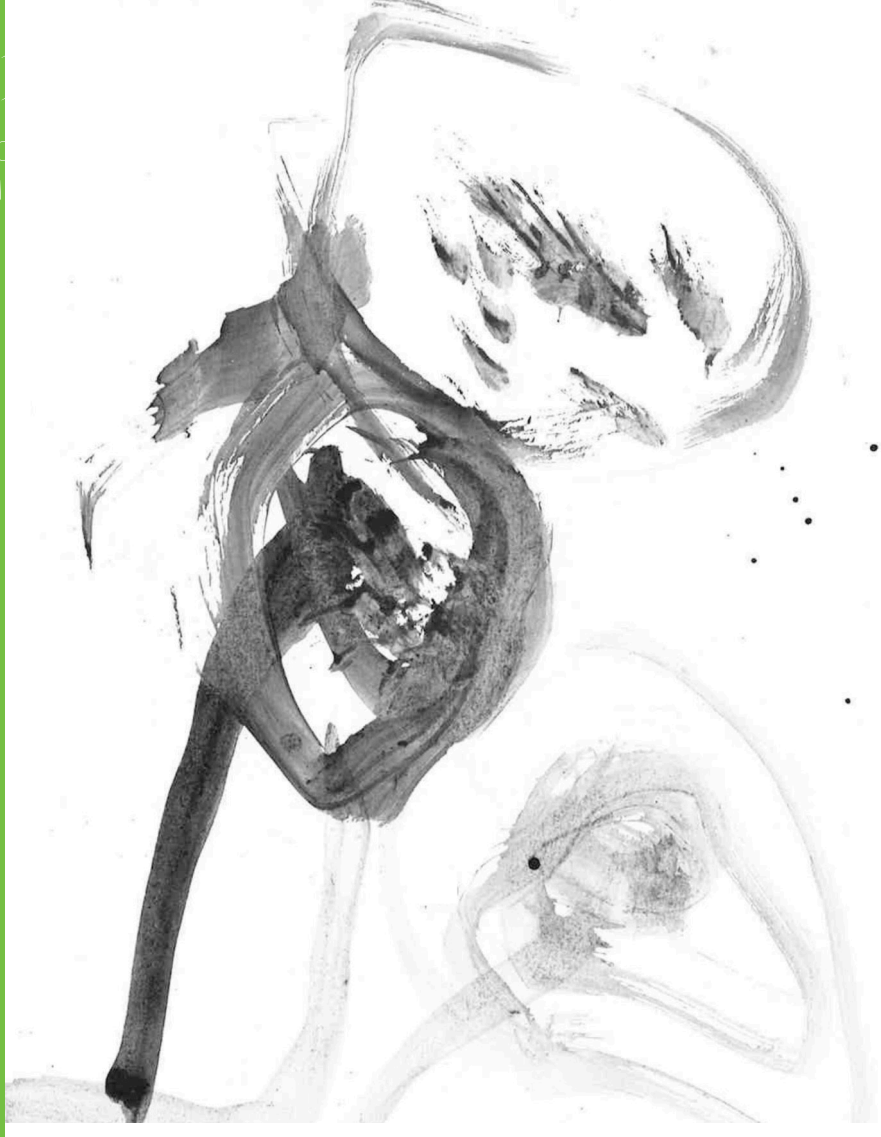

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Dissertation
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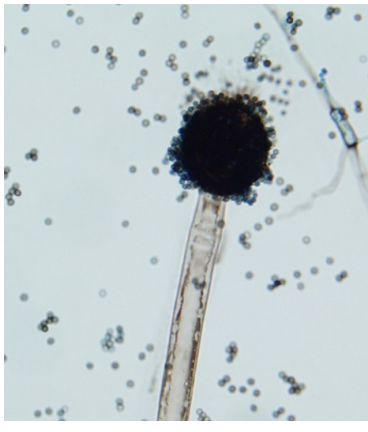
Metabolic engineering of the fungal D-galacturonate pathway

Joosu Kuivanen



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VTT SCIENCE 106

Metabolic engineering of the fungal D-galacturonate pathway

Joosu Kuivanen

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Abstract

Industrial biotechnology is one of the enabling technologies for biorefineries, where biomass is converted into value-added products. In addition to biofuels, several platform and fine chemicals can be produced from biomass using biotechnological routes taking advantage of metabolic pathways in the cell. Some of these metabolic pathways exist naturally in the cells that are used as production hosts. However, many of the desired chemical products are not naturally produced by the cellular metabolism. Consequently, genetic engineering is needed to redirect the cellular metabolism towards a product of interest. In this thesis, one of these metabolic pathways – the catabolic D-galacturonate pathway in filamentous fungi – was engineered and redirected to desired end products. D-Galacturonic acid is the main monomer of pectin, which is a common heteropolysaccharide in certain biomasses. Two examples of pectin-rich biomasses are citrus processing waste and sugar beet pulp from agro-industry. These residual biomasses are often poorly utilised.

Biotechnological production of L-galactonic acid, a potential platform chemical, was demonstrated in this thesis for first time. The production was obtained in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*) strains by deleting the second gene, encoding a dehydratase, from the fungal D-galacturonate pathway. Overexpression of the first gene, encoding a D-galacturonate reductase, in the pathway improved the initial production rate in *A. niger*. In addition, production at low pH resulted in higher productivity and titres in cultivations with the engineered *A. niger* strains. Final titres between 7 and 9 g L-galactonic acid l^{-1} and product yields close to 100% were observed from pure D-galacturonic acid with both of the production hosts.

In addition to L-galactonic acid production from pure D-galacturonic acid, a consolidated bioprocess from citrus processing waste, a pectin-rich biomass, to L-galactonic acid was investigated using the engineered strains of *A. niger*. Two different bioprocess types, submerged and solid state fermentation, were compared. As a result, similar final titres and product yields were observed to those obtained in the process from pure D-galacturonic acid. The highest product yield, approaching 90% of the theoretical maximum, was achieved in the solid state fermentation.

The second reaction in the fungal D-galacturonate pathway is dehydration of L-galactonic acid by the action of an L-galactonate dehydratase. Deletion of the gene *gaaB* encoding this enzyme in *A. niger* is crucial for L-galactonic acid production. Despite the deletion of *gaaB*, product yields (L-galactonic acid per consumed D-galacturonic acid) have remained below the theoretical maximum. In addition, catabolisation of mucic acid, an industrially potential dicarboxylic acid

that can be produced via an engineered D-galacturonate pathway, has been observed in the earlier studies. We hypothesised that catabolisation of L-galactonic acid and mucic acid may be due to other dehydratases. For these reasons, all the five putative dehydratase-encoding genes from *A. niger* were expressed in yeast and the resulting enzymes were characterised. The current study revealed the substrate specifities for four of the studied dehydratases, whereas one of the putative dehydratases was apparently not in fact an active dehydratase. In addition to GaaB, two dehydratases with activity towards D-galactonic acid and one with activity towards L-rhamnonic acid were identified. GaaB was the only dehydratase with activity towards L-galactonic acid. Although GaaB has broad substrate specificity, neither it nor any other dehydratase showed activity towards mucic acid or its lactone. In summary, undesired L-galactonic acid or mucic acid catabolisation was not explained by these dehydratases.

L-Galactonate-5-dehydrogenase, a bacterial enzyme oxidising L-galactonic acid to D-tagaturonic acid, was also studied in this thesis. This enzyme activity has been demonstrated earlier from crude extract of *Escherichia coli*. Later on, the corresponding gene encoding the enzyme was suggested to be *yjjN*, although without characterisation of the enzyme. In this work, it was shown that *yjjN* does indeed encode an L-galactonate-5-dehydrogenase. The K_m and k_{cat} for L-galactonic acid were 19.5 mM and 0.51 s^{-1}, respectively. In addition, the YjjN enzyme was applied in a colorimetric assay for L-galactonic and L-gulonic acids with detection limits of 1.65 µM and 10 µM, respectively.

L-Galactonic acid can be lactonised and further oxidised to L-ascorbic acid (vitamin C) via chemical or biochemical routes. Synthetic L-ascorbic acid is widely used as a nutrient and preservative in several industries. Currently, it is produced in a process combining chemical and biochemical steps. In this thesis, an *A. niger* strain was engineered for direct conversion of D-galacturonic acid to L-ascorbic acid. In addition to the deletion of *gaaB*, two heterologous genes, encoding L-galactono-1,4-lactone lactonase and L-galactono-1,4-lactone dehydrogenase from a plant biosynthetic L-ascorbic acid pathway, were introduced into *A. niger*. In addition, a gene encoding an unspecific L-gulono-1,4-lactone lactonase from a mammalian biosynthetic L-ascorbic acid pathway was tested instead of the plant lactonase. The lactonase enzyme activity was not observed in any of the engineered *A. niger* strains. However, the resulting strains were capable of L-ascorbic acid production from pure D-galacturonic acid or citrus processing waste with final titres up to 170 mg l^{-1}.

