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Author(s)	Joosu Kuivanen, Dominik Mojzita, Yanming Wang, Satu Hilditch, Merja Penttilä, Peter Richard, Marilyn G. Wiebe
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Engineering filamentous fungi for the conversion of D-galacturonic acid to L-galactonic acid

Joosu Kuivanen, Dominik Mojzita, Yanming Wang, Satu Hilditch, Merja Penttilä, Peter Richard, Marilyn G. Wiebe[#]

VTT Technical Research Centre of Finland, Tietotie 2, Espoo, Finland

Running title: Fungal conversion of D-galacturonate to L-galactonate

[#] Address correspondence to Marilyn G. Wiebe, Marilyn.wiebe@vtt.fi

Abstract

D-galacturonic acid, the main monomer of pectin, is an attractive substrate for bioconversions, since pectin rich biomass is abundantly available and pectin is easily hydrolysed. L-Galactonic acid is an intermediate in the eukaryotic pathway for D-galacturonic acid catabolism, but extracellular accumulation of L-galactonic acid has not been reported. By deleting the gene encoding L-galactonic acid dehydratase (*lgd1* or *gaaB*) in two filamentous fungi, strains were obtained that converted D-galacturonic acid to L-galactonic acid. Both *Trichoderma reesei* Δ*lgd1* and *Aspergillus niger* Δ*gaaB* produced L-galactonate at yields of 0.6 to 0.9 g per g substrate consumed. While *T. reesei* Δ*lgd1* could produce L-galactonate at pH 5.5, lower pH was necessary for *A. niger* Δ*gaaB*. Provision of a co-substrate improved the production rate and titre in both strains. Intracellular accumulation of L-galactonate (40 to 70 mg [g biomass]$^{-1}$) suggested that export may be limiting. Deletion of the L-galactonate dehydratase from *A. niger* was found to delay induction of D-galacturonate reductase and overexpression of the reductase improved initial production rates. Deletion of the L-galactonate dehydratase from *A. niger* also delayed or prevented induction of the putative D-galacturonate transporter An14g04280. In addition, *A. niger* Δ*gaaB* produced L-galactonate from polygalacturonate as efficiently as from the monomer.

Introduction

D-galacturonic acid is the principal component of pectin, a major constituent of sugar beet pulp and citrus peel which are abundant and inexpensive raw materials. The annual worldwide production of sugar beet and citrus fruit is about 250 x 10^6 and 115 x 10^6 metric tons respectively. After beet processing, 5-10% of the sugar beet remains as dried sugar beet pulp. This pulp contains about 25% pectin (5). Citrus peel contains about 20% pectin on a dry mass basis. Sugar beet pulp and citrus peel are mainly used as cattle feed or they are dumped. The use

as cattle feed requires that pulp and peel are dried since otherwise they rot rapidly. Disposal of the material is problematic because of the bad odour generated at the dumping sites. In the case of sugar beet pulp the energy consumption for drying and pelleting are 30% to 40% of the total energy used for beet processing (5). This process is only economical when done in large scale and when energy costs are low. Other products, such as pectin and limonene, may be extracted from citrus peel. Pectin is used as a gelling agent in the food industry; limonene as a flavour compound. These are limited markets and with increasing energy costs and alternative animal feed sources reducing the revenues from pectin-rich biomass for cattle feed sales, it is desirable to find new ways to convert this biomass to other useful products. This may be accomplished by microbial fermentation (16). Genetically modified bacteria have been used to produce ethanol from pectin rich biomass (6, 7). Using genetically modified fungi, D-galacturonic acid has been converted to galactaric acid (14) or to 2-keto-3-deoxy-L-galactonic acid (20).

Using fungi to valorise D-galacturonic acid is attractive since many species can use D-galacturonic acid efficiently for growth, indicating that these species have efficient D-galacturonic acid uptake. Filamentous fungi, especially *Aspergillus niger*, may also efficiently produce pectinases, enabling simultaneous hydrolysis and conversion of the pectin rich biomass. Other advantages are that many fungi are robust, low pH tolerant organisms with simple nutritional requirements.

In fungi, D-galacturonic acid is catabolised through a pathway (Fig. 1) which includes reactions catalysed by D-galacturonic acid reductase (10), L-galactonate dehydratase (9), 2-keto-3-deoxy galactonate aldolase (8) and L-glyceraldehyde reductase (11); the intermediates are L-galactonate, 2-keto-3-deoxy-L-galactonate (3-deoxy-L-*threo*-hex-2-ulosonate) and L-glyceraldehyde and the products of the pathway are pyruvate and glycerol. D-galacturonic acid

61 can induce pectinolytic and D-galacturonic acid catabolic genes in *A. niger*, regardless of
62 whether D-galacturonic acid is metabolised or not (4, 14).

63 By disrupting the native D-galacturonic acid catabolic pathway it is possible to engineer
64 fungal strains for alternative D-galacturonic acid conversions (14, 20). In the case of galactaric
65 acid production, the gene encoding D-galacturonic acid reductase was deleted and a gene
66 encoding a D-galacturonic acid dehydrogenase expressed (14). Strains lacking the reductase
67 were unable to grow on D-galacturonic acid and the strains also expressing the dehydrogenase
68 converted D-galacturonic acid to galactaric acid. To produce 2-keto-3-deoxy-L-galactonic acid,
69 it was only necessary to delete the gene for the 2-keto-3-deoxy-L-galactonic acid aldolase (20).
70 The resulting strain did not grow on D-galacturonic acid (8), but converted D-galacturonic acid
71 to 2-keto-3-deoxy-L-galactonic acid. The pathway for D-galacturonic acid catabolism in fungi
72 can also be interrupted at the L-galactonate dehydratase step. A strain of *Trichoderma reesei*
73 (anamorph of *Hypocrea jecorina*) in which the L-galactonate dehydratase, *lgd1*, was deleted was
74 unable to grow on D-galacturonic acid (9). In the present communication we show that deletion
75 of the gene encoding L-galactonate dehydratase, i.e. *lgd1* in *T. reesei* and *gaaB* in *A. niger*,
76 results in strains that convert D-galacturonic acid to L-galactonic acid which is excreted into the
77 medium.

78 L-galactonic acid is currently expensive and not widely used, but has the potential to be
79 used more widely once it is available at a low price. The physico-chemical properties are similar
80 to those of D-gluconic acid, which is widely used as a chelator, in the pharmaceutical, cosmetic,
81 and other industrial (e.g. dyes, detergents, solvents, paints) sectors and as an acidifier in food. L-
82 Galactonic acid is also a precursor for L-ascorbic acid (vitamin C) synthesis. The L-galactono-

1,4-lactone which forms from L-galactonic acid at acidic pH can be oxidised to L-ascorbic acid chemically (3) or in a fermentative process (17).

Materials and Methods

Strains. The deletion of the *lgd1* in *Trichoderma reesei* (anamorph of *Hypocrea jecorina*) was described previously (9).

