saccharomyces cerevisiae* strain that consumes L-arabinose and produces ethanol. Applied and Environment Microbiology 69, 4144–4150.
Björkqvist, S., Ansell, R., Adler, L., Lidén, G., 1997. Physiological response to anaerobicity of glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. Applied and Environment Microbiology 63, 128–132.
Brat, D., Boles, E., Wiedemann, B., 2009. Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. Applied and Environment Microbiology 75, 2304–2311.
Burke, D., Dawson, D., Stearns, T., Laboratory, C.S.H., 2000. Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual. Cold Spring Harbor Laboratory Press.
Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., Hieter, P., 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene 110, 119–122.
Cynkin, M.A., Ashwell, G., 1960. Uronic acid metabolism in bacteria. 4. Purification and properties of 2-keto-3-deoxy-D-gluconokinase in *Escherichia coli*. Journal of Biological Chemistry 235, 1576–1579.
Doran, J.B., Cripe, J., Sutton, M., Foster, B., 2000. Fermentations of pectin-rich biomass with recombinant bacteria to produce fuel ethanol. Applied Biochemistry and Biotechnology 84 (6), 141–152.
Edwards, M., Doran-Peterson, J., 2012. Pectin-rich biomass as feedstock for fuel ethanol production. Applied Microbiology and Biotechnology, http://dx.doi.org/10.1007/s00253-012-4173-2 (epub ahead of print).
Ehrlich, F., Guttmann, R., 1934. Zur Kenntnis der *d*-Galakturonsäure, II. Mitteil.: Ihre Umlagerung in 5-Keto-*l*-galaktonsäure. Berichte der Deutschen Chemischen Gesellschaft 67, 573–589.
Entian, K.D., Kötter, P., 2007. Yeast genetic strain and plasmid collections. In: Stansfield, I., Stark, M.J.R. (Eds.), Methods in Microbiology. Academic Press, pp. 629–666.
Gárdonyi, M., Hahn-Hägerdal, B., 2003. The *Streptomyces rubiginosus* xylose isomerase is misfolded when expressed in *Saccharomyces cerevisiae*. Enzyme and Microbial Technology 32, 252–259.
Grohmann, K., Bothast, R.J., 1994. Pectin-rich residues generated by processing of citrus fruits, apples, and sugar beets – enzymatic hydrolysis and biological conversion to value-added products. ACS Symposium Series 566, 372–390.
Guadalupe Medina, V.G., Almering, M.J.H., Van Maris, A.J.A., Pronk, J.T., 2010. Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. Applied and Environment Microbiology 76, 190–195.
Güldener, U., Heck, S., Fiedler, T., Beinhauer, J., Hegemann, J.H., 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Research 24, 2519–2524.
Hahn-Hägerdal, B., Karhumaa, K., Jeppsson, M., Gorwa-Grauslund, M., 2007. Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. In: Olsson, L. (Ed.), Biofuels. Springer, Berlin/Heidelberg, pp. 147–177.
Hensing, M.C.M., Bangma, K.A., Raamsdonk, L.M., Hulster, E., Dijken, J.P., Pronk, J.T., 1995. Effects of cultivation conditions on the production of heterologous α-galactosidase by *Kluyveromyces lactis*. Applied Microbiology and Biotechnology 43, 58–64.
Hickman, J., Ashwell, G., 1960. Uronic acid metabolism in bacteria. 2. Purification and properties of D-altronic acid and D-mannonic acid dehydrogenases in *Escherichia coli*. Journal of Biological Chemistry 235, 1566–1570.
Hilditch, S., Berghall, S., Kalkkinen, N., Penttila, M., Richard, P., 2007. The missing link in the fungal D-galacturonate pathway; identification of the L-threo-3-deoxy-hexulosonate aldolase. Journal of Biological Chemistry 282, 26195–26201.
Hong, K.-K., Nielsen, J., 2012. Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries. Cellular and Molecular Life Sciences, 1–20.
Huisjes, E.H., De Hulster, E., Van Dam, J.C., Pronk, J.T., Van Maris, A.J.A., 2012. Galacturonic acid inhibits growth of *Saccharomyces cerevisiae* on galactose, xylose and arabinose. Applied and Environment Microbiology, http://dx.doi.org/10.1128/aem.07617-11 (epub ahead of print).
Janke, C., Magiera, M.M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E., Knop, M., 2004. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast 21, 947–962.
Kovachevich, R., Wood, W.A., 1955. Carbohydrate metabolism by *Pseudomonas fluorescens*. IV. Purification and properties of 2-keto-3-deoxy-6-phosphogluconate aldolase. Journal of Biological Chemistry 213, 757–767.