Pectin-rich biomass has potential as a raw material for the production of renewable chemicals. This thesis presents new ways to utilise this residual biomass by using industrial biotechnology. In addition, the thesis broadens basic understanding of the fungal catabolic D-galacturonate pathway and how it can be engineered for production of useful chemicals.

Keywords filamentous fungi, *Aspergillus niger*, pectin, D-galacturonic acid, L-galactonic acid, L-ascorbic acid, metabolic engineering

Tiivistelmä

Teollinen biotekniikka on yksi tärkeistä teknologioista, jotka mahdollistavat biomassan jalostamisen erilaisiksi lopputuotteiksi. Bioteknologiaa käyttäen biomassasta voidaan biopolttoaineiden lisäksi tuottaa useita eri kemikaaleja hyödyntämällä solujen metaboliareittejä. Osa näistä hyödyllisistä metaboliareiteistä on luonnostaan soluissa. Sen sijaan osa halutuista lopputuotteista ei syntetisoidu luonnostaan tuotto-organismeissa solujen metaboliareittien kautta. Näissä tapauksissa voidaan hyödyntää solujen geneettistä muokkausta, jotta solun metabolia saadaan ohjattua halutun yhdisteen tuottamiseen. Tässä työssä yksi solun metaboliareitti – mikrosienten D-galakturonihapon kataboliareitti – oli geneettisen muokkauksen kohteena ja se ohjattiin haluttujen yhdisteiden tuottoon. D-galakturonihappo on pektiinin pääkomponentti. Pektiini taas on yleinen kasvibiomassan heteropolysakkaridi. Sitrushedelmien ja sokerijuurikkaan prosessoinnista jäljelle jäävät kuori- ja puristusjäte ovat esimerkkejä pektiinipitoisista biomassoista. Nämä jäännösbiomassat ovat usein vajavaisesti hyödynnettyjä.

L-galaktonihappo on kemikaali, jota voidaan potentiaalisesti hyödyntää moniin eri tarkoituksiin. Tässä työssä sen bioteknologinen tuotanto osoitettiin ensimmäisen kerran hyödyntäen *Aspergillus niger* ja *Hypocrea jecorina* (*Trichoderma reesei*) -homeita, joista D-galakturonihapporeitin toinen, dehydrataasientsyymiä koodaava geeni oli poistettu. D-galakturonihapporeitin ensimmäisen, D-galakturonihapporeduktaasia koodaavan geenin ekspressointi paransi alkuvaiheen tuottonopeutta *A. niger* -homeessa. Lisäksi matala pH paransi tuottoa muokatuissa *A. niger* -kannoissa. Puhtaasta D-galakturonihaposta saavutettiin parhaimmillaan 7–9 g l^{-1} L-galaktonihapon tuotto saannon ollessa lähellä 100 %.

L-galaktonihapon tuoton lisäksi tämän työn tutkimuskohteena oli bioprosessi suoraan pektiinipitoisesta biomassasta L-galaktonihapoksi. Kahta eri bioprosessityyppiä, nestemäistä ja kiinteän tilan kasvatusta, vertailtiin käyttäen muokattuja *A. niger* -kantoja. L-galaktonihapon loppupitoisuudet ja saannot pektiinipitoisesta biomassasta olivat verrannollisia arvoihin, joita saavutettiin puhtaasta D-galakturonihaposta. Korkein saanto, joka oli lähes 90 % teoreettisesta maksimista, saavutettiin kiinteän tilan prosessissa.

Sienien D-galakturonihapon kataboliareitin toinen reaktio on L-galaktonihapon dehydratointi L-galaknonihappodehydrataasientsyymillä. Kyseistä entsyymiä koodaavan geenin deleetio on välttämätön L-galaktonihapon tuoton saavuttamiseksi. Deleetiosta huolimatta saannot (tuotettu L-galaktonihappo per kulutettu D-galakturonihappo) ovat jääneet alle teoreettisen maksimiarvon. Tämän lisäksi toisen D-galakturonihaposta tuotettavan yhdisteen, teollisesti potentiaalisen kemikaalin galaktaarihapon on havaittu katabolisoituvan sen tuottoon muokatuissa *A. niger* -kannoissa. Tässä työssä hypoteesinä edellä mainittujen yhdisteiden eitoivotulle katabolialle olivat mahdolliset muiden dehydrataasientsyymien reaktiot.

Näin ollen kaikki viisi putatiivista dehydrataasigeeniä *A. nigerin* genomista ekspressoitiin hiivassa ja niiden proteiinituotteiden dehydrataasiaktiivisuudet karakterisoitiin. Entsyymikarakterisoinnin avulla määritettiin neljän dehydrataasin substraattispesifisyydet, kun taas yksi putatiivisista dehydrataasigeeneistä ei todennäköisimmin koodaa toiminnallista dehydrataasia. GaaB-L-galaktonihappodehydrataasin lisäksi identifioitiin kaksi dehydrataasia, joilla on aktiivisuus D-galaktonihappoa, ja yksi dehydrataasi, jolla on aktiivisuus L-rhamnonihappoa kohtaan. GaaB oli ainoa tutkituista dehydrataaseista, jolla oli aktiivisuus L-galaktonihappoa kohtaan. Yhdelläkään tutkituista dehydrataaseista ei ollut aktiivisuutta limahappoa tai sen laktonimuotoa kohtaan. Näin ollen L-galaktonihapon ja limahapon kataboliaa ei voitu yhdistää tutkittuihin dehydrataaseihin.

L-galaktonihappo-5-dehydrogenaasi on bakteereissa esiintyvä entsyymi, joka hapettaa L-galaktonihapon D-tagaturonihapoksi. Tämän entsyymin aktiivisuus on aikaisemmissa tutkimuksessa osoitettu *Escherichia coli* -bakteerin soluekstraktista. Myöhemmin kyseisen entsyymin ehdotettiin olevan *yjjN*-geenin koodaama, mutta kyseisessä tutkimuksessa ei karakterisoitu geenin koodaamaa proteiinia. Tässä työssä osoitettiin, että yjjN-geeni koodaa todellakin L-galaktonihappo-5-dehydrogenaasia. K_m ja k_{cat} -arvoiksi L-galaktonihappoa kohtaan määritettiin 19,5 mM ja 0,51 s^{-1}. Tämän lisäksi YjjN-entsyymiä hyödynnettiin kolorimetrisessä analyysimenetelmässä L-galaktoni- ja L-gulonihapon pitoisuuksien määrittämiseen. Havaittavien pitoisuuksien alarajaksi määritettiin L-galaktonihapolle1,65 µM ja L-gulonihapolle 10 µM.

L-galaktonihappo voidaan laktonisoida ja edelleen hapettaa L-askorbiinihapoksi (vitamiini C) käyttäen kemiallisia tai biokemiallisia reaktioita. Synteettinen L-askorbiinihappo on laajalti käytössä ravinteena ja säilöntäaineena eri teollisuudenaloilla. Tällä hetkellä käytössä oleva valmistusmenetelmä yhdistää useita kemiallisia ja biokemiallisia vaiheita. Tässä työssä *A. niger* -home muokattiin tuottamaan L-askorbiinihappoa D-galakturonihaposta. *gaaB*-geenin poistamisen lisäksi kasvien L-askorbiinihapon synteesireitiltä ekspressoitiin L-galaktono-1,4-laktoni laktonaasia ja L-galaktono-1,4-laktoni dehydrogenaasia koodaavat geenit *A. nigerissä*. Vaihtoehtoisesti epäspesifi L-gulono-1,4-laktoni laktonaasia koodaava geeni eläinten L-askorbiinihapon synteesireitiltä ekspressoitiin kasviperäisen laktonaasin sijasta. Laktonaasientsyymiaktiivisuutta ei pystytty kuitenkaan havaitsemaan yhdestäkään muokatuista *A. niger* -kannoista. Tästä huolimatta kannat pystyivät tuottamaan L-askorbiinihappoa puhtaasta D-galakturonihaposta tai sitrushedelmien prosessijäännösbiomassasta. Korkein havaittu L-askorbiinihapon pitoisuus kasvatuksissa oli 170 mg l^{-1}.

Pektiinipitoinen biomassa on potentiaalinen uusiutuva raaka-aine kemikaalien tuottamiseksi. Tämä väitöskirja tuo esille uusia tapoja, joilla jäännösbiomassa voidaan hyödyntää entistä tehokkaammin käyttäen teollista bioteknologiaa. Lisäksi väitöskirja laajentaa perusymmärrystä sienien D-galakturonihapon metaboliareitistä ja siitä, kuinka sitä voidaan muokata tuottamaan hyödyllisiä kemikaaleja.