Aspergillus niger ATCC 1015 Δ*pyrG*, with the gene encoding the orotidine-5'-phosphate decarboxylase (*pyrG*) deleted (14), was used to construct the *gaaB* deletion strain. The cassette for deletion of *gaaB* contained 1550 bp from the *A. niger gaaB* promoter, 1533 bp from the *A. niger gaaB* terminator, and a 1920 bp fragment containing the *pyrG* gene flanked with its native promoter and terminator. These fragments were obtained by PCR of *A. niger* ATCC1015 genomic DNA using primers gaaB-5-F, gaaB-5-R, gaaB-3-F, gaaB-3-R, pyrG-del-F_n, and pyrG-del-R_n (Table 1), and the proofreading DNA polymerase Phusion (Finnzymes). Plasmid pRSET-A (Invitrogen) was digested with *Eco*RI and *Pvu*II (both NEB) and the terminator fragment (*gaaB-3*) with *Eco*RI to produce an intermediary construct by ligation using T4 DNA ligase (NEB). This intermediary construct was digested with *Xho*I (NEB) and *Ecl*136II (Fermentas) and ligated to the *Xho*I digested promoter fragment (*gaaB-5*). The resulting vector was digested with *Ecl*136II and treated with phosphatase. The *pyrG* DNA fragment, after digestion with *Sma*I, was inserted between the two *gaaB* flanking regions. The deletion cassette, 5006 bp containing the *gaaB* flanking regions and the *pyrG* gene, was released by *Eco*RI + *Xho*I digestion and introduced into *A. niger* ATCC1015 Δ*pyrG* as described previously (14). Transformants were selected by ability to grow in the absence of uracil. Strains with a correct deletion were verified by PCR and tested for growth on D-galacturonate as a sole carbon source.

105 The cassette for the overexpression of *A. niger* D-galacturonate reductase (*gaaA*) contained
106 the native *gaaA* gene between the *gpdA* promoter and *trpC* terminator from *A. nidulans*,
107 following the hygromycin B phosphotransferase (*hph*) gene under the *gpdA* promoter. The *gaaA*
108 fragment was obtained by PCR from ATCC1015 genomic DNA using the primers gaaA-exp-F
109 and gaaA-exp-R (Table 1). The plasmid (JKp1-*hph*) containing the *gpdA-trpC-hph* fragment was
110 derived from pRS426 (ATCC). Both JKp1-*hph* and the PCR-amplified *gaaA* fragment were
111 digested with *Sac*I and *Xma*I (both NEB), followed by ligatation using T4 DNA ligase to
112 generate the intermediary construct JKp1-*hph-gaaA*. JKp1-*hph-gaaA* was digested with *Bsp*HI
113 and *Psi*I (both NEB) and the fragment containing the *gpdA-gaaA-trpC-hph* cassette was
114 introduced into *A.niger* ATCC1015 *gaaB*Δ strain by transformation. Transformants were
115 screened for integration of the *gpdA-gaaA-trpC-hph* cassette by growth in the presence of 400 μg
116 ml^{-1} hygromycin B (Calbiochem). Integration of the transformed cassette into the genome was
117 confirmed by PCR with the primers gpdA-F and gaaA-exp-R (Table 1).

118 **Media.** The defined medium of Vogel (19), modified as described by Mojzita et al. (14),
119 was used to assess L-galactonate production in flasks and bioreactors. D-Xylose (2 to 11 g l^{-1})
120 was provided as carbon source and ammonium sulphate (1.65 or 3.3 g l^{-1}) as nitrogen source. D-
121 galacturonate (approximately 10 g l^{-1}; prepared as sodium salt), or polygalacturonate (15 g l^{-1};
122 prepared as sodium salt and containing 11 g D-galacturonic acid l^{-1} plus 1 g l^{-1} combined D-
123 xylose, D-galactose and D-mannose when hydrolysed) were used as substrates in production
124 media. Alternatively, the *A. nidulans* defined minimal medium of Barratt *et al*. (1) was used for
125 *A. niger* cultures with 20 g D-galacturonate l^{-1} and 5 g D-xylose l^{-1}. The pH of production media
126 was adjusted between 3.0 and 6.0 with NaOH.

127 Medium (modified from Vogel (19)) for pre-cultures contained 20 g xylose l^{-1} and was
128 supplemented with 1 g bactopeptone l^{-1} to provide more rapid growth in this chemically defined
129 medium. *A. niger* pre-cultures also contained 4 g agar l^{-1} or 30 g gelatin l^{-1}, so that growth would
130 be more filamentous. Agar was used in pre-cultures for bioreactor cultures, since it was not
131 metabolised by *A. niger*, and thus the biomass received the same nutrients as the *T. reesei*
132 precultures. For studies of gene expression, pre-cultures of *A. niger* were grown in medium
133 containing 10 g yeast extract l^{-1}, 20 g peptone l^{-1} (YP) and 30 g gelatin l^{-1}.

134 **Cultural conditions.** Small scale cultures were grown in 250 ml Erlenmeyer flasks
135 containing 50 ml medium and incubated at 30°C, 200 rpm. Pre-culture flasks were inoculated
136 with conidial suspensions (final concentrations 5.3 x 10^5 conidia ml^{-1}) and production flasks with
137 mycelium from the pre-cultures. *T. reesei* pre-cultures were allowed to grow for approximately
138 24 h before being harvested by vacuum filtration through disks of sterile, disposable cleaning
139 cloth (X-tra, 100% viscose household cleaning cloth, Inex Partners Oy, Helsinki) and rinsed with
140 sterile H_2O (> 2 volumes) to remove residual peptone and D-xylose. *A. niger* was grown for 24 h
141 in pre-culture medium containing 4 g agar l^{-1} or 30 g gelatin l^{-1} to reduce formation of pellets.
142 Mycelium (5 ml) from agar-containing pre-cultures was transferred to fresh pre-culture medium
143 lacking agar (50 ml) and incubated for 18 h to reduce the agar content in the cultures and provide
144 inoculum consisting of very small (<2 mm diam.) pellets for D-galacturonate conversion which
145 could be filtered and washed in the same manner as the *T. reesei* pre-cultures. Alternatively,
146 gelatin-containing pre-cultures were harvested by vacuum filtration and rinsed with sterile H_2O
147 warmed to 37 °C to remove gelatin, then with cold H_2O. Washed mycelium was aseptically
148 transferred to production medium.

149 For larger scale cultures, mycelium was grown in bioreactors in 500 ml (Multifors, max
150 working volume 500 ml, Infors HT, Switzerland). Cultures were maintained at 30°C, 800 rpm,
151 with 1.6 volume gas [volume culture]$^{-1}$ min^{-1} (vvm). Culture pH was kept constant at pH 4.5, 4.9
152 or 5.5 by the addition of sterile 1 M KOH or 1 M H_3PO_4. Polypropylene glycol (mixed molecular
153 weight (21)) was added to control foam production.
154 The initial biomass concentration in *T. reesei* cultures was 0.3 g l^{-1} and in *A. niger* cultures
155 0.4 g l^{-1} in bioreactors and 0.7 to 1.4 g l^{-1} in flasks.
156 **Chemical analyses.** Samples (1 to 60 ml, depending on the culture scale and density of
157 biomass) were removed at intervals and mycelium was separated from the supernatant by
158 filtration through cloth. For analysis of intracellular L-galactonate concentrations, biomass which
159 had been washed first with an equal volume 9 g NaCl l^{-1}, then with distilled water, was frozen at
160 -20°C and subjected to freeze-drying. After weighing, L-galactonate in the dried biomass was
161 extracted in 5 mM H_2SO_4, as described previously for extraction of intracellular 2-keto-3-deoxy-
162 L-galactonate (20). Intracellular amounts are given as mg per g dry biomass, but concentration
163 may be estimated by assuming the volume (ml) of cytoplasm per g dry biomass would be similar
164 to that of *Penicillium chrysogenum*, which has been determined to be 2.86 ml per g dry biomass
165 (15).
166 The concentration of D-xylose, D-galacturonate and L-galactonate was determined by
167 HPLC using a Fast Acid Analysis Column (100 mm x 7.8 mm, BioRad Laboratories, Hercules,
168 CA) linked to an Aminex HPX-87H organic acid analysis column (300 mm x 7.8 mm, BioRad
169 Laboratories) with 2.5 or 5.0 mM H_2SO_4 as eluant and a flow rate of 0.5 ml min^{-1}. The column
170 was maintained at 55°C. Peaks were detected using a Waters 410 differential refractometer and a
171 Waters 2487 dual wavelength UV (210 nm) detector.

