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Galacturonic Acid Inhibits the Growth of *Saccharomyces cerevisiae* on Galactose, Xylose, and Arabinose

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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 pK_a value of galacturonic acid (3.51), the addition of 10 g · liter^{-1} galacturonic acid did not affect galactose fermentation kinetics and growth. In cultures grown at pH 3.5, the addition of 10 g · liter^{-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 nondissociated 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.

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 (19, 39). 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 (31).

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 (16, 32, 39). 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 hydrolyzed and used as a substrate for fermentation (10, 14). 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 (14) but instead contain a significant amount of pectin (14, 29). Pectin is a complex polysaccharide that consists of a backbone of galacturonic acid residues and can have side chains containing various neutral sugars (30). In addition, the polymer can be methylesterified and acetylated (30).

Hydrolysates of sugar beet pulp and citrus peel consist of predominantly glucose, galactose, arabinose, xylose, and galacturonic acid (29) (Table 1). Glucose and galactose are consumed sequentially by wild-type *S. cerevisiae* strains (see, e.g., reference 20). Galactose is metabolized via the Leloir pathway (11), which is repressed by glucose and induced by galactose (13, 26, 28). The inability of some *S. cerevisiae* strains to switch from anaerobic glucose-limited growth to galactose consumption illustrates the energetic costs associated with the expression of the Leloir pathway enzymes (37). Although *S. cerevisiae* cannot naturally ferment the pentose sugars xylose and arabinose, this limitation has been alleviated through various metabolic engineering strategies (4, 22–24, 44). 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 (pK_a) of 3.51 (21). 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 nondissociated forms (1, 3, 32, 33). 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. cerevi-*

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

Substrate	Weight fraction of feedstock (%)	
	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
Noncarbohydrate		
Lignin	1.5	3.0
Protein	5.8	6.3
Ash	8.2	4.0

[a] Data adapted from reference 14.

siae. 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 (45).

MATERIALS AND METHODS

Strains and maintenance. Stock cultures of *S. cerevisiae* laboratory reference strain CEN.PK 113-7D were grown in shake flasks in 100 ml medium containing 1% (wt/vol) Bacto yeast extract, 2% (wt/vol) Bacto peptone, and 2% (wt/vol) glucose. Pentose-fermenting *S. cerevisiae* strain IMS0010 (45) was cultivated in synthetic medium (41) containing 2% (wt/vol) 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 (41) was performed at 30°C in an orbital shaker (200 rpm). The pH of the medium was set to 6.0 with 2 M KOH prior to sterilization. Precultures were prepared by the inoculation of 100 ml medium with a 1-ml glycerol stock. Galactose (2%, wt/vol) was used as a carbon and energy source for *S. cerevisiae* CEN.PK 113-7D, and 2% (wt/vol) 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 · liter^{-1} glucose and 10 g · liter^{-1} galactose or 20 g · liter^{-1} glucose, 10 g · liter^{-1} arabinose, and 10 g · liter^{-1} xylose was supplemented with 0.3 g · liter^{-1} silicone antifoam (antifoam C; Sigma-Aldrich, St. Louis, MO) as well as with the anaerobic growth factors ergosterol (0.01 g · liter^{-1}) and Tween 80 (0.42 g · liter^{-1}) dissolved in ethanol. If galacturonic acid was added to cultures, this was filter sterilized separately. Cultures with 10 g · liter^{-1} glucose and 10 g · liter^{-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 2 M KOH. Cultures were stirred at 800 rpm and sparged with 0.5 liters · 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. (35). 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 analyzed by high-performance liquid chromatography (HPLC) analysis on a Waters Alliance 2690 HPLC instrument (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 · liter^{-1} H_2SO_4 at a flow rate of 0.6 ml · 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. (15).

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 · liter^{-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 analyzer (Rosemount Analytical, Orrville, OH). The exhaust gas flow rates and carbon dioxide production rates were determined as described previously (40). 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. (5).