Avainsanat filamentous fungi, *Aspergillus niger*, pectin, D-galacturonic acid, L-galactonic acid, L-ascorbic acid, metabolic engineering

Preface

This study was carried out at the VTT Technical Research Centre of Finland Ltd in the Metabolic Engineering and Synthetic Biology teams, during the years 2010–2014. The work was financially supported by the Academy of Finland under the research program Sustainable Energy (grants 131869 and 271025). This financial support is warmly acknowledged.

I sincerely would like to thank Vice President Professor Anu Kaukovirta-Norja, Head of Research Area, Docent Kirsi-Marja Oksman-Caldentey and former Technology Manager Dr. Tiina Nakari-Setälä for providing excellent working facilities and for the opportunity to prepare my thesis at VTT. My former and current Team Leaders, Dr. Laura Ruohonen and Docent Jussi Jäntti are deeply acknowledged for their supportive attitude towards my work.

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I have truly enjoyed my time working in the yeast-mould lab at VTT. This is mostly due to skilful, but above all kind-hearted people whom I have had a privilege to have as colleagues and workmates. I would like to thank you all for your support during these years. Outi, Yvonne, Mari, Anssi, Kiira, Mira, Pekka and Eero, thank you for all the discussions, lunches, coffee breaks (when some goodies were served), room sharing and other activities inside and outside working hours. I truly appreciate Outi's support and help in all matters during these years, thank you a lot. I thank Yvonne for accepting me as a roommate at work and for all the fruitful discussions from science to sport during these years.

Finally I would like to thank everybody in my family, especially my dear wife Veera for your love and support (both mental and practical). In the end I would thank my true sources of inspiration, my wonderful little daughters Saimi and Saana. Thank you for taking me to another world every day. Special thanks to my older daughter Saimi (3 yrs.) for permitting her artistic view of *Aspergillus niger* conidiophores being presented as a cover picture in this thesis.

Espoo, August 2015
Joosu Kuivanen

Academic dissertation

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Opponent	Associate Professor Uffe Hasbro Mortensen Department of Systems Biology DTU, Technical University of Denmark

List of publications

This thesis is based on the following original publications, which are referred to in the text as I–V. The publications are reproduced with kind permission from the publishers.

I Kuivanen J., Mojzita D., Wang Y., Hilditch S., Penttilä M., Richard P., Wiebe M.G. 2012. Engineering filamentous fungi for conversion of D-galacturonic acid to L-galactonic acid. Applied and Environmental Microbiology 78(24): 8676–8683. doi: 10.1128/AEM.02171-12.

II Kuivanen J., Dantas H., Mojzita D., Mallmann E., Biz A., Krieger N., Mitchell D., Richard P. 2014. Conversion of orange peel to L-galactonic acid in a consolidated process using engineered strains of *Aspergillus niger*. AMB Express 4:33. doi: 10.1186/s13568-014-0033-z.

III Motter F.A., Kuivanen J., Keränen H., Hilditch S., Penttilä M., Richard P. 2014. Categorisation of sugar acid dehydratases in *Aspergillus niger*. Fungal Genetics and Biology 64:67–72. doi: 10.1016/j.fgb.2013.12.006.

IV Kuivanen J., Richard P. 2014. The *yjjN* of *E. coli* codes for an L-galactonate dehydrogenase and can be used for quantification of L-galactonate and L-gulonate. Applied Biochemistry and Biotechnology 173(7): 1829–1835. doi: 10.1007/s12010-s.

V Kuivanen J., Penttilä M., Richard P. 2015. Metabolic engineering of the fungal D-galacturonate pathway for L-ascorbic acid production. Microbial Cell Factories 14:2.

Author’s contributions

Publication I

Joosu Kuivanen participated in the design of the experimental work, carried out the *Aspergillus* strain construction, transcriptional analysis and some of the shake flask cultivations, participated in the data analysis and collaborated with the other authors to write the article.

Publication II

Joosu Kuivanen participated in the design of the experimental work, carried out all the submerged cultivations, supervised a student who carried out the solid state fermentations, analysed the data, drafted the article and is the corresponding author.

Publication III

Joosu Kuivanen participated in the design of the experimental work, carried out the cloning of the *gaaB* expression plasmid, participated in the supervision of a student who carried out most of the experimental work, and collaborated with the other authors to write the article.

Publication IV

Joosu Kuivanen participated in the design of the experimental work, carried out all the experimental work, analysed the data, drafted the article and is the corresponding author.

Publication V

Joosu Kuivanen designed the experimental work, carried out all the experimental work, analysed the data, drafted the article and is the corresponding author.

Contents

List of symbols

ALase	Aldonolactonase
AP	Apiogalacturonan
ATP	Adenosine triphosphate
BTX	Benzene, toluene and xylene
CAZY	Carbohydrate-active enzymes
CPW	Citrus processing waste
D-galUA	D-Galacturonic acid
dha	2-Keto-3-deoxy-D-lyxo-heptulosaric acid
DHAP	Dihydroxyacetone phosphate
DW	Dry weight
EMP	Embden-Meyerhof-Parnas
ER	Endoplasmic reticulum
FAD(H_2)	Flavin adenine dinucleotide
GALDH	L-Galactono-1,4-lactone dehydrogenase
GRAS	Generally recognised as safe
HG	Homogalacturonan
HPLC	High performance chromatography
INT	p-Iodonitrotetrazolium
kdo	2-Keto-3-deoxy-D-manno-octulosonic acid
L-AA	L-Ascorbic acid
L-galA	L-Galactonic acid
L-galL	L-Galactono-1,4-lactone

LB	Luria Broth
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
NHEJ	Non-homologous end joining
ORF	Open reading frame
P	Promoter
PCR	Polymerase chain reaction
PD	Potato dextrose
PEG	Polyethylene glycol
PPP	Pentose phosphate pathway
RG	Rhamnogalacturonan
SBP	Sugar beet pulp
SEM	Standard error of the mean
SmF	Submerged fermentation
SSF	Solid state fermentation
T	Terminator
TBA	Thiobarbituric acid
TCA	Tricarboxylic acid
UDH	Uronate dehydrogenase
XGA	Xylogalacturonan
5-FOA	5-Fluoro-orotic acid

1. Introduction

Increasing world population, our carbon intensive society and the growth-based economic system will continue to increase carbon consumption for fuels, chemicals and materials in the near future. In the industrial revolution about 300 years ago, the principal carbon source for fuels was shifted from biomass to fossil resources such as coal. Later, economic growth during the 20th century was driven by petroleum, which also provided the raw material for many new materials such as plastics. In the 21st century the sufficiency of petroleum reservoirs has been called into question, even though the use of the alternative fossil resources such as shale gas and shale oil is increasing. However, disadvantages such as environmental issues and lack of energy self-sufficiency are associated with the use of fossil resources. Consequently, a shift from fossil resources back to a biomass-based society has become a topical issue and the vision is accordingly named "bioeconomy".

In an ideal bioeconomy, the use of fossil resources would be replaced by the use of renewables such as sunlight, wind and biomass. Instead of oil refineries, biomass would be processed in biorefineries producing energy, fuels, chemicals and materials. Some of the greatest challenges facing the use of biomass instead of fossil resources are its chemical heterogeneity, high oxygen and low energy content. In addition, issues such as competition with food production or the negative effects of biomass transportation may possibly lead to unsustainable biorefining processes. In addition, fluctuating petroleum prices and unstable government subsidy policies complicate the biorefinery business. Thus, it is crucial to define suitable biomass resources and feasible products from biorefining.

In biorefining, conversion processes can be coarsely divided into three categories, namely thermal, chemical and biochemical processes. Thermal conversions include methods such as pyrolysis, gasification and traditional burning of biomass. In chemical conversion methods, e.g. gasified biomass can be condensed into a liquid through Fischer-Tropsch synthesis or biobased fatty acids can be converted to biodiesel by transesterification or hydrogenation reactions. In biochemical methods, biomass is converted using enzymatic reactions, commonly inside microbial cells which are in this context often referred to as microbial cell factories. This approach is also called industrial biotechnology or white biotechnology.

Diesel and gasoline are the major transport fuels derived from petroleum. Renewable replacements for diesel are commonly refined from vegetable oils or animal fats using chemical conversion methods. In the case of gasoline, the renewable substitute is typically ethanol, which is already widely used as an additive in gasoline. Industrial biotechnology is an efficient way to convert biomass into ethanol. Ethanol production is generally classified on the basis of the raw material, and the same classification applies to all industrial biotechnology processes. In first generation bioethanol the raw material is commonly D-glucose obtained from sugar cane or corn starch. These two feedstocks are typical examples of biomass resources which compete with food production. In second generation bioethanol the raw material is typically more complex and heterogenous lignocellulosic biomass, such as wood, straw, corn stover or sugar cane bagasse, containing cellulose, hemicellulose and lignin. Lignocellulosic biomass is a more sustainable raw material for biochemical conversions in biorefineries. However, physical or chemical pretreatment and enzymatic hydrolysis to monomeric sugars is often needed prior to microbial conversion, which causes additional costs for these processes. In addition, the monomeric sugars include pentoses, such as D-xylose and L-arabinose, which are not naturally utilised by some of the common production hosts. Thus, it would be beneficial if the production host would itself be capable of hydrolysing polysaccharides and utilising all the resulting monomeric sugars. The direct microbial conversion of untreated biomass into a product is also called consolidated bioprocessing.