Expression analysis. Samples (1 ml) were collected from flask cultures and the mycelium was harvested by vacuum filtration. The filtered mycelium was immediately frozen with liquid nitrogen and stored at -80°C. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and 1 µg of total RNA was used for cDNA synthesis with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche), following the manufacturer's instructions. cDNA samples were diluted 1:10 with RNase free water (Roche) and 5 µl of diluted cDNA was used for quantitative PCR (qPCR) using a LightCycler II with the LightCycler SYBR green I Master mix (both Roche). The expression of *gaaA*, An03g01620, An07g00780, An14g04280 and actin were quantified using corresponding primers listed in (Table 1). The level of expression of *gaaA* and the genes encoding the putative transporters was normalized to actin using the accompanying software (Advance Relative Quantification tool).

Results

Conversion of D-galacturonate to L-galactonate by *T. reesei* and *A. niger* (at pH 5.5). Deletion of *T. reesei lgd1* (9) and *A. niger gaaB* resulted in drastically reduced growth of the corresponding strains on D-galacturonic acid when this was provided as the sole carbon source (data not shown). Preliminary experiments demonstrated that both *T. reesei* Δ*lgd1* (1.8 g l^{-1}) and *A. niger* Δ*gaaB* (5.9 ± 0.1 g l^{-1}) produced L-galactonate when incubated for 120 h in flasks initially containing 10 g l^{-1} D-galacturonate and 2 g l^{-1} D-xylose as co-substrate (initial pH 5.1). Less L-galactonate (2.0 ± 0.1 g l^{-1}) was produced by *A. niger* Δ*gaaB* when no D-xylose was provided and D-xylose was included as co-substrate in all further experiments.

When cultivated in a bioreactor, *T. reesei* Δ*lgd1* L-galactonate production and D-galacturonate utilisation increased with the provision of increasing concentrations of D-xylose as co-substrate (Fig. 2). Up to 7.2 g L-galactonate l^{-1} were produced in the culture provided 11 g D-

xylose l^{-1}. The initial production rate was 0.07 to 0.12 g L-galactonate l^{-1} h^{-1} and final yields were 0.60 to 0.85 g L-galactonate per g D-galacturonate consumed (Fig. 2). Although initial yields of 0.9 to 1.0 g L-galactonate per g D-galacturonate were observed, the yield decreased during the production phase. The biomass concentration also increased with increasing provision of D-xylose (yield 0.5 g biomass [g D-xylose]$^{-1}$), and the specific L-galactonate production rate was lower when 11 g D-xylose l^{-1} was provided than with 3 g l^{-1} (Fig. 2F).

Extracellular L-galactonate was not observed in *T. reesei* Δ*lgd1* until D-xylose had been consumed, but L-galactonate was present intracellularly prior to this (Fig. 2). During the production phase there was 40 to 70 mg intracellular L-galactonate [g biomass]$^{-1}$. Intracellular D-galacturonate remained below 2 mg [g biomass]$^{-1}$ (data not shown).

A. niger Δ*gaaB* produced only 1.4 - 1.9 g L-galactonate l^{-1} when cultivated in bioreactors at pH 5.5 (Fig. 2), although 5.9 g l^{-1} had been produced in the preliminary flask experiment. Biomass concentrations were similar to those of *T. reesei* Δ*lgd1* (yield 0.56 g biomass [g D-xylose]$^{-1}$), as were intracellular concentrations of L-galactonate (Fig. 2). D-Galacturonate (10-30 mg [g biomass]$^{-1}$) was also detectable in mycelia from the cultures which received 6 or 11 g D-xylose l^{-1}. An initial assessment indicated that *gaaA* expression in this strain was low (data not shown).

Production of L-galactonate by *A. niger* is sensitive to culture pH. The modified Vogel's medium used here is not well buffered and thus pH in flask cultures decreased as ammonium was consumed and increased when D-galacturonate was taken up from the medium without release of L-galactonate from the hyphae. Data from the preliminary *A. niger* flask cultures indicated that the highest L-galactonate production rates were observed when pH was low (Fig. 3), and suggested that pH 5.5 may be too high for L-galactonate production by *A.*

218 *niger*. Indeed, L-galactonate production decreased with increasing pH above 5.0 in flask cultures,
219 but was generally high (5-6 g l^{-1}) at pH values below 5 (Fig. 3). L-Galactonate production was
220 further improved at pH 3 to 4 by cultivating the strain in buffered medium with 20 g D-
221 galacturonate l^{-1} and 5 g D-xylose l^{-1} (Fig. 3).

222 When *A. niger* Δ*gaaB* was grown in a pH controlled bioreactor at pH 4.8 with 10 g D-
223 galacturonate l^{-1} and 6 to 7 g D-xylose l^{-1}, 2.7 g L-galactonate l^{-1} were produced within 72 h at a
224 rate of 0.04 g l^{-1} h^{-1} (yield 0.7 g L-galactonate [g D-galacturonate consumed]$^{-1}$, Fig. 4). An
225 additional pulse of 8 g D-xylose l^{-1} was added after 127 h to compensate for the decreasing
226 biomass and a further 2.5 g L-galactonate were produced at the same rate to give a final
227 concentration of 5.4 g l^{-1} (yield 0.9 g [D-galacturonate consumed]$^{-1}$, Fig. 4) when the culture was
228 harvested at 171 h. Intracellular L-galactonate accumulation (56 ± 2 mg [g biomass]], Fig. 4) was
229 similar to that observed at pH 5.5 (Fig. 2), but decreased after the addition of D-xylose. D-
230 Galacturonate (<1.6 mg [g biomass]$^{-1}$) did not accumulate in the mycelia (data not shown).

231 **Bioconversion of polygalacturonate to L-galactonate**. *A. niger* Δ*gaaB* converted
232 polygalacturonate to L-galactonate at a similar rate (initial rate 0.04 g l^{-1} h^{-1}, increasing to 0.07 g
233 l^{-1} h^{-1} after addition of extra D-xylose) and titre (2.5 g L-galactonate l^{-1} within 72 h) as it
234 converted the monomer D-galacturonate (Fig. 4). L-Galactonate (1.2 g l^{-1}) was present in the
235 culture supernatant after 26 h, but did not accumulate above 2.8 g l^{-1} at any time during the
236 cultivation. Addition of D-xylose after 127 h resulted in a total of 6.5 g L-galactonate l^{-1} (yield
237 0.85 g L-galactonate [g D-galacturonate consumed]$^{-1}$) after 171 h, increasing to 7.6 g l^{-1} after 195
238 h. The intracellular concentration of L-galactonate (52 ± 4 mg [g biomass]$^{-1}$) was similar to that
239 observed in other L-galactonate producing cultures and also decreased after the addition of D-

240 xylose (Fig. 4). Low concentrations of D-galacturonate (0.2 to 4.3 mg [g biomass]$^{-1}$) were also
241 extracted from mycelia incubated in polygalacturonate (data not shown).