Please cite this article in press as: Huisjes, E.H., et al., Toward pectin fermentation by *Saccharomyces cerevisiae*: Expression of the first two steps of a bacterial pathway for D-galacturonate metabolism. J. Biotechnol. (2012), http://dx.doi.org/10.1016/j.jbiotec.2012.10.003

Kuorelahti, S., Jouhten, P., Maaheimo, H., Penttila, M., Richard, P., 2006. l-Galactonate dehydratase is part of the fungal path for D-galacturonic acid catabolism. Molecular Microbiology 61, 1060–1068.
Kuorelahti, S., Kalkkinen, N., Penttila, M., Londesborough, J., Richard, P., 2005. Identification in the mold *Hypocrea jecorina* of the first fungal D-galacturonic acid reductase. Biochemistry 44, 11234–11240.
Liepins, J., Kuorelahti, S., Penttila, M., Richard, P., 2006. Enzymes for the NADPH-dependent reduction of dihydroxyacetone and d-glyceraldehyde and l-glyceraldehyde in the mould *Hypocrea jecorina*. FEBS Journal 273, 4229–4235.
Linster, C.L., Van Schaftingen, E., 2004. A spectrophotometric assay of d-glucuronate based on *Escherichia coli* uronate isomerase and mannonate dehydrogenase. Protein Expression and Purification 37, 352–360.
Martens-Uzunova, E.S., Schaap, P.J., 2008. An evolutionary conserved D-galacturonic acid metabolic pathway operates across filamentous fungi capable of pectin degradation. Fungal Genetics and Biology 45, 1449–1457.
Mata-Gilsinger, M., Ritzenthaler, P., 1983. Physical mapping of the exuT and uxaC operators by use of exu plasmids and generation of deletion mutants in vitro. Journal of Bacteriology 155, 973–982.
Micard, V., Renard, C.M.G.C., Thibault, J.F., 1996. Enzymatic saccharification of sugar-beet pulp. Enzyme and Microbial Technology 19, 162–170.
Moes, C.J., Pretorius, I.S., Zyl, W.H., 1996. Cloning and expression of the Clostridium thermosulfurogenes D-xylose isomerase gene (xyLA) in *Saccharomyces cerevisiae*. Biotechnology Letters 18, 269–274.
Mumberg, D., Müller, R., Funk, M., 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156, 119–122.
Naesby, M., Nielsen, S., Nielsen, C., Green, T., Tange, T., Simon, E., Knechtle, P., Hansson, A., Schwab, M., Titiz, O., Folly, C., Archila, R., Maver, M., Van Sint Fiet, S., Boussemghoune, T., Janes, M., Kumar, A., Sonkar, S., Mitra, P., Benjamin, V., Korrapati, N., Suman, I., Hansen, E., Thybo, T., Goldsmith, N., Sorensen, A., 2009. Yeast artificial chromosomes employed for random assembly of biosynthetic pathways and production of diverse compounds in *Saccharomyces cerevisiae*. Microbial Cell Factories 8, 45.
Nevoigt, E., 2008. Progress in metabolic engineering of *Saccharomyces cerevisiae*. Microbiology and Molecular Biology Reviews 72, 379–412.
Nijkamp, J., Van den Broek, M., Datema, E., De Kok, S., Bosman, L., Luttik, M., Daran-Lapujade, P., Vongsangnak, W., Nielsen, J., Heijne, W., Klaassen, P., Paddon, C., Platt, D., Kotter, P., Van Ham, R., Reinders, M., Pronk, J., De Ridder, D., Daran, J.-M., 2012. De novo sequencing, assembly and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology. Microbial Cell Factories 11, 36.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29, e45.
Piper, M.D.W., Daran-Lapujade, P., Bro, C., Regenberg, B., Knudsen, S., Nielsen, J., Pronk, J.T., 2002. Reproducibility of oligonucleotide microarray transcriptome analyses. Journal of Biological Chemistry 277, 37001–37008.
Postma, E., Verduyn, C., Scheffers, W.A., Van Dijken, J.P., 1989. Enzymic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Applied and Environment Microbiology 55, 468–477.
Pronk, J.T., 2002. Auxotrophic yeast strains in fundamental and applied research. Applied and Environment Microbiology 68, 2095–2100.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406–425.
Sarthy, A.V., McConaughy, B.L., Lobo, Z., Sundstrom, J.A., Furlong, C.E., Hall, B.D., 1987. Expression of the *Escherichia coli* xylose isomerase gene in *Saccharomyces cerevisiae*. Applied and Environment Microbiology 53, 1996–2000.
Schmitt, M.E., Brown, T.A., Trumpower, B.L., 1990. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Research 18, 3091–3092.