In addition to biofuels, many bulk and fine chemicals can be prepared from biomass. Taking into account all the biomass conversion methods, the annual production of renewable chemicals and polymers including cellulose and starch derivatives exceeds 50 million tons [1]. By contrast, the scale of fossil-based production of chemicals and polymers is about 330 million tons, accounting for about 9% of the total use of fossil resources (including energy reqired for production) [1]. The production of fossil-based chemicals is focused on a few building block compounds, including ethylene, propylene, butadiene, methanol and the aromatics benzene, toluene and xylene (BTX) [1]. These building blocks are then converted further to other chemicals and polymers. In order to replace the current use of fossil feedstocks with biomass in the chemical industry, these building blocks, their derivatives or direct substitutes must be produced in biorefineries. The U.S. Department of Energy has listed the top value-added chemicals that can be derived from biomass using different biorefining methods. The first report [2] listed 12 most potential chemical building block chemicals that can be derived from biomass carbohydrates, whereas the second report [3] focused on lignin utilisation. Later on the list of building block chemicals was updated [4] to include 10 compounds or groups. The updated list includes some organic acids, such as lactic, succinic and hydroxypropionic acid that are used as building blocks in polymers. These acids and many other organic compounds (e.g. fatty acids and hydrocarbons) occur naturally as intermediates in cellular metabolism, which means that they can potentially be produced using industrial biotechnology.

In principle, industrial biotechnology enables processes for production of many chemical compounds or their substitutes that are currently produced from fossil resources in the chemical industry. For example, some microbial organisms secrete byproducts from metabolic processes (e.g. ethanol and lactic acid) or have evolved naturally to acidify the surrounding environment by producing organic acids (e.g. gluconic acid and citric acid). Some of these microbes are already utilised in traditional industrial biotechnology processes, such as lactic acid production with bacteria or citric acid production with filamentous fungi. Some of the natural production hosts in industrial biotechnology were improved further by using classical strain development strategies including random mutagenesis with UV or chemicals. More recently, molecular biology techniques have revolutionised microbial strain development, offering tools for targeted genetic modifications, also called genetic engineering. In a single microbial cell, countless numbers of biochemical reactions are taking place which offer a huge potential for production of different chemical compounds. The approach to manipulate natural cellular metabolism to produce compounds of interest or generate catabolic pathways for new substrates is called metabolic engineering.

In this work one of the traditional production hosts – the filamentous fungus *Aspergillus niger* – was used as a platform for metabolic engineering. The aim was to engineer the catabolic D-galacturonate (D-galUA) pathway, redirecting it to useful chemical compounds. D-GalUA is the main constituent of pectin, which is a cell wall polysaccharide. Pectin is found in many non-woody biomass types, such as fruit peels or sugar beet pulp, which are typical agricultural waste streams. Currently these pectin-rich agricultural waste streams are insufficiently utilised, providing potential raw material for second generation biorefining. The production host used in this work is capable of hydrolysing complex natural polysaccharides such as pectin and utilises efficiently the resulting mixture of monomeric sugars, enabling consolidated bioprocessing. Thus, it is a well-suited organism for second generation industrial biotechnology processes.

1.1 *Aspergillus* – a diverse genus of filamentous fungi

The genus *Aspergillus* comprises a broad range of about 250 filamentous fungal species belonging to the phylum of *Ascomycota* [5]. *Aspergillus* species are saprotrophs (extracellularly digest organic matter) and are found from various aerobic environments such as decaying biomass. They are also common contaminants in food. Different *Aspergilli* (plural of *Aspergillus*) can grow over a wide range of extreme conditions, such as at pH 2–11, temperatures from 10 °C to 50 °C and in low or high osmolarity [6]. They exhibit a mould type of growth, forming multicellular filaments on the surface of substrate particles. A single branching filament is also called a hypha and the network of hyphae is known as mycelium. The haploid stage predominates among *Aspergilli* and they reproduce typically by forming asexual spores called conidia. The structures carrying conidia are known as conidiophores. In addition to asexual reproduction, some *Aspergillus* species, such as

Aspergillus nidulans, possess sexual stages forming fruiting bodies for reproduction. However, the sexual stages are not referred to as *Aspergillus*. For instance, in the case of *A. nidulans*, the sexual stage is known as *Emericella nidulans*.

In the asexual life cycle, fungal conidia germinate and form hyphae and mycelia which are the principal structures in vegetative growth. In *Aspergillus* species the hypha is divided into cells which are separated with a wall structure known as a septum. However, the cells are typically connected to each other through small pores in the septa. *Aspergilli*, in common with many fungi, have evolved to utilise decomposing organic matter. Biomass polysaccharides, such as cellulose, hemicellulose and pectin, are first degraded to smaller units outside the cell by the fungus using a set of secreted biomass-degrading enzymes that are also known as "Carbohydrate-Active enZYmes" (CAZY). Released oligo- and monosaccharides are then transported into the cells and catabolised further through metabolic pathways producing energy and providing building blocks for biomass. *Aspergilli* are known for their versatile capacity to degrade and catabolise a wide range of different polysaccharides and sugars as carbon sources. Moreover, they can utilise several different nitrogen sources, such as nitrate, nitrite, amides, purines, ammonia and amino acids [7].

The *Aspergillus* genus includes species ranging from human pathogens to industrial workhorses with GRAS status (generally recognised as safe) that are widely used in commercial organic acid and enzyme production processes. One of the best known and studied species of *Aspergillus* is *A. nidulans*. This species has a long history as a eukaryotic model organism and it has been used e.g. to study cell cytoskeleton microtubules [8,9]. Its genome sequence was released already in 2003 and the annotated genome with the analysis was published at the same time as those of two other *Aspergilli* – *A. oryzae* and *A. fumigatus* – in 2005 [10,11,12].

Some of the *Aspergillus* species are also opportunistic pathogens. The most common human pathogen is *A. fumigatus,* which typically causes fungal infections in lungs, known as aspergilliosis. Another important species in the context of human health is *A. flavus,* which is known as a contaminant in grains. *A. flavus* contamination can result in the production of aflatoxin (the name is derived from ***A****s-pergillus* ***fla****vus*), which is a severely carcinogenic chemical [13].

Several species among the *Aspergilli* are exploited in food processing and industrial biotechnology. *A. oryzae* has a long history in Japanese, Chinese and Korean cuisines. Culinary applications include soy bean fermentations for production of soy sauce, hydrolysis of rice polysaccharides for sake fermentation and production of rice vinegars [14]. *A. oryzae* is also used for production of enzymes, heterologous proteins [15] and organic acid acids [16]. Another species investigated for production of commondity chemicals is *A. terreus*, which has been reported to be a potential producer of secondary metabolites for pharmaceutical purposes [17]. Production of itaconic acid – an organic acid used in polymers – has also been investigated and commercially implemented using *A. terreus* [18]. However, probably the most widely used *Aspergillus* in commercial protein and organic acid production is the black-spored member of the *Aspergilli* – *A. niger*.

1.1.1 *Aspergillus niger* – a widely used workhorse in biotechnology

A. niger is one of the most used host organisms in industrial biotechnology. Several commodities including enzymes and organic acids are produced commercially in *A. niger* fermentations. It is also a common contaminant in fruits and vegetables, especially in onions, causing a disease called black mould.

A. niger is an aggregate of different strains that are difficult to differentiate on the basis of morphological characteristics [19]. In contrast to *A. nidulans*, only asexual reproduction and haploid states are observed among *A. niger* strains. The genome of *A. niger* consists of eight linear chromosomes. The first genome sequence, from the strain CBS 513.88 (a platform strain for protein production), was published in 2007 [20]. Later on, the genome sequence of a traditionally used citric acid-producing strain ATCC 1015, the strain used in this work, was published by The U.S. Department of Energy Joint Genome Institute and the genome sequences of the two strains were compared [21]. The comparison revealed high genetic variation between the two strains. The genome size of CBS 512.88 is 34.02 Mb whereas ATCC 1015 has a genome of 34.85 Mb. The predicted number of genes in CBS 512.88 is 14 082, including on average 3.6 exons per gene. In ATCC 1015 the predicted gene number is 11 200, with an average of 3.1 exons per gene. The overall single-nucleotide polymorphism between the strains was 8 bp per kb, which is an exceptionally high amount within a single species. Genotypic differences between these two strains are concentrated in metabolic pathways that are involved in protein synthesis and acid production, as expected [21].