242 **Overexpression of *A. niger gaaA*.** Since *gaaA* expression appeared low in the Δ*gaaB*
243 strain, the galacturonate reductase coding gene, *gaaA*, was overexpressed in *A. niger* Δ*gaaB*. *A.*
244 *niger* ATTC1015, Δ*gaaB* and the overexpression strain (Δ*gaaB-gaaA*) were grown in modified
245 Vogel's medium with 10 g D-galacturonate l^{-1} and 2 g D-xylose l^{-1} at initial pH 3 in flasks.
246 Expression of *gaaA* in *A. niger* Δ*gaaB* was considerably lower compared to the wild type after 6
247 h (Table 3). In contrast, in *A. niger* Δ*gaaB-gaaA* expression of *gaaA* was much higher at 0 and 6
248 h, as expected (Table 3). After 24 h, *gaaA* expression in *A. niger* Δ*gaaB* and *A. niger* Δ*gaaB-*
249 *gaaA* was similar, whereas its expression in the wild type had decreased (Table 3), probably due
250 to D-galacturonate depletion.

251 Approximate L-galactonate production rates were determined for the flask cultures. During
252 the first 24 h after inoculation, *A. niger* Δ*gaaB-gaaA* produced L-galactonate at a significantly (p
253 < 0.05) higher rate (0.070 g L-galactonate l^{-1} h^{-1}) than *A. niger* Δ*gaaB* (0.048 g L-galactonate l^{-1}
254 h^{-1}, Table 4). After 24 h the difference in the production rates of the Δ*gaaB* and Δ*gaaB-gaaA*
255 strains decreased, and after 48 h, when L-galactonate production by both strains was decreasing,
256 their production rates were similar ($p > 0.05$, 0.046 and 0.054 g L-galactonate l^{-1} h^{-1},
257 respectively, Table 4).

258 The final L-galactonate titres of Δ*gaaB* and Δ*gaaB-gaaA* strains were compared in both
259 modified Vogel's and *A. nidulans* minimal medium in flasks (Table 2). Both L-galactonate titre
260 and yield were generally higher for *A. niger* Δ*gaaB-gaaA* than for *A. niger* Δ*gaaB* when grown at
261 pH 3 or 4 in either medium (Table 2). At pH 5 in *A. nidulans* minimal medium, the final L-
262 galactonate titre was notably lower than at pH 4 for both strains and there was no difference

between the strains. However, the yield of L-galactonate on D-galacturonate for *A. niger* Δ*gaaB* -*gaaA* was higher than for *A. niger* Δ*gaaB* also at pH 5 (Table 2).

Transciption of putative transporter genes in *A. niger* Δ*gaaB*. The relative transcript levels of 3 genes which have been identified as possible transporters of D-galacturonate (An07g00780, An14g04280, and An03g01620, (12)) were assessed in *A. niger* ATCC1015 and *A. niger* Δ*gaaB* 3, 6 and 24 h after transfer to D-galacturonic acid containing medium at pH 3 (Table 5). Both An14g04280 and An03g01620 were strongly induced in ATC1015 within 3 hours of the transfer, whereas induction of An07g00780 was only seen 24 h after the transfer. In contrast, no induction of An14g04280 was observed in *A. niger* Δ*gaaB*. Transcription of An03g1620 and An07g00780 in *A. niger* Δ*gaaB* was similar to that observed in the control strain.

Discussion

Deletion of the gene for the L-galactonate dehydratase, *lgd1* in *T. reesei* or *gaaB* in *A. niger*, resulted in strains that converted D-galacturonate to L-galactonate, which was secreted to the culture supernatant (Figs. 2 to 5). This confirmed that D-galacturonate was still taken up in the deletion strains, as was also the case when either the D-galacturonate reductase (*gar1* or *gaaA* in *T. reesei* and *A. niger*, respectively, (14)) or the 2-keto-3-deoxy-L-galactonate aldolase, *lga1* or *gaaC*, (20) were deleted. In *T. reesei*, the conversion of D-galacturonate to L-galactonate occurred at similar rates (0.07 to 0.12 g L-galactonate l^{-1} h^{-1}) as previously reported for the conversion to keto-deoxy-L-galactonate (0.10 to 0.14 g l^{-1} h^{-1}, (20)), but was faster than the conversion to galactarate (0.024 to 0.046 g l^{-1} h^{-1}, (14)). In *A. niger*, on the other hand, the conversion of D-galacturonate to L-galactonate (0.04 to 0.07 g L-galactonate l^{-1} h^{-1}) was much

slower than the conversion to keto-deoxy-L-galactonate (0.27 to 0.33 g l^{-1} h^{-1}, (20)), suggesting that the disruption of the pathway at the earlier step created additional constraints in this strain.

The yield of L-galactonate from D-galacturonate was 0.6 to 0.8 g g^{-1} for *T. reesei* Δ*lgd1* and 0.7 to 0.9 for *A. niger* Δ*gaaB*. Thus, the yields were only slightly lower than the theoretical yield (1.0 g L-galactonate [g D-galacturonate]$^{-1}$), but still indicated that some of either the D-galacturonate or the produced L-galactonate were consumed in unidentified metabolic reaction(s). Futile consumption of D-galacturonate has been observed previously in strains deleted of *gaaA*/*gar1* or *gaaC*/*lga1* (14, 20), but the fate of the carbon remains unclear since there is no measureable production of biomass from D-galacturonate in these strains.

Although production of both L-galactonate and keto-deoxy-L-galactonate require NADPH as a co-factor for the D-galacturonate reductase, L-galactonate production was more dependent on the addition of D-xylose as a co-substrate (Fig. 2) to obtain good production than was the production of the keto-deoxy derivative. This may reflect a greater need for energy in the export of L-galactonate, since we observed that the intracellular concentration of L-galactonate (40 to 70 mg L-galactonate [g biomass]$^{-1}$ in both *T. reesei* and *A. niger*) was higher than the maximum intracellular concentrations of keto-deoxy-L-galactonate (35 to 45 mg L-galactonate [g biomass]$^{-1}$) in the corresponding strains (20). After provision of additional co-substrate to *A. niger* Δ*gaaB* cultures at pH 4.5 to 4.8 the intracellular L-galactonate concentration decreased to around 23 mg [g biomass]$^{-1}$ (Fig. 4), supporting the hypothesis that energy is needed for export.

Assuming the volume of cytoplasm to be approximately 2.86 times the dry biomass (10), the average intracellular concentration of L-galactonate was ~20 g l^{-1} and was much higher than the L-galactonate concentration in the medium. This also suggests that export may be a bottleneck in extracellular production. In addition, the high intracellular concentration of L-

308 galactonate may limit the rate of D-galacturonate conversion by feedback inhibition and/or
309 providing substrate for the reverse reaction, which has been shown to occur with both the *T.*
310 *reesei* gar1 (10) and the *A. niger* gaaA (13) D-galacturonate reductases. The K_m for L-
311 galactonate of *T. reesei* gar1 is 4 mM (0.8 g l^{-1}) (10), which is much lower than the intracellular
312 L-galactonate concentrations observed. Thus, accumulation of L-galactonate may limit the
313 reaction more than accumulation of keto-deoxy-L-galactonate, since the action of the L-
314 galactonate dehydratase is irreversible (9). Generation of intracellular D-galacturonate may also
315 have affected uptake of the substrate, about which little is known in filamentous fungi.
316 Intracellular D-galacturonate was, however, only observed in *A. niger* and not in *T. reesei*.

317 In contrast to keto-deoxy-L-galactonate production (20), L-galactonate production was
318 more efficient in *T. reesei* than in *A. niger* at pH 5.5, producing higher titres at higher rates (Fig.
319 2). *T. reesei* was also found to be more effective than *A. niger* in the production of galactarate
320 (14), and these results confirm that *T. reesei* is an interesting and useful host for organic acid
321 production, even though it is not known as a high producer of organic acids, nor tolerant to very
322 low culture pH.