Sedlak, M., Ho, N.W.Y., 2001. Expression of *E. coli* araBAD operon encoding enzymes for metabolizing L-arabinose in *Saccharomyces cerevisiae*. Enzyme and Microbial Technology 28, 16–24.
Sikorski, R.S., Hieter, P., 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122, 19–27.
Smiley, J.D., Ashwell, G., 1960. Uronic acid metabolism in bacteria. 3. Purification and properties of D-altronic acid and D-mannonic acid dehydrases in *Escherichia coli*. Journal of Biological Chemistry 235, 1571–1575.
Souffriau, B., den Abt, T., Thevelein, J.M., 2012. Evidence for rapid uptake of D-galacturonic acid in the yeast *Saccharomyces cerevisiae* by a channel-type transport system. FEBS Letters, http://dx.doi.org/10.1016/j.febslet.2012.06.012 (epub ahead of print).
Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences of the United States of America 101, 11030–11035.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28, 2731–2739.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22, 4673–4680.
Van Dijken, J.P., Scheffers, W.A., 1986. Redox balances in the metabolism of sugars by yeasts. FEMS Microbiology Letters 32, 199–224.
Van Hoek, P., Van Dijken, J.P., Pronk, J.T., 2000. Regulation of fermentative capacity and levels of glycolytic enzymes in chemostat cultures of *Saccharomyces cerevisiae*. Enzyme and Microbial Technology 26, 724–736.
Van Hylckama Vlieg, J.E.T., Rademaker, J.L.W., Bachmann, H., Molenaar, D., Kelly, W.J., Siezen, R.J., 2006. Natural diversity and adaptive responses of *Lactococcus lactis*. Current Opinion in Biotechnology 17, 183–190.
Van Maris, A., Winkler, A., Kuyper, M., De Laat, W., Van Dijken, J., Pronk, J., 2007. Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose isomerase as a key component. In: Olsson, L. (Ed.), Adv. Biochem. Eng./Biotechnol. Springer, Berlin/Heidelberg, pp. 179–204.
Van Maris, A.J.A., Abbott, D.A., Bellissimi, E., Van den Brink, J., Kuyper, M., Luttik, M.A.H., Wisselink, H.W., Scheffers, W.A., Van Dijken, J.P., Pronk, J.T., 2006. Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Van Leeuwenhoek 90, 391–418.
Verduyn, C., Postma, E., Scheffers, W.A., Van Dijken, J.P., 1990. Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. Journal of General Microbiology 136, 395–403.
Verduyn, C., Postma, E., Scheffers, W.A., Van Dijken, J.P., 1992. Effect of benzoic-acid on metabolic fluxes in yeasts – a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8, 501–517.
Walfridsson, M., Bao, X., Anderlund, M., Lilius, G., Bülow, L., Hahn-Hägerdal, B., 1996. Ethanolic fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus xylA* gene, which expresses an active xylose (glucose) isomerase. Applied and Environment Microbiology 62, 4648–4651.
Weber, C., Farwick, A., Benisch, F., Brat, D., Dietz, H., Subtil, T., Boles, E., 2010. Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels. Applied Microbiology and Biotechnology 87, 1303–1315.
Wiedemann, B., Boles, E., 2008. Codon-optimized bacterial genes improve L-arabinose fermentation in recombinant *Saccharomyces cerevisiae*. Applied and Environment Microbiology 74, 2043–2050.
Wisselink, H.W., Cipollina, C., Oud, B., Crimi, B., Heijnen, J.J., Pronk, J.T., Van Maris, A.J.A., 2010. Metabolome, transcriptome and metabolic flux analysis of arabinose fermentation by engineered *Saccharomyces cerevisiae*. Metabolic Engineering 12, 537–551.
Wisselink, H.W., Toirkens, M.J., Del Rosario Franco Berriel, M., Winkler, A.A., van Dijken, J.P., Pronk, J.T., Van Maris, A.J.A., 2007. Engineering of *Saccharomyces cerevisiae* for efficient anaerobic alcoholic fermentation of L-arabinose. Applied and Environment Microbiology 73, 4881–4891.
Wisselink, H.W., Toirkens, M.J., Wu, Q., Pronk, J.T., Van Maris, A.J.A., 2009. Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. Applied and Environment Microbiology 75, 907–914.

Please cite this article in press as: Huisjes, E.H., et al., Toward pectin fermentation by *Saccharomyces cerevisiae*: Expression of the first two steps of a bacterial pathway for D-galacturonate metabolism. J. Biotechnol. (2012), http://dx.doi.org/10.1016/j.jbiotec.2012.10.003