Being a filamentous fungus, *A. niger* can be cultivated either in submerged (SmF) or solid state (SSF) fermentations. In SmFs the important cultivation parameters, such as aeration, substrate feed, pH and temperature are easy to measure and control during the process. In a typical industrial biotechnology process *A. niger* is cultivated in SmFs. However, in SSF the surrounding habitat is closer to the natural environment of the organism and in some cases, such as production of organic acids, SSF can result in higher productivity [22]. The drawback of SSF processes is that the process parameters are more difficult to control and monitor.

A. niger is an efficient producer of secreted biomass-degrading enzymes. Several native biomass-degrading enzymes, such as glucoamylases, α-amylases, glucose oxidases and pectinases, are produced commercially in SmF or SSF processes with exceptionally high final titres approaching 30 g l^{-1} [15,23,24]. In addition to its outstanding secretion capacity, *A. niger* possesses a suitable glycosylation machinery for production of heterologous proteins [15]. Thus, several heterologous proteins, including therapeutic human proteins such as interferons, are produced with recombinant *A. niger* strains. However, production levels of heterologous proteins have remained significantly lower when compared with the native secreted enzymes [7,15].

Another commercially significant field in *A. niger* biotechnology is the production of citric acid (E330) in SmF. Citric acid occurs as a primary metabolite in the

tricarboxylic acid (TCA) cycle in the cell. It is widely used as a preservative and flavour compound in beverage and food industries and as a buffering or metal ion chelating agent in other applications. The first report on citric acid production in *A. niger* fermentations was published almost one hundred years ago [25] and commercial production with *A. niger* was started in 1923 by the pharmaceutical company Pfizer [26]. Most of the commercial citric acid is currently produced with *A. niger* fermentations. The annual production of citric acid is about 2 million tons, with a market price of around 1 USD per kg [26,27]. The raw material for the production is commonly corn starch derived sugars. Citric acid production with *A. niger* fermentations is strictly dependent on culture conditions – high sugar concentration, low pH (<3), manganese depletion and efficient aeration are needed for high productivity [28]. Product yields close to 100% and final titres of above 200 g l^{-1} can be achieved, enabling economically feasible processes.

If the pH value in a D-glucose fermentation with *A. niger* is elevated to around 4.5-6.5, production shifts from citric acid to D-gluconic acid (E574) [29]. The production is based on extracellular oxidation of D-glucose by secreted glucose oxidases. Electrons from D-glucose are shuttled to oxygen, resulting in formation of glucono-1,5-lactone and hydrogen peroxide. The lactone spontaneously hydrolyses or is enzymatically converted to D-gluconic acid in the fermentation. D-Gluconic acid production using *A. niger* fermentations has a long history and both yields and titres are comparable with those of citric acid production. Application areas for D-gluconic acid and its derivatives are mainly in the cement (D-gluconic acid, sodium gluconate), food (sodium and calcium gluconate) and pharmaceutical (calcium gluconate, lactone) industries. The market price depends on the type of derivative and field of application, being generally between 1.20 and 8.50 USD per kg [30]. However, the market volume is less than 5% compared to citric acid production [30].

Besides the commercial processes for enzyme, heterologous protein and organic acid production, other hitherto commercially less significant application areas using *A. niger* include production of secondary metabolites, fungal biotransformations, bioremediation and bioleaching. Filamentous fungi including *A. niger* are known for their diverse capacity to produce and secrete secondary metabolites. In contrast to primary metabolites, such as citric acid, secondary metabolites are organic compounds, which are not directly connected to growth, and are resulting from metabolic pathways that are commonly activated only in certain specific conditions. An example of secondary metabolites in filamentous fungi is penicillins, which were first discovered from *Penicillium* species [31]. In the case of *A. niger*, several interesting biologically active secondary metabolites have been identified, including compounds belonging to classes such as polyketides and terpenoids (isoprenoids) [6,32]. In addition to commercially interesting secondary metabolites, some natural isolates of *A. niger* have been reported to produce the carcinogenic secondary metabolite ochratoxin A. However, this does not apply to industrial strains [24,33].

Biotransformation refers to biochemical reactions in which a chemical compound is modified *in vivo* with high stereo-specificy by native enzymatic activities

of an organism. As an example, *A. niger* has been reported to be capable of biotransformation of alpha pinene to verbenone, which is a terpene used as an odorant in some applications [34]. Another interesting field, partially overlapping with biotransformations, is bioremediation, referring to removal or neutralization of toxic compounds from different materials using microorganisms [6]. The detoxification can be based on biosorption or degradation of a toxic compound. Some *A. niger* strains have been investigated for biosorption of metal ions such as copper (II), lead (II) and mercury or methyl-mercury ions from waste waters [6,7]. In addition, some researchers are trying to use *A. niger* in bioleaching processes aiming at metal recovery, in which metal ions are mobilized due to organic acids (citric and oxalic acid) secreted by the fungus [35].

1.2 Genetic engineering of *Aspergillus niger*

Many current commercial industrial biotechnology processes are carried out using wild type or randomly mutagenized microbial strains. However, genetic engineering enables targeted and more rational genome modifications for strain improvement. In genetic engineering a piece of genomic DNA, such as a gene, is removed or a new fragment of recombinant DNA is introduced to a host organism.

Construction of recombinant DNA is an important part of genetic engineering. Different types of DNA constructs, such as gene deletion and expression cassettes, are used in genetic engineering. Polymerase chain reaction (PCR), restriction endonucleases and DNA ligases are the conventional molecular biology tools that are used for DNA construction. Afterwards, advanced technologies such as Gibson assembly, Golden Gate cloning, MoClo and chemically synthesized and codon optimized genes have facilitated recombinant DNA construction.

In *Aspergilli*, many of the tools for genetic engineering were originally developed for the model species *A. nidulans*. Many of those tools are also applicable to *A. niger* and several enzyme-producing engineered strains were established already in the 1990s [24]. However, compared to *E. coli* or *S. cerevisiae*, genetic engineering of *Aspergilli* is challenging. One of the limiting factors is the lack of stable episomal plasmids for gene expression. In addition, as a multicellular organism, genetic transformation is more demanding due to the necessity for preparation of protoplasts by enzymatic removal of the cell wall. In the next sections, the molecular tools that are used for genetic engineering in *A. niger* are introduced. In addition, some examples of the approaches are presented in which genetic engineering is used in *A. niger* for protein production or metabolic engineering.

1.2.1 Genetic transformation

After the construction of recombinant DNA, the next step in genetic engineering is to deliver the DNA into the cell. *A. niger* transformations are routinely carried out using the so-called protoplast method. In this method, the cell wall of germinated conidia or young multicellular mycelium is enzymatically degraded, resulting in

protoplasts. The cell membrane is then destabilised using polyethylene glycol (PEG) and $CaCl_2$ and the DNA is taken in by the protoplasts. Due to the missing cell wall, the protoplast must be osmotically stabilised during the transformation process. Other reported transformation methods for *Aspergilli* are electroporation, *Agrobacterium* transformation and biolistic methods [36]. In the case of *A. niger,* protoplast transformation is clearly the predominant method.

1.2.2 Tools for genetic engineering

Gene deletions are created by replacing a target gene with a deletion cassette. Typically, deletion cassettes contain homologous flanking regions, which are targeted for the gene of interest, and a selection marker between the flanks. Due to the fact that homologous recombination is relatively inefficient in *A. niger*, the homologous flanks in the cassette must be rather long (around 1500 bp). Even then, most of the transformed colonies that contain the cassette are randomly integrated into the genome by non-homologous end joining (NHEJ), which is the dominating DNA repair mechanism in *A. niger* [37]. This means that several resulting colonies must be screened for the deleted target gene. Deletion of the *kusA* gene, which encodes one of the proteins acting in the NHEJ-mechanism, led to an *A. niger* strain with significantly increased frequency of homologous recombination [37]. However, due to the disrupted DNA repair mechanism, the resulting strain is more sensitive to UV radiation and X-ray irradiation when compared with wild type strain.

There are several nutritional and antibiotic selection markers available for *A. niger*. The most widely used nutritional selection marker is the gene *pyrG* encoding an orotidine 5-phosphate decarboxylase involved in the biosynthesis of pyrimidine ribonucleotides for RNA [38]. Deletion of the homologous *pyrG* gene results in a uridine/uracil auxotrophic *A. niger* strain. The *pyrG* selection system allows counter-selection using 5-fluoro-orotic acid (5-FOA), which is converted to toxic 5-fluorouracil by the action of functional PyrG. Other nutritional selection markers that have been reported to function in *A. niger* are *amdS* (acetamide utilization), *argB* and *agaA* (arginine auxotrophy), *trpC* (tryptophan auxotrophy), *niaD* (nitrate utilization) and *sC* (sulphate utilization) [36,39]. The gene *hph* encoding for hygromycin B phosphotransferase, giving resistance against hygromycin, is probably the most widely used antibiotic marker in *A. niger*. Other reported antibiotic markers for *A. niger* are the *oliC* (oligomycin) and *bar* (phosphinothricin) genes [36].