Enzyme activity assays. Cell extracts for galactokinase activity assays were prepared from exponentially growing shake flask cultures with galactose as the carbon source and analyzed for protein content as described previously (35). Galactokinase was assayed with freshly prepared cell extracts according to methods described previously (37), 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 · liter^{-1} solution of galacturonic acid in 1 M 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 · liter^{-1} galacturonic acid.

Measurement of galacturonic acid derivatives. Two independent batch cultures with 10 g · liter^{-1} glucose, 10 g · liter^{-1} galactose, and 10 g · liter^{-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 (7). 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) (36). 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 (Fig. 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 off 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 (Fig. 1A) and those grown at pH 3.5 (Fig.

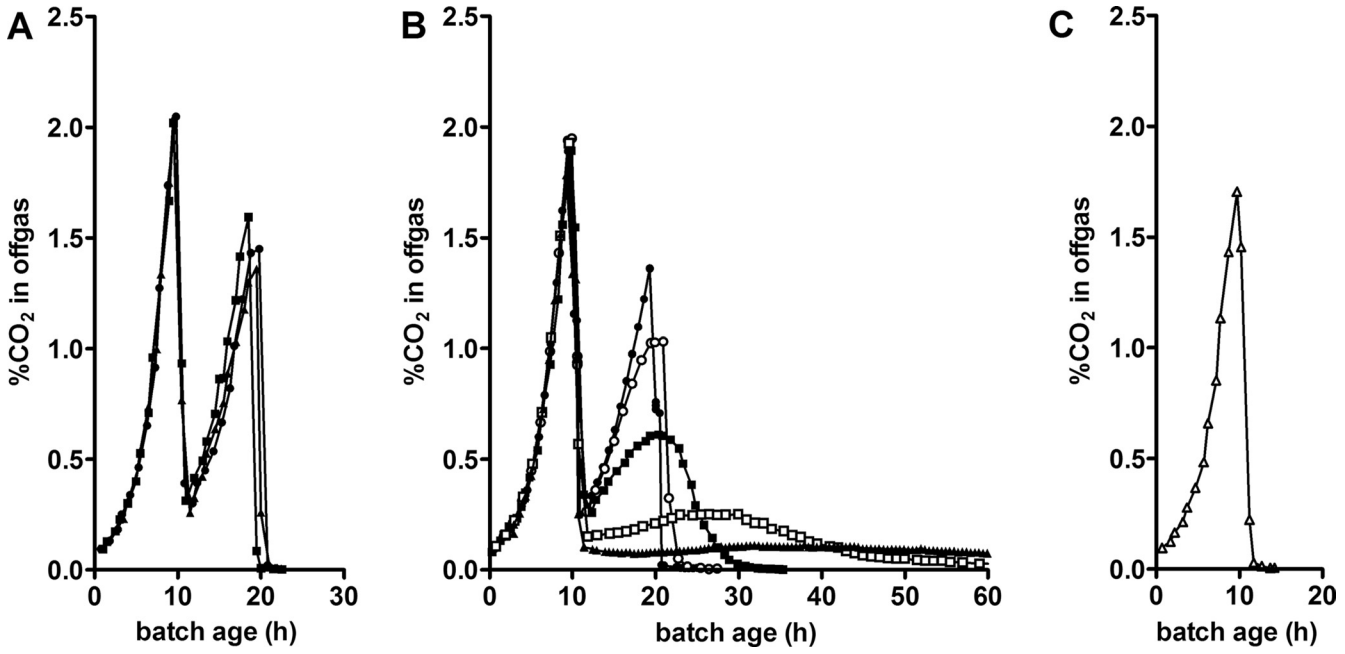

FIG 1 Impact of galacturonic acid on performance of *S. cerevisiae* CEN.PK 113-7D during growth on glucose-galactose mixtures in batch fermentations. The result of one representative batch experiment is shown for each condition. Replicate experiments yielded essentially the same results. Fermentation performance is indicated by the CO_2 (percent) in the exhaust gas of anaerobic batch cultures of *S. cerevisiae* CEN.PK 113-7D, which were flushed with nitrogen gas at a constant rate of 0.5 liters · liter^{-1} · h^{-1}. (A) Cultures grown at pH 5.0 on a mixture of 10 g · liter^{-1} glucose and 10 g · liter^{-1} galactose with either 0 g · liter^{-1} (●), 5 g · liter^{-1} (■), or 10 g · liter^{-1} (▲) galacturonic acid. (B) Cultures grown at pH 3.5 on a mixture of 10 g · liter^{-1} glucose, 10 g · liter^{-1} galactose, and either 0 g · liter^{-1} (●), 2.5 g · liter^{-1} (○), 5 g · liter^{-1} (■), 7.5 g · liter^{-1} (□), or 10 g · liter^{-1} (▲) galacturonic acid. (C) Cultures grown at pH 3.5 on a mixture of 10 g · liter^{-1} glucose and 10 g · liter^{-1} glucuronic acid (△).

1B). The first peak in the CO_2 profile, which represents the glucose consumption phase (Fig. 2), 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 · liter^{-1} each) with galacturonic acid concentrations of up to 10 g · liter^{-1}. At pH 5.0, where 97% of the galacturonic acid is present as the anion, the addition of either 5 or 10 g · liter^{-1} galacturonate did not influence the glucose consumption phase and had only a minor

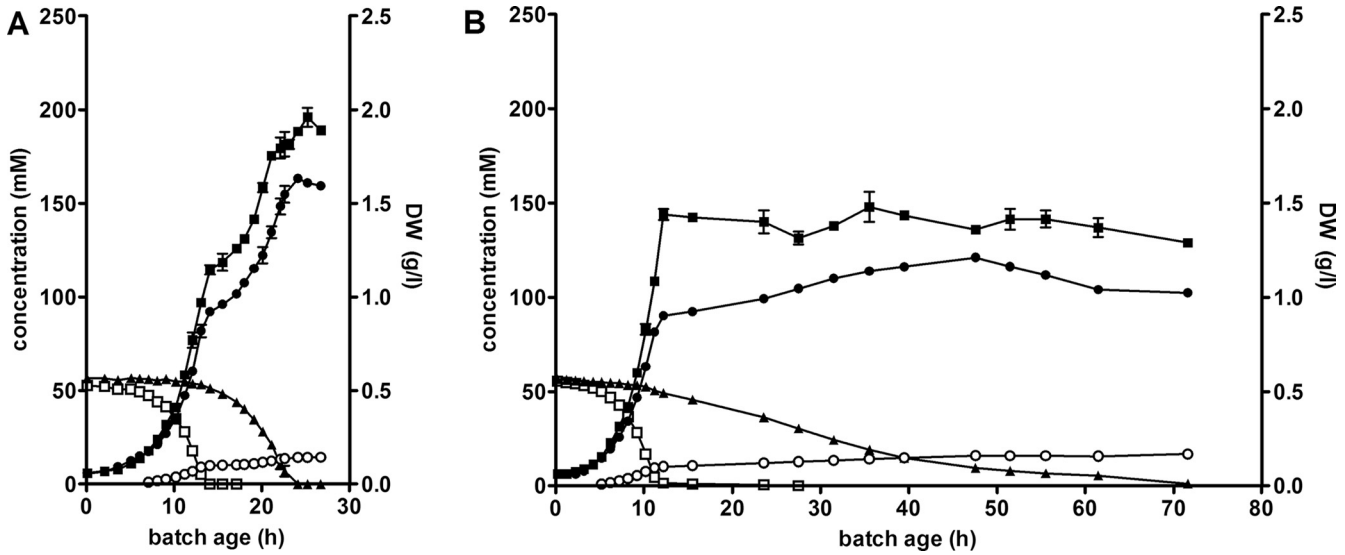

FIG 2 Growth and metabolite production in anaerobic batch cultures of *S. cerevisiae* CEN. PK113-7D cultivated in duplicate at pH 3.5 on a mixture of 10 g · liter^{-1} glucose (□) and 10 g · liter^{-1} galactose (▲) in the absence of galacturonic acid (A) and in the presence of 10 g · liter^{-1} galacturonic 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.PK 113-7D grown on a mixture of 10 g · liter^{-1} glucose and 10 g · liter^{-1} galactose in the presence and absence of 10 g · liter^{-1} galacturonic acid

Culture	Phase	Avg μ (h^{-1}) ± SD	Avg q_s (mmol · g^{-1} · h^{-1}) ± SD	Avg $q_{ethanol}$ (mmol · g^{-1} · h^{-1}) ± SD	Avg Y_{sx} (g · g^{-1}) ± 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
With 10 g · liter^{-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.

influence on the CO_2 profiles during the galactose consumption phase (Fig. 1A).