323 Low galactarate production by *A. niger* Δ*gaaA-udh* was attributed to subsequent
324 metabolism of the galactarate (14). Metabolism of L-galactonate appeared negligible (Fig. 4) or
325 limited (Fig. 2) in *A. niger* Δ*gaaB*, rather L-galactonate production by *A. niger* was found to be
326 pH-dependent, with the highest production rates and titres observed at pH values below 5.0 and
327 no reduction in production even at pH 3.0 (Fig. 3). At pH 4.5 to 4.8, production of L-galactonate
328 by *A. niger* Δ*gaaB* was as good as that of *T. reesei* Δ*lgd1* at pH 5.5. At low extracellular pH,
329 more of the product is protonated to L-galactonic acid (pKa ~3.5) creating a greater difference in
330 concentration between the dissociated intra- and extracellular L-galactonate pools. If the

protonated organic acid is not re-imported to the cytoplasm, then a low extracellular pH can provide the dominant driving force for organic acid export, as has been predicted for citrate export from *A. niger* (2). Further, low extracellular pH may influence the transport of D-galacturonic acid (pKa=3.51). However, *A. niger* transported D-galacturonate at much higher rates when producing keto-deoxy-L-galactonate at pH 5.5 (0.12 to 0.56 g l^{-1} h^{-1}, (20)) or galactarate at pH 5.0 (0.21 to 0.46 g l^{-1} h^{-1}, (14)) than observed during L-galactonate production at any pH (0.04 to 0.15 g l^{-1} h^{-1}, Fig. 2 and data not shown). Thus improved uptake at low pH is unlikely to explain the improved L-galactonate production observed.

D-galacturonate is an inducer of the D-galacturonate pathway genes *gaaA*, *gaaB* and *gaaC* in *A. niger* ATCC1015, CBS120.49 and Δ*gaaA* strain (12, 14). In ATCC1015, the transcription of these three genes was induced simultaneously within 4 hours of transfer to D-galacturonate and induction of *gaaB* and *gaaC* remained similar in *A. niger* Δ*gaaA* compared to ATCC1015 (14). In this study, we observed that *gaaA* was not induced in *A. niger* Δ*gaaB* even 6 hours after exposure to D-galacturonate (Table 3), although transcription had increased after 24 hours. In ATCC1015, *gaaA* expression was already reduced after 24 h incubation, due to D-galacturonate depletion. Induction of the gene encoding the third enzyme of the pathway, *gaaC*, was similarly delayed in *A. niger* Δ*gaaB* (J. Kuivanen, unpublished data), suggesting that the induction of the entire pathway was affected by the deletion of *gaaB*. The similar transcriptional response of *gaaA* and *gaaC* might be expected since these genes share a common bidirectional promoter (13). The altered transcription profiles of the genes in the Δ*gaaB* strain suggest that L-galactonate, keto-deoxy-L-galactonate or L-galactonate dehydratase itself may have roles in transcriptional regulation of the D-galacturonate pathway genes. Regardless of the regulatory

353 mechanism, the delayed induction of *gaaA* in the Δ*gaaB* strain would account for low initial
354 rates of D-galacturonate conversion.

355 In order to eliminate *gaaA* induction as a rate limiting factor for L-galactonate production,
356 *gaaA* was overexpressed under the *gpdA* promoter in *A. niger* Δ*gaaB*. The L-galactonate
357 production rate was initially significantly ($p<0.002$) higher in *A. niger* Δ*gaaB-gaaA* compared to
358 *A. niger* Δ*gaaB* in flasks at pH 3 (Table 4), indicating that low *gaaA* expression was indeed a rate
359 limiting factor. However, *gaaA* was expressed under the *gpdA* promoter, which gives less
360 induction in the absence of a metabolisable carbon source (here D-xylose), even though it is
361 generally described as constitutive. Thus, expression of *gaaA* decreased during the expression
362 studies. After 24 h, when *gaaA* expression had been induced in the *gaaB* deletion strain, the
363 production rates of *A. niger* Δ*gaaB-gaaA* and *A. niger* Δ*gaaB* were similar (Table 4). The initial
364 improved production resulted in 24 to 39% more L-galactonate being produced at pH 3 when
365 *gaaA* was overexpressed than when it was not, with corresponding improvements in the
366 conversion efficiency and yield (Table 2). Interestingly, the benefit of overexpression of *gaaA*
367 was pH dependent even though *gaaA* expression was not (J. Kuivanen, unpublished data), with
368 the greatest benefit at pH 3, although smaller improvements in yield were also observed at higher
369 pH values (Table 2).

370 D-Xylose was previously found to be a good co-substrate in the production of keto-deoxy-
371 L-galactonate (20), but D-Galacturonate did not appear to be taken up while D-xylose was being
372 consumed (Fig. 2). Limited D-galacturonate uptake during the time when *gaaA* expression was
373 high in *A. niger* Δ*gaaB-gaaA* probably limited the improvement in L-galactonate production
374 which could be achieved by this strain. In addition, only two of the three putative D-
375 galacturonate transporters (12) were induced in the Δ*gaaB* strain (Table 5). The roles of these

putative transporters is not known, but the limited D-galacturonate transport in *A. niger* Δ*gaaB* and *A. niger* Δ*gaaB-gaaA* may indicate that the protein encoded by An14g04280 has a dominant role.

Despite the fact that production of L-galactonate with *A. niger* Δ*gaaB* required more investigation and additional strain development than with *T. reesei* Δ*lgd1*, *A. niger* is more suitable for development of a consolidated L-galactonate production process, which would use less processed polymeric substrates, such as polygalacturonate, pectin, or even raw, untreated biomass. *A. niger* produces a more complex spectrum of pectinases than *T. reesei*, which is unable to degrade pectin (20). Using the current *A. niger* Δ*gaaB* strain, production of L-galactonate from polygalacturonate was found to be as efficient as production from the D-galacturonate monomer (Fig. 4). Thus, a high concentration of extracellular D-galacturonate was not necessary to sustain its uptake and the slow release of monomer may be beneficial in providing continual induction of the native *gaaA* gene. Polygalacturonate was used as a substrate here, but these results suggest that L-galactonate could also be produced directly from pectin, which would require less processing and would also provide the co-substrates (e.g. D-galactose, D- xylose, L-arabinose) for the initial production of biomass and NADPH. A more gradual provision of co-substrate in a fed-batch or continuous process may also be useful, since this would ensure that production rates did not decrease as a result of cell lysis after the co-substrate was consumed and for the Δ*gaaB-gaaA* strain would sustain higher expression levels of *gaaA*.

D-Galactonate has been produced in high concentration from D-galactose using *Gluconobacter oxydans* (18), but this is the first report of extracellular production of L-galactonate in gram quantities from D-galacturonic and polygalacturonic acids. Its production has led to further insights into D-galacturonate metabolism in *A. niger*, while further

399 enhancement in production by both strain engineering and process development may provide an
400 efficient source of L-galactonate for e.g. microbial ascorbic acid production and other
401 applications.
402

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466 **TABLE 1** Primers used to generate vectors for deletion of *gaaB* and incorporation of *gaaA* in *A.*

467 *niger* ATCC1015 Δ*pyrG*, to confirm integration and for qPCR.