In addition to an open reading frame (ORF), a DNA reading frame coding for a protein in a gene, cassettes for gene expression contain a promoter (P), terminator (T) and selection marker. As in the case of gene deletions, transformation and genetic integration of the expression cassettes is enforced with the use of selection markers. The same marker genes are used for expression cassettes that were used with the deletion cassettes. In genetic engineering, different promoters have a central role and are used as regulatory elements tuning a suitable transcription level for the gene of interest. In an ideal case orthogonal promoters, which are

promoters independent of the native cellular regulatory mechanisms, would be used in genetic engineering. In *A. niger*, the availability of orthogonal promoters is rather limited – only one example, a tetracycline inducible promoter, has been reported in the literature [40]. Some of the most widely used promoters in the genetic engineering of *A. niger* are described in the following examples.

The most commonly used promoter for enzyme and protein production in *A. niger* is the native promoter of the glucoamylase gene *glaA* (*PglaA*). *PglaA* is an inducible promoter, the activity of which is induced in the presence of maltose or starch. In addition, three CCAAT sites have been identified in the 5'UTR of *PglaA* that are essential for high expression [15]. The activity of *PglaA* was improved when additional CCAAT sites were introduced into the promoter [41]. *PglaA* is under the control of carbon catabolite repression mediated by CreA and the expression is low or prevented when easily metabolized carbon sources such as D-glucose and D-xylose are present [15]. Other inducible promoters used in protein production with *A. niger* include e.g. promoters of *aldA* (alcohol dehydrogenase; ethanol and threonine induced) and *sucA* (sucrase A, sucrose and inulin induced) [15]. The most used constitutive promoter in protein-producing *A. niger* strains is *PgpdA* (the promoter of glyceraldehyde-3-phosphate dehydrogenase) [15].

In addition to promoter strength, the copy number of integrated genes is one of the variables affecting the gene transcription and protein production. However, it has been observed that the protein production is not necessary improved by high copy numbers. This may be due to titration of transcription factors or the negative effects of several random integrations [15]. Other factors affecting the transcription are the activity of the genomic locus in which the target gene is integrated, and mRNA stability after the transcription. Little research has been published on mRNA stability in *A. niger*, although the currently used heterologous genes are often codon-optimized for the host, thus improving the transcription and mRNA stability.

1.2.3 Engineering for recombinant protein production

A. niger is a widely used host organism in commercial protein and enzyme production. Although detailed information concerning the strains developed by commercial producers is often not available, the development process of one enzyme-producing *A. niger* strain within a company (DSM) was described by van Dijck et al in 2003 [24]. In the past at DSM, an expression cassette containing *Pgla*, the corresponding terminator (*TglaA*) and the gene of interest was transformed into a host strain, resulting in random integration of the cassettes into one or several loci by the NHEJ-mechanism. Transformed strains were then exposed to mutagenesis and the best strains were selected. Due to the unexpected effects of random integrations, currently used expression cassettes are typically designed for targeted integration into several *glaA* loci that have been introduced in the platform strain. The commonly used selection marker is *amdS,* which is subsequently removed by using counter-selection on fluoroacetate, which is converted to a toxic compound

by the action of AmdS. This strategy enables reuse of the marker in further engineering steps and final production strains which are free of the selection marker.

In extracellular protein production, after the transcription and translation of a gene of interest, the resulting polypeptide chain with the secretion signal enters the endoplasmic reticulum (ER), where it is folded and modified. In the secretion pathway the next destination is the Golgi apparatus, which is reached through vesicle transport mediated by SNARE-proteins. The final step is the vesicular trafficking between the Golgi and plasma membrane, again with the help of SNAREs ending in the secretion of the protein from the tips of hyphae. In one approach to improve secretion, an *A. niger* strain was engineered for alternative protein secretion: *A. niger* v-SNARE protein was fused with a peroxisomal membrane protein, enabling the secretion of proteins with a peroxisomal targeting signal [42]. The resulting secretion pathway, called peroxicretion, was described to be useful especially for the production of intracellular proteins. After the secretion of heterologous proteins, their survival may be adversely affected by the several proteases that are naturally secreted into the environment by the fungus. Thus, there has been considerable effort to minimize unwanted proteolysis by protease gene deletions [15].

1.2.4 Metabolic engineering for chemicals production

Compared to recombinant protein production, there is much less scientific literature about engineering of *A. niger* for production of chemicals. Citric acid production with *A. niger* is a widely used process, but the production hosts were mainly generated by classical strain development. This is probably partially due to the negative attitude towards genetically modified organisms in the food industry, where most of the citric acid is used. In addition, the current processes are relatively efficient. However, there are some examples in the literature in which it was attempted to improve production of citric acid or other chemicals using genetic engineering in *A. niger*.

In the current processes for citric acid production with *A. niger*, productivities and product yields are on a high level. However, one of the challenges is the dependence of the production on specific cultivation conditions such as depletion of trace manganese. The optimal cultivation conditions lead to a gene expression pattern favouring citrate accumulation in the cells. In one of the few metabolic engineering approaches in *A. niger*, several TCA cycle enzymes that are crucial for citrate accumulation were introduced and expressed under the constitutive *gpdA* promoter [43]. In contrast to the parental strain, the resulting strain was capable of citrate production in the presence of trace manganese. In addition to citric acid, production of two C4-dicarboxylic acids from the TCA cycle – succinic and malic acids – was attempted by engineering *A. niger* strains. In one approach, the glyoxylate cycle enzyme isocitrate lyase was constitutively expressed [44]. As a result, although succinate and malate were not produced from D-glucose, production of fumaric acid, another dicarboxylic acid in the TCA cycle, was observed.

In another approach for succinic acid production in *A. niger*, the native gene encoding ATP citrate lyase was deleted [45]. The ATP citrate lyase activity was predicted to be inhibitory for succinate by using a stoichiometric metabolic model. The gene deletion resulted in a significant increase in succinate production. Furthermore, metabolic engineering of *A. niger* has been applied to produce or increase the production of itaconic acid [46,47,48] and oxalic acid [49].

In addition to the altered TCA cycle metabolism, another target for metabolic engineering in *A. niger* has been the catabolic pathway of the pectin constituent D-galacturonic acid (D-galUA). In sections 1.3 and 1.4, pectin and D-galUA metabolism in *A. niger* is reviewed in more detail and some metabolic engineering approaches for altered D-galUA metabolism are described.

1.3 Pectin

1.3.1 Structure

Pectins are a group of complex polysaccharides that are found in plant primary cell walls and middle lamellae. Pectins are abundant in the cell walls as gel-like flexible polymers around growing or dividing plant cells and soft plant tissues such as fruit peels. The common feature in all pectic polysaccharides is the main monomer D-galUA, accounting for about 70% of pectin monomers [50]. Based on their structure, pectins can be divided into homogalacturonan (HG), substituted HGs and more complex rhamnogalacturonan I (RG-I) (Fig. 1). HG is also referred to as smooth pectin, while substituted HGs and RG-I are referred to as hairy pectin.

HG is a homopolymer of α-1,4-linked D-galUA units that are partially methylesterified and acetylated [51]. It is the most common pectin type, representing about 65% of pectic polysaccharides [50]. The group of substituted HGs contains pectic heteropolysaccharides with an α-1,4-linked D-galUA backbone attached to different side chains. Substituted HGs are classified on the basis of their side chains. The most abundant of the substituted HGs, representing about 10% of pectin, is rhamnogalacturonan II (RG-II) [52]. In RG-II complex, side chains are composed of 12 different monomers including sugars and the sugar acids L-rhamnose, L-arabinose, D-galactose, L-galactose, D-apiose, D-xylose, L-fucose, D-galUA, D-glucuronic acid, 2-keto-3-deoxy-D-lyxo-heptulosaric acid (dha), 2-keto-3-deoxy-D-manno-octulosonic acid (kdo) and L-aceric acid [50]. In addition to RG-II, substituted HGs include the less common heteropolysaccharides xylogalacturonan (XGA) and apiogalacturonan (AP), with D-xylose or D-apiofuranose side chains, respectively [50]. The third type of pectic polysaccharides, RG-I, accounts for about 20-35% of pectin [50]. In contrast to HG and substituted HGs, the backbone of RG-I is made up of alternating α-1,4-linked D-galUA and α-1,2-linked L-rhamnose units. In addition, rhamnosyl residues in the backbone are often attached by side chains consisting of D-galactose and L-arabinose [53,54].