At pH 3.5, half of the galacturonic acid (pK_a = 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 · liter^{-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 (Fig. 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 · liter^{-1}, galacturonic acid increased the duration of the galactose consumption phase by 27% (from 11 h to 14 h). At 5.0 g · liter^{-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 · liter^{-1} and 10 g · liter^{-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 (2).

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 · liter^{-1} glucose and 10 g · liter^{-1} galactose with and without 10 g · liter^{-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 (Fig. 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]$^{-1}$ · h^{-1}; $P < 0.09$).

Consistent with the strongly reduced rates of CO_2 production (Fig. 1), the addition of 10 g · liter^{-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)$^{-1}$ · h^{-1} (Table 2). In the presence of 10 g · liter^{-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 (Fig. 2). Glycerol formation (at a low osmotic pressure) is coupled to the reoxidation of excess NADH (38), 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 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 · liter^{-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 (45), 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 · liter^{-1} glucose, 10 g · liter^{-1} xylose, and 10 g · liter^{-1} arabinose in anaerobic bioreactors in the presence and absence of 10 g · liter^{-1} galacturonic acid at pH 3.5 (Fig. 3). In the absence of galacturonic acid, growth and metabolism were essentially the same as those described previously for growth at pH 5.0 (45): first, glucose was consumed, followed by the simultaneous and complete consumption of xylose and arabinose within 40 h (Fig. 3A). The addition of 10 g · liter^{-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 · liter^{-1}, part of the xylose (28%) and part of the arabinose (14%) were coconsumed, 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 (Fig. 3B), clearly indicating that galacturonic acid also inhibits pentose fermentation in *S. cerevisiae* IMS0010.