Primer	**Sequence**
gaaB-5-F	TATACTCGAGAGTTCCTCGATCAGGAACGA
gaaB-5-R	TATAGAGCTCGCAATCTAGTTGCAATGC
gaaB-3-F	TATAGAGCTCGCATTACATTGGTTATGTGGG
gaaB-3-R	TATAGAATTCAGACATTAGTCCCCGAGAA
pyrG-del-F_n	TATACCCGGGTGATTGAGGTGATTGGCGAT
pyrG-del-R_n	TATACCCGGGTTATCACGCGACGGACAT
gaaB-ORF-F	AGATCACAAGTTTCACCACGA
gaaB-ORF-R	GCCCCTCCAGAATGGTCTT
gaaA-exp-F	ATGAATTCGAGCTCCACAATGGCTCCCCCAG
gaaA-exp-R	AGGCGCGCCCGGGCTACTTCAGCTCCCACTTTC
gpdA-F	AAGTGGAAAGGCTGGTGTGC
gaaA_qPCR_F	AGGACACGATTACTCTACTTGTG
gaaA_qPCR_R	GAGCCCATATAATGGAAGTACTG
act_qPCR_F	CAACATTGTCATGTCTGGTGG
act_qPCR_R	GGAGGAGCAATGATCTTGAC
An07g00780_qPCR_F	CTATCATCAATGCCGCCTCC
An07g00780_qPCR_R	CCACTGACGAAGCCATAGAC
An14g04280_qPCR_F	GTATGTGAGCGAGATCTTCCC
An14g04280_qPCR_R	TTTCCTTGGCGAAGACAATGAC

An03g01620_qPCR_F	GGAATACGAAGAAGTGCAGGA
An03g01620_qPCR_R	GGTGTTTCCAGACATGCCAG

468

469

TABLE 2 L-Galactonate (L-GalA) production at 144 h by *A. niger* Δ*gaaB* and the Δ*gaaB* strain overexpressing *gaaA* (Δ*gaaB-gaaA*) in buffered *A. nidulans* minimal medium (MM) with 20 g D-galacturonate l^{-1} and 5 g D-xylose l^{-1} and in modified Vogel's medium with 10 g D-galacturonate l^{-1} and 2 g D-xylose l^{-1} in flasks at initial pH 3, 4 or 5. Mean ± SEM (n = 3). The conversion and yield on D-galacturonate (D-GalUA) are also shown.

Medium	Initial pH	Strain	L-GalA (g l^{-1})	Conversion (g g^{-1}) L-GalA [D-$GalUA_{initial}$]$^{-1}$	Yield (g g^{-1}) L-GalA [D-$GalUA_{consumed}$]$^{-1}$
A. nidulans **MM**	5	Δ*gaaB*	4.1 ± 0.2	0.20	0.82
		Δ*gaaB-gaaA*	4.1 ± 0.3	0.20	0.97
	4	Δ*gaaB*	7.2 ± 0.8	0.35	0.95
		Δ*gaaB-gaaA*	7.8 ± 0.4	0.38	0.97
	3	Δ*gaaB*	6.3 ± 0.1	0.31	0.86
		Δ*gaaB-gaaA*	8.7 ± 0.2	0.43	1.00
modified Vogel's	4	Δ*gaaB*	4.2 ± 0.1	0.41	0.70
		Δ*gaaB-gaaA*	5.0 ± 0.1	0.49	0.75
	3	Δ*gaaB*	4.9 ± 0.1	0.47	0.70
		Δ*gaaB-gaaA*	6.2 ± 0.3	0.59	0.82

476 **TABLE 3** Relative expression of *gaaA* in *A. niger* ATCC1015, Δ*gaaB* and Δ*gaaB-gaaA* when
477 grown in flasks in modified Vogel's medium with 10 g D-galacturonate l^{-1} and 2 g D-xylose l^{-1} at
478 initial pH 3.0. Average ± SEM (n = 3).

	Relative transcription of *gaaA*		
Time (h)	**ATCC1015**	**Δ*gaaB***	**Δ*gaaB-gaaA***
0	0.2 ± 0.0	0.4 ± 0.0	14.0 ± 0.0
3	3.6 ± 0.6	0.1 ± 0.0	16.6 ± 0.6
6	2.6 ± 0.1	0.1 ± 0.0	9.5 ± 0.6
24	0.2 ± 0.1	1.5 ± 0.1	1.5 ± 0.2

479

480 **TABLE 4** L-Galactonate production rates for *A. niger* Δ*gaaB* and Δ*gaaB-gaaA* when grown in
481 flasks in modified Vogel's medium with 10 g D-galacturonate l^{-1} and 2 g D-xylose l^{-1} at initial
482 pH 3.0. Values (average ± SEM, n = 3) in the same row with different superscripts [a] or [b] differed
483 significantly ($p < 0.05$).

	LGalactonate production rate (g l^{-1} h^{-1})	
Time interval (h)	**Δ*gaaB***	**Δ*gaaB-gaaA***
0-24	0.048 ± 0.001 [a]	0.070 ± 0.002 [b]
24-48	0.064 ± 0.001 [a]	0.075 ± 0.002 [b]
48-78	0.046 ± 0.000 [a]	0.054 ± 0.002 [a]

484

485 **TABLE 5** Relative expression of putative transporters An07g00780, An14g04280, and
486 An03g01620 in *A. niger* ATCC1015 and Δ*gaaB* when grown in flasks in modified Vogel's
487 medium with 10 g D-galacturonate l^{-1} and 2 g D-xylose l^{-1} at initial pH 3.0. Average ± SEM (n =
488 3), n.d. = no data.

		Relative transcription	
Putative transporter	**Time (h)**	**ATCC1015**	**Δ*gaaB***
An07g00780	0	0.4 ± 0.0	0.1 ± 0.0
	3	0.1 ± 0.0	n.d.
	6	0.3 ± 0.0	0.2 ± 0.1
	24	1.1 ± 0.3	1.9 ± 1.0
An14g04280	0	0.0 ± 0.0	0.0 ± 0.0
	3	2.1 ± 0.2	0.1 ± 0.0
	6	0.9 ± 0.0	0.1 ± 0.0
	24	0.1 ± 0.0	0.1 ± 0.0
An03g01620	0	0.0 ± 0.0	0.0 ± 0.0
	3	2.0 ± 0.5	3.4 ± 0.4
	6	0.3 ± 0.0	0.2 ± 0.1
	24	0.0 ± 0.0	0.0 ± 0.0

489

Figure legends

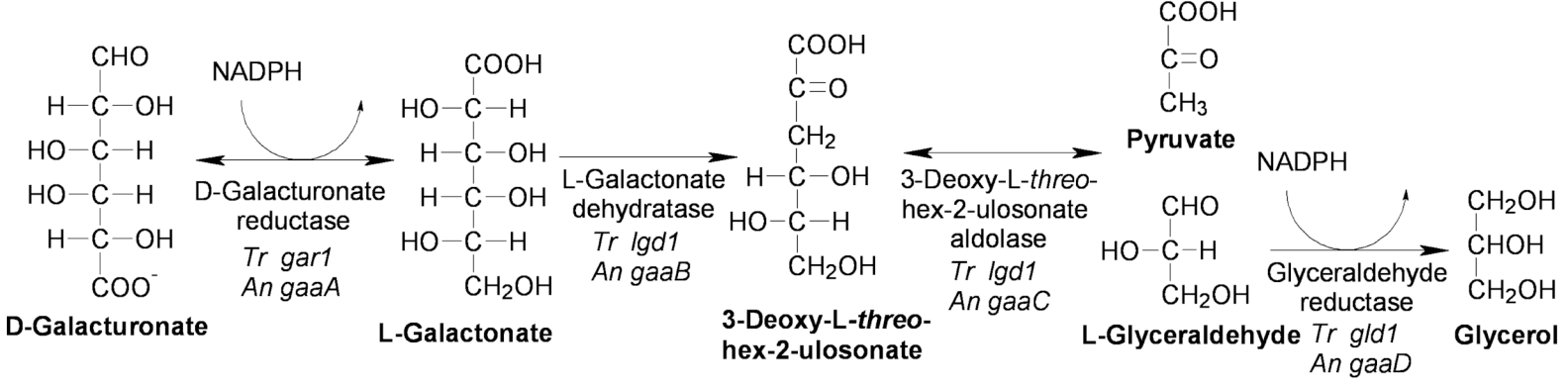
FIG 1 The fungal D-galacturonic acid pathway. The genes encoding the enzymes in *T. reesei* and *A. niger* are indicated. The deletion of *lgd1* in *T. reesei* and *gaaB* in *A. niger* disrupted the pathway and generated strains which accumulated L-galactonate.