According to research literature, the pectic polysaccharides are synthesized in the Golgi lumen and transported to plant cell walls via vesicle trafficking [50]. In

the cell wall, different pectin polymers are interconnected most probably by covalent bonds from their main chains [55]. In addition, it has been suggested that pectic polysaccharides interact tightly with other cell wall polysaccharides such as xylans [50,56].

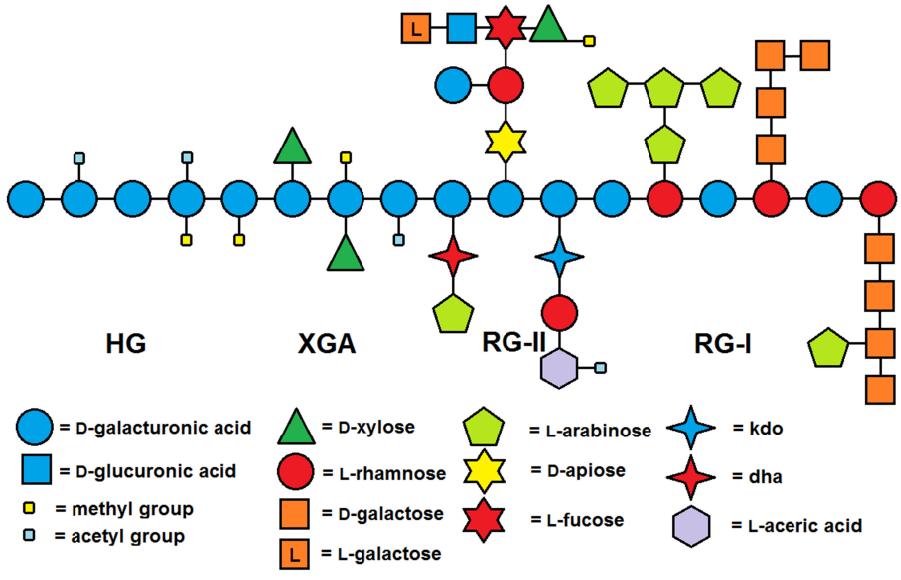

Figure 1. Pectin structure: homogalacturonan (HG), xylogalacturonan (XGA) rhamnogalacturonan II (RG-II) and rhamnogalacturonan I (RG-I).

1.3.2 Fungal pectin degradation

Pectins are abundant polysaccharides in biomass, and therefore many microorganisms have developed the capacity to degrade pectic polysaccharides and to utilise the resulting monomers as carbon source. As described above, the structure of pectins is very diverse and thus a complex set of pectin-degrading enzymes must be produced and secreted by fungi in order to release the monomers for utilisation. These enzymes are collectively referred to as pectinases or pectic enzymes.

Pectic enzymes that degrade the main chains of pectic polysaccharides (polygalacturonate in HG and substituted HGs or alternating D-galUA and L-rhamnose units in RG-I) can be classified into hydrolases and lyases. Pectic hydrolases include exo- and endopolygalacturonan hydrolases and rhamnogalacturonan hydrolases, which are also known as polygalacturonases and rhamnogalacturonases. Exopolygalacturonases hydrolyse terminal D-galUA units at the non-reducing end of the polygalacturonate chain, whereas endopolygalacturonases hydrolyse internal α-1,4-bonds in the chain [57,58]. The group of rhamnogalac-

turonases also includes exo- and endo-acting enzymes, which are responsible for hydrolysis of the RG-I backbone [58]. In the case of XGA, specific xylogalacturonan hydrolases are acting in the hydrolysis [59]. Another enzyme class degrading pectin main chains includes pectin, pectate and rhamnogalacturonan lyases. These lyases cleave the HG or RG-I main chain through a β-elimination mechanism, forming unsaturated non-reducing ends [58].

In addition to enzymes degrading the main pectin chain, an extensive set of accessory enzymes is needed for complete pectin degradation. As described earlier, D-galUA and L-rhamnose units in pectin are often methylated or acetylated and thus different pectin methyl [60] and acetyl [61] esterases are required for removing these groups. A diverse set of different side chains occurs in RG-I and RG-II and thus accessory enzymes including arabinases, arabinofuranosidases, β-xylosidases, galactanases, β-galactosidases, α-rhamnosidases and glucuronyl hydrolases are needed for their degradation [58].

A. niger is known to be efficient in pectin degradation, and many of the commercial pectic enzymes are derived from this species. Pectic enzymes are utilised e.g. in the beverage industry, where they are used to improve the juice yields or clarity of the final product. The genome of *A. niger* contains 66 putative genes that are possibly involved in pectin degradation [57]. Of these genes, 46 were observed to be upregulated in the presence of pectin or monomeric pectin substituents [62]. In addition, it has been shown that *A. niger* is capable of growing on pectin-rich biomass (sugar beet and citrus pulp), pure pectin and on most of the monomeric pectin substituents [63]. From the perspective of pectin degradation, *A. niger* is a suitable host organism for engineering a cell factory aiming at utilisation of pectin-rich biomass.

1.4 Cellular sugar catabolism

Most of the monomeric sugars resulting from biomass hydrolysis, such as D-glucose, D-xylose, D-galactose and L-arabinose, are oxidised to pyruvate via catabolic pathways generating cellular energy in the form of ATP and reducing equivalents. The ultimate factor determining whether an overall reaction in a metabolic pathway is possible or not, is thermodynamic feasibility. Catabolic sugar pathways are exergonic (energy-releasing), providing the necessary energy, reducing power and building blocks for energy-requiring functions and endergonic metabolic pathways (energy absorbing, anabolic pathways) in the cell.

The most central catabolic pathways in the cell are the Embden-Meyerhof-Parnas pathway (EMP, commonly referred to as glycolysis), the pentose phosphate pathway (PPP), the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (Fig. 2). The cellular energy that drives thermodynamically uphill reactions is commonly derived from these pathways in the form of adenosine triphosphate (ATP) and the cellular electron transfer cofactors (i.e. reducing equivalents) nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and flavin adenine dinucleotide (FAD), or NADH, NADPH and

$FADH_2$ when in their reduced form. These electron transfer cofactors shuttle electrons between chemical species in redox reactions which include oxidation (loss of electrons) and reduction reactions (gain of electrons). The energy that is bound in NADH, NADPH and $FADH_2$ is partially converted into ATP in oxidative phosphorylation when oxygen is available. The correct balance of the electron transfer cofactors (i.e. redox balance) is crucial for the cellular metabolism. For example, in the case of the ethanol fermentation pathway, one molecule of D-glucose is oxidised to two pyruvate molecules via glycolysis while the electrons from the oxidation are carried by two NADH molecules. When the two pyruvates are converted to two molecules of ethanol via acetaldehyde, the electrons from NADH are transferred to acetaldehyde, reducing it to ethanol. Due to the fact that three molecules of two-carbon ethanol would be more reduced than one six-carbon D-glucose, one-third of the carbon from D-glucose must be oxidised to carbon dioxide. This takes place in the reaction converting pyruvate to acetaldehyde, resulting in a redox neutral pathway. This example illustrates the tight interconnectedness between redox-balance and stoichiometric balance in metabolic pathways.

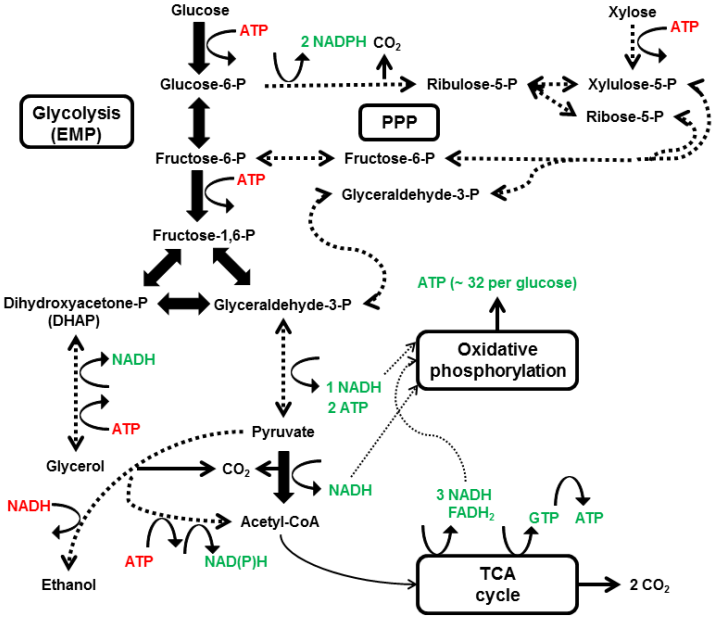

Figure 2. The central pathways of carbohydrate catabolism in the cell: Embden-Meyerhof-Parnas pathway (EMP), pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle and oxidative phosphorylation. Solid arrows represent single enzymatic reactions, dotted line arrows represent simplified steps of several reactions.

1.4.1 Fungal catabolic D-galacturonate pathway

After the pectic polysaccharides have been extracellularly degraded to sugars and sugar acids, the resulting monomers are transported into the fungal cell and catabolised further. Being a sugar acid, D-galUA (the main monomer in pectins) is more oxidised than the common biomass sugars and thus is catabolised through a specific metabolic pathway. The fungal catabolic pathway for D-galUA begins with a reduction reaction resulting in L-galactonic acid (L-galA) (Fig. 3). In the second reaction, a water molecule is removed from L-galA by the action of L-galA dehydratase, resulting in 2-keto-3-deoxy-L-galA (3-deoxy-L-*threo*-hex-2-ulosonate), which is then split to pyruvate and L-glyceraldehyde in the third reaction by an aldolase. In the final step, L-glyceraldehyde is reduced to glycerol, resulting in the overall reaction of the fungal D-galUA pathway as presented in Equation 1. If glycerol is considered to be oxidized further to dihydroxyacetone phosphate (DHAP) by the actions of glycerol kinase and cytosolic NAD-dependent or mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase [64], and oxidised further to pyruvate in glycolysis (Equation 2), the overall reaction for D-galUA catabolism is redox neutral, producing one ATP per D-galUA (Equation 3).

$$D-galUA + 2NAD(P)H \rightarrow pyruvate + glycerol + H_2O + 2NAD(P) \quad (1)$$

$$glycerol + 2NAD/FAD + ADP \rightarrow pyruvate + 2NADH/FADH_2 + ATP \quad (2)$$

$$D-galUA + ADP \rightarrow 2pyruvate + H_2O + ATP \quad (3)$$

D-galUA reductase is the first enzyme in the fungal D-galUA pathway. The first fungal D-galUA reductase GAR1 was described and characterised from *Hypocrea jecorina* (*Trichoderma reesei*) [65]. In the genome of *A. niger* an orthologous gene for *H. jecorina gar1* is also found, although it does not appear to be the principal D-galUA reductase in the *A. niger* pathway [66]. Instead, an analogous D-galUA reductase GAAA functions in *A. niger* having slighty different properties compared to GAR1. GAAA has a K_m of 0.175 mM for D-galUA (with NADPH) and it accepts both NADH and NADPH as cofactors, whereas GAR1 is strictly NADPH dependent with a K_m of 6 mM for D-galUA [65,66].

The second enzyme in the pathway, L-galA dehydratase, is encoded by the orthologous genes *lgd1* [67] and *gaaB* [66] in *H. jecorina* and *A. niger*, respectively. The third reaction splits 2-keto-3-deoxy-L-galA to pyruvate and L-

glyceraldehyde by the aldolases encoded by *lga1* in *H. jecorina* [68] and *gaaC* in *A. niger* [66]. An interesting detail is the fact that the *A. niger* genes *gaaA* and *gaaC* are clustered in the genome and they share a common bidirectional promoter region. The fourth reaction, reducing L-glyceraldehyde to glycerol, is catalysed by the NADPH-dependent reductase encoded by *gld1* in *H. jecorina* [69] and by *gaaD* in *A. niger* [66]. In addition to its function in the D-galUA pathway in *A. niger*, the gene *gaaD* is also known by the name *larA* and it is part of the catabolic L-arabinose pathway, reducing L-arabinose to L-arabitol in the first step [70].

In order to take part in the catabolic pathway, D-galUA must first get into the cell prior to catabolism. In general, only little data is available on D-galUA transporter proteins in fungi. There are three putative D-galUA transporter genes in *A. niger* that were upregulated in the presence of D-galUA [66,71]. However, the only characterised fungal D-galUA transporter is GAT1 (NCU00988) from *Neurospora crassa,* which has 40% identity in protein sequence with one of the putative D-galUA transporters (JGI41809; An14g04280) in *A. niger* [72]. GAT1 was reported to be most probably an H^+/D-galUA symporter and it was suggested that GAT1 may have a function in the signalling pathway sensing D-galUA and pectin in *N. crassa*. This claim was based on transcriptional data according to which a Δ*gat1* strain showed impaired pectinase induction in comparison to the wild type strain [72]. However, there is no existing data on transcription factors regulating D-galUA or HG metabolism in fungi – the only known pectin-related transcription factor is RhaR from *A. niger*, which regulates the genes related to RG and L-rhamnose catabolism [73].

1.4.2 Other metabolic pathways for D-galacturonate

In addition to the fungal D-galUA pathway, some bacterial pathways for D-galUA catabolism have been described in the literature. In *E. coli*, D-galUA is metabolised through the isomerase pathway (Fig. 3). The name orginates from the first enzyme in the pathway, which is a uronate isomerase converting D-galUA to D-tagaturonic acid [74]. In addition, *E. coli* has an enzyme oxidising L-galA to D-tagaturonic acid [75]. After the isomerisation, D-tagaturonic acid is reduced to D-altronic acid, which is converted via three steps to pyruvate and D-glyceraldehyde-3-phosphate [76,77,78,79]. The overall reaction in the isomerase pathway is presented in Equation 4. Oxidation of D-glyceraldehyde-3-phosphate to pyruvate via glycolysis generates one NADH and two ATPs. As a result, catabolisation of D-galUA through the isomerase pathway leads to an overall reaction which is the same as in the case of the fungal pathway (Equation 3). An alternative bacterial pathway for D-galUA catabolism is active in *Pseudomonas* and *Agrobacterium* species and is called the oxidative D-galUA pathway (Fig 3). In the oxidative pathway, the first reaction is oxidation of D-galUA to meso-galactaric acid (also known as mucic acid), or more precisely its lactone form [80,81]. The oxidation is followed by dehydration, decarboxylation and oxidation steps resulting in 2-keto-glutarate,

which is a key metabolite in the TCA cycle [82,83]. The overall reaction in the oxidative pathway is presented in Equation 5.

$$D - galUA + NADH + ATP \rightarrow pyruvate + D - glyceraldehyde - 3 - phosphate + H_2O + NAD + ADP \quad (4)$$

$$D - galUA + 2NAD \rightarrow 2 - keto - glutarate + CO_2 + 2H_2O + 2NADH \quad (5)$$

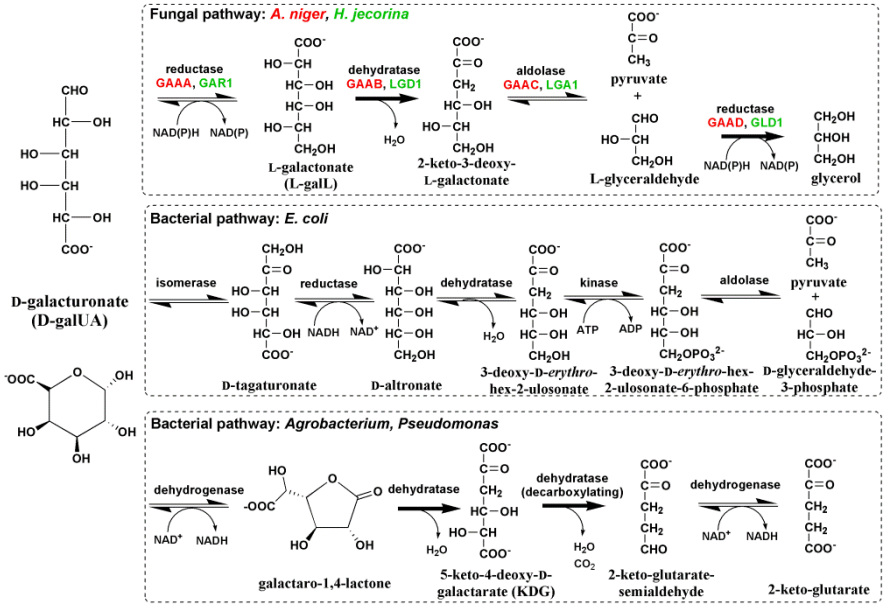

Figure 3. Fungal and bacterial pathways for D-galUA catabolism.

In plants, D-galUA is synthesized as its activated form of UDP-D-galUA from UDP-glucuronic acid, which is in turn synthesized either through the inositol oxygenation pahway or by oxidation of UDP-glucose [84]. As described earlier, the main function of D-galUA in plants is as the principal monomer in pectic polysaccharides. However, D-galUA has been shown to be a precursor for L-ascorbic acid (L-AA), also known as vitamin C, in one of the biosynthetic L-AA pathways in plants (Fig. 4). The L-AA pathway from D-galUA starts with the reduction of D-galUA to L-galA by the action of a reductase, which is the same reaction as is found in the

fungal pathway. Several different D-galUA reductases have been described in plants, and