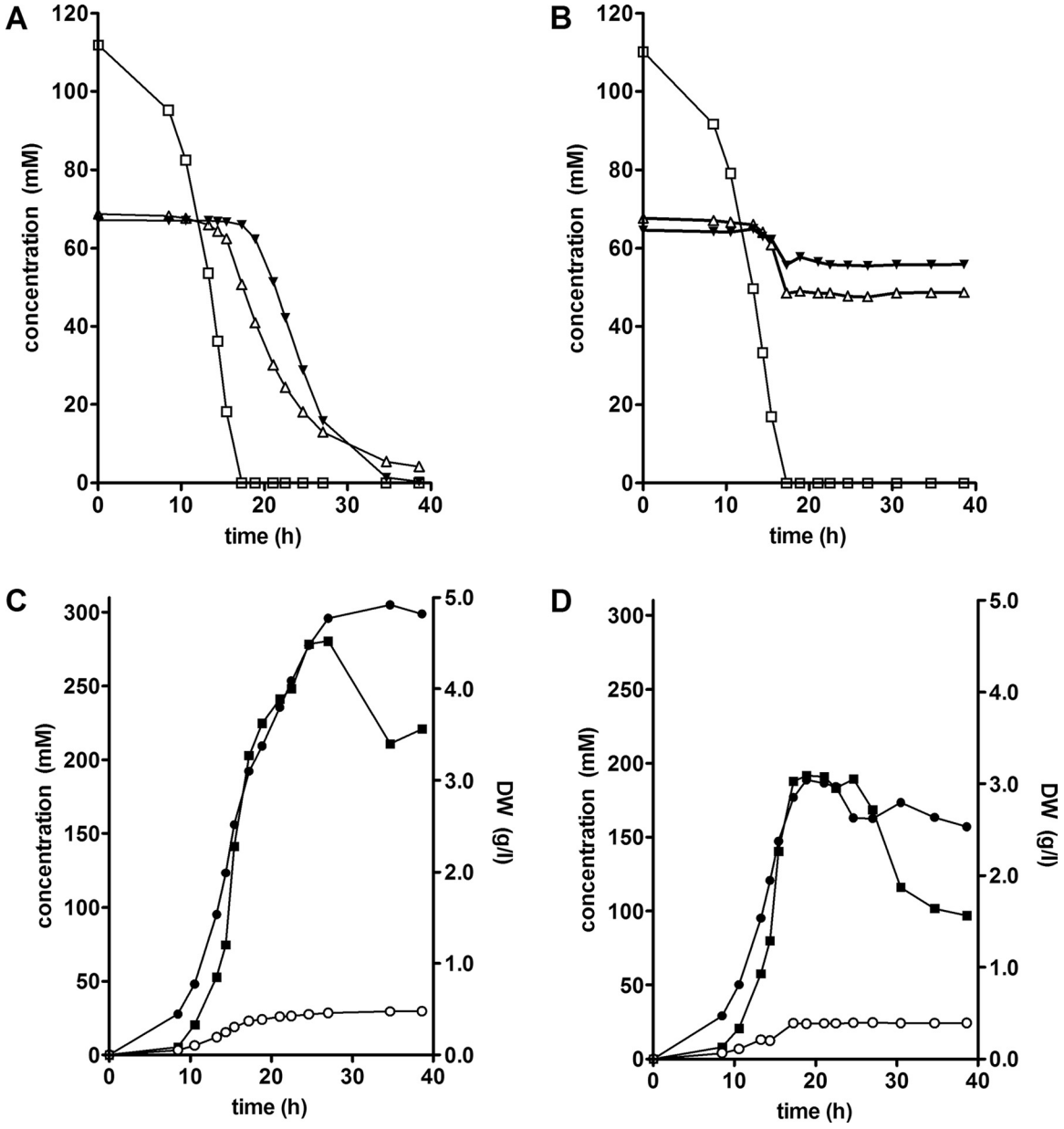

FIG 3 Growth and metabolite production in anaerobic batch cultures of *S. cerevisiae* IMS0010 grown at pH 3.5 on a mixture of 20 g · liter^{-1} glucose (□), 10 g · liter^{-1} xylose (△), and 10 g · liter^{-1} arabinose (▼) in the absence of galacturonic acid (A and C) and in the presence of 10 g · liter^{-1} 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.

Glucuronic acid does not inhibit glucose fermentation. Glucuronic acid (pK_a of 3.28) is the uronic acid derived from glucose (21). 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, anaerobic batch cultures were grown at pH 3.5 on 10 g · liter^{-1} glucose and 10 g · liter^{-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 (Fig. 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 (13, 26, 28) and in IMS0010 cells growing on arabinose (43). 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 · liter^{-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 · 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 · 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 · liter^{-1} glucose and 10 g · liter^{-1} galactose in the presence of 10 g · liter^{-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 μmol · 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 catalyzed by galactose-1-phosphate uridyltransferase (Gal7p), did not differ from that of the control and remained below 13 nmol · 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 (43), 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 (Fig. 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 (E. H. Huisjes et al., unpublished data). However, the fact that galacturonic acid also inhibits the fermentation of xylose, which is not (solely) transported by Gal2p (17, 47), 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 (33). 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} (6). The ATP production associated with the observed galactose consumption rate at pH 3.5 in the presence of 10 g · liter^{-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 · liter^{-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 impacts 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 μmol · 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 (7). Although not previously described for galacturonic acid 1-phosphate, the inhibitory effects of other phosphorylated compounds have been well documented (8, 12, 18, 27, 34). High levels of UDP-sugars can also have toxic effects (9), 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 (42). 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 (25, 45). 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 (3). 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 (45) and the fermentation kinetics of engineered strains for the nonnative substrate xylose in the presence of acetic acid (3, 46). 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 (14, 39), there are many advantages that make yeast the desired organism for bioethanol production from pectin-rich second-generation feedstocks (39). The implications of the implementation of metabolic routes from other microorganisms that can naturally consume galacturonic acid in *S. cerevisiae* were described previously (39). 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.

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