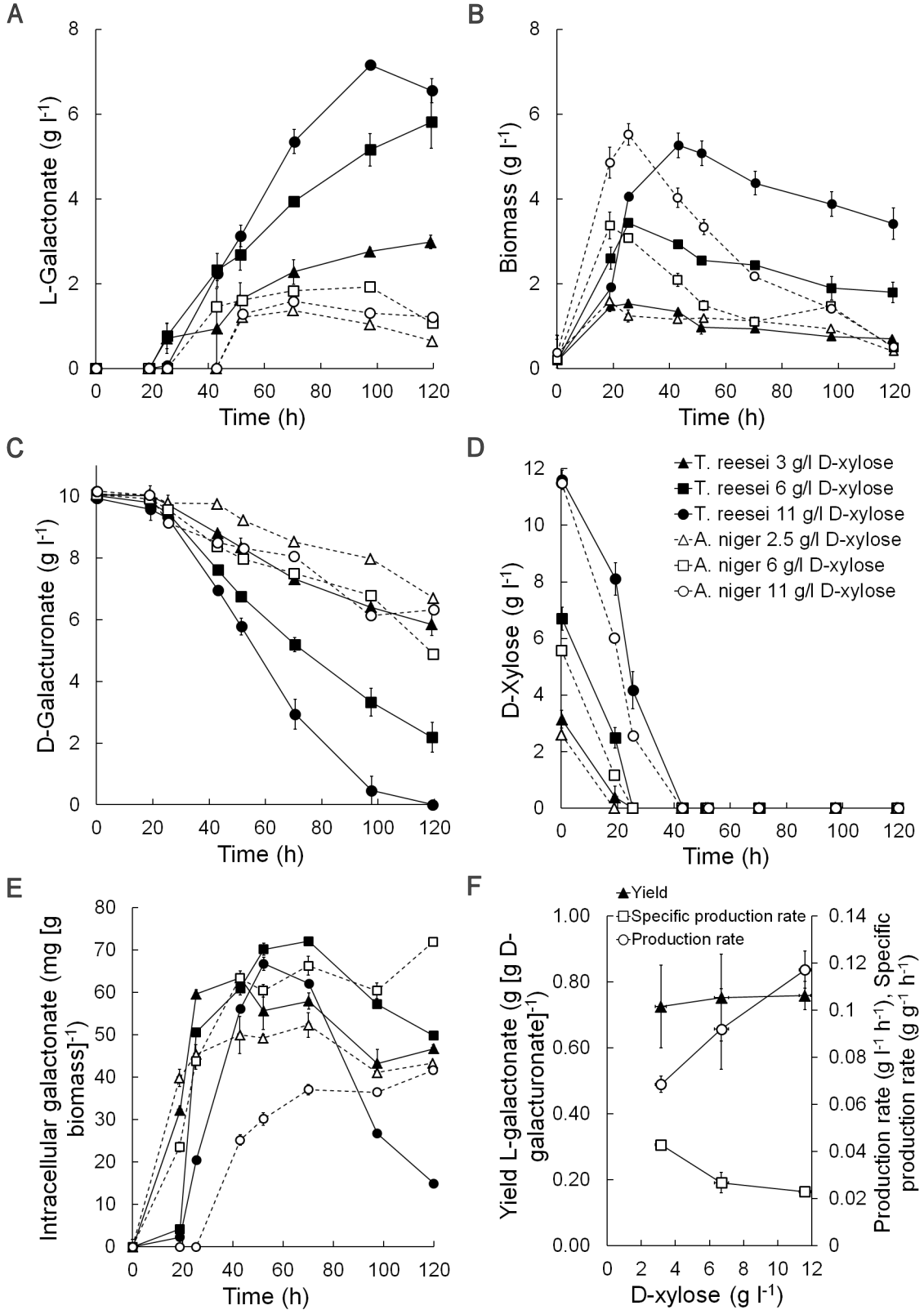
FIG 2 Concentrations of extracellular A) L-galactonate, B) biomass, C) D-galacturonate, D) D-xylose, and intracellular E) L-galactonate from *T. reesei* Δ*lgd1* (solid symbols) and *A. niger* Δ*gaaB* (open symbols) in modified Vogel's medium initially containing 10 g D-galacturonate l^{-1}, and 2.5 or 3, 6 or 11 g D-xylose l^{-1}, as indicated, at pH 5.5, 800 rpm, 1.6 vvm aeration, 30°C. F) The effect of D-xylose concentration on the yield of L-galactonate on D-galacturonate consumed, and the volumetric production and specific production rates of L-galactonate for *T. reesei* Δ*lgd1*. Error bars represent ± SEM (n = 2).

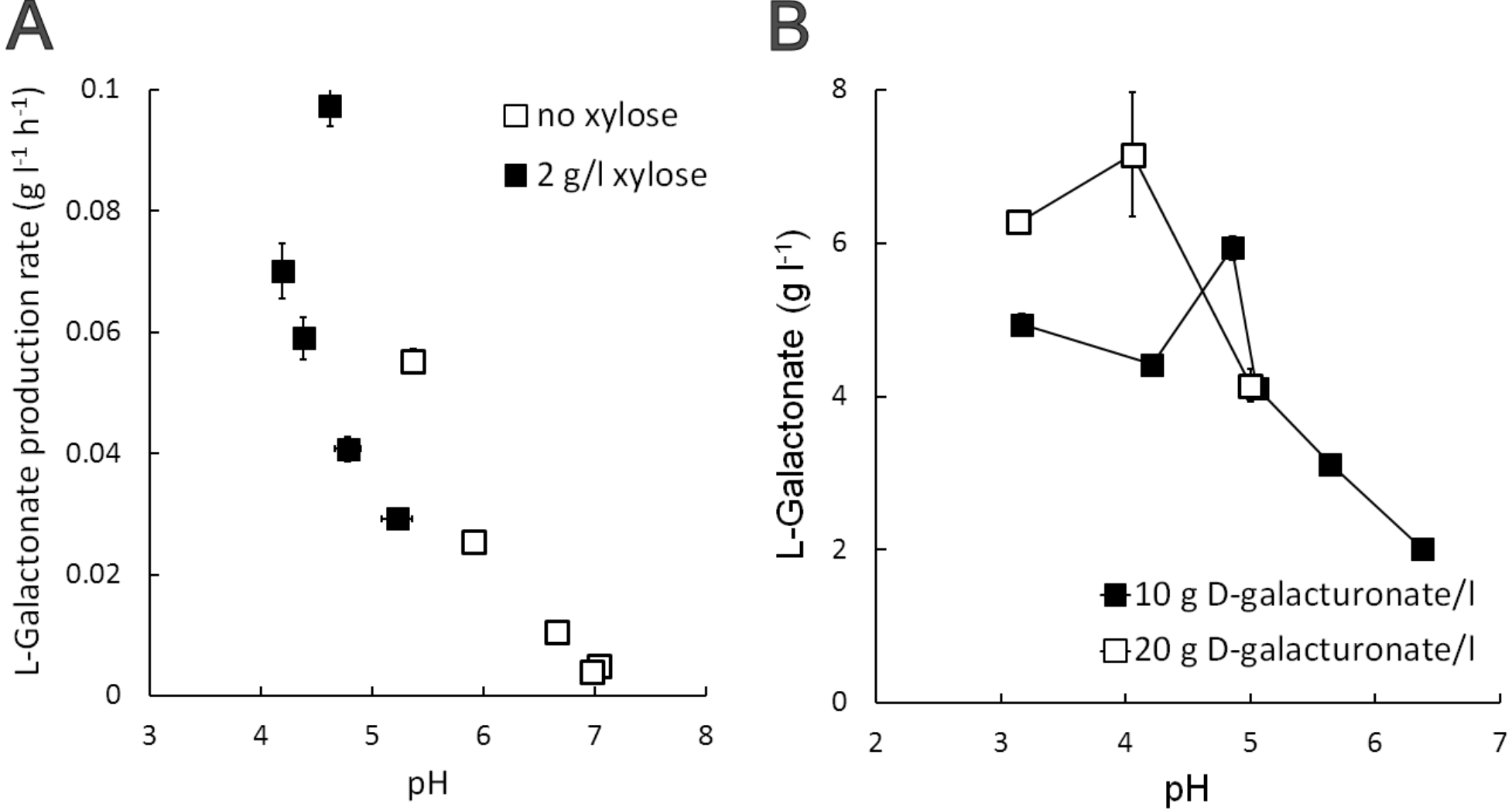
FIG 3 L-Galactonate production by *A. niger* Δ*gaaB* in flasks. A) L-Galactonate production rate as a function of pH for unbuffered cultures provided 10 g D-galacturonate l^{-1} at initial pH 5.2, with no (open symbols) or 2 g l^{-1} D-xylose (solid symbols) provided for growth. Error bars represent ± SEM, n = 3. B) Concentration of L-galactonate produced in 120-144 h in unbuffered modified Vogel's medium containing 10 g D-galacturonate l^{-1} and 2 g D-xylose l^{-1} (solid symbols) and in buffered *A. nidulans* medium containing 20 g D-galacturonate l^{-1} and 5 g D-xylose l^{-1} (open symbols). The pH of the media was initially adjusted to 3, 4, 5 or 6, but average culture pH is shown. Error bars represent ± SEM for 3 to 6 replicate cultures and where not visible are smaller than the symbol.

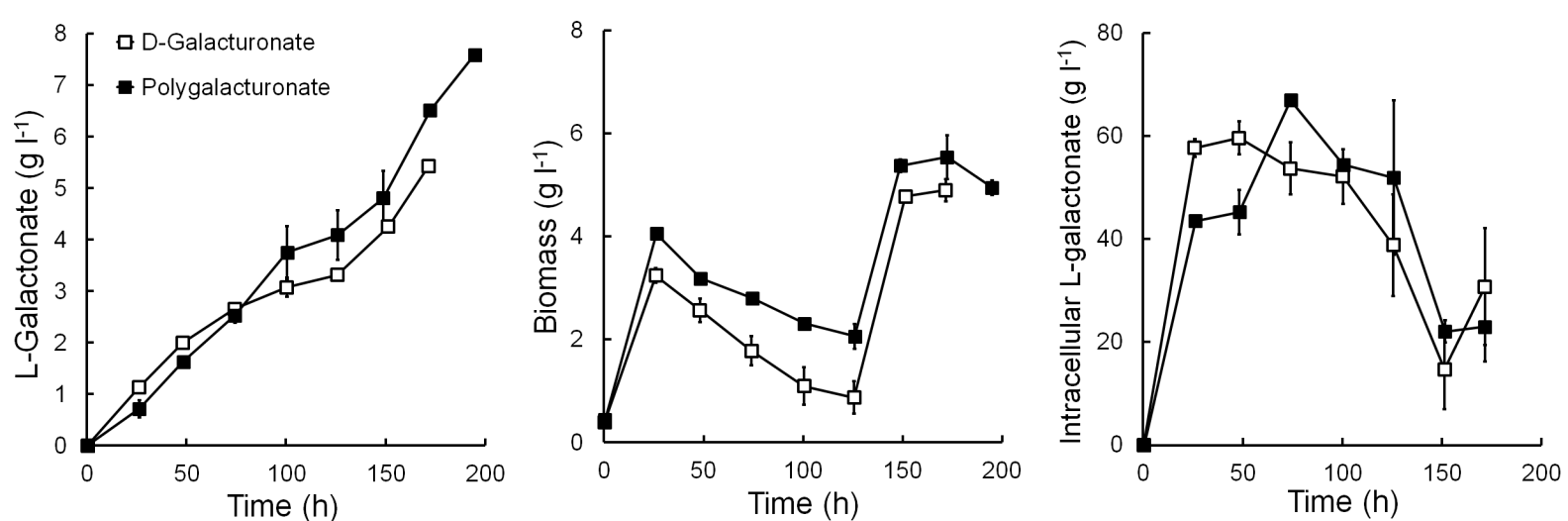
FIG 4 Concentration of L-galactonate, biomass and intracellular L-galactonate in cultures of *A. niger* Δ*gaaB* in modified Vogel's medium with 5 g D-xylose l^{-1} and containing 10 g D-

galacturonate l^{-1} (open symbols, pH 4.8) or 15 g polygalacturonate l^{-1} (solid symbols pH 4.5). Cultures were maintained at 30°C, 800 rpm, 1.6 vvm aeration and were given an additional 9 g D-xylose l^{-1} at 127.8 h. Error bars represent ± SEM (n = 2) and where not visible are smaller than the symbol.


CHO
H—C—OH
HO—C—H
HO—C—H
H—C—OH
COO⁻
D-Galacturonate
NADPH
D-Galacturonate reductase
Tr gar1
An gaaA
COOH
HO—C—H
H—C—OH
H—C—OH
HO—C—H
CH2OH
L-Galactonate
L-Galactonate dehydratase
Tr lgd1
An gaaB
COOH
C=O
CH2
H—C—OH
HO—C—H
CH2OH
3-Deoxy-L-threo-hex-2-ulosonate
3-Deoxy-L-threo-hex-2-ulosonate aldolase
Tr lgd1
An gaaC
COOH
C=O
CH3
Pyruvate
CHO
HO—C—H
CH2OH
L-Glyceraldehyde
NADPH
Glyceraldehyde reductase
Tr gld1
An gaaD
CH2OH
CHOH
CH2OH
Glycerol


A
L-Galactonate (g l-1)
Time (h)
B
Biomass (g l-1)
Time (h)
C
D-Galacturonate (g l-1)
Time (h)
D
D-Xylose (g l-1)
Time (h)
T. reesei 3 g/l D-xylose
T. reesei 6 g/l D-xylose
T. reesei 11 g/l D-xylose
A. niger 2.5 g/l D-xylose
A. niger 6 g/l D-xylose
A. niger 11 g/l D-xylose
E
Intracellular galactonate (mg [g biomass]-1)
Time (h)
F
Yield
Specific production rate
Production rate
Yield L-galactonate (g [g D-galacturonate]-1)
Production rate (g l-1 h-1), Specific production rate (g g-1 h-1)
D-xylose (g l-1)


A
B
L-Galactonate production rate (g l-1 h-1)
0.1
0.08
0.06
0.04
0.02
0
no xylose
2 g/l xylose
3
4
5
6
7
8
pH
L-Galactonate (g l-1)
8
6
4
2
0
10 g D-galacturonate/l
20 g D-galacturonate/l
2
3
4
5
6
7
pH


D-Galacturonate
Polygalacturonate
L-Galactonate (g l-1)
Time (h)
Biomass (g l-1)
Time (h)
Intracellular L-galactonate (g l-1)
Time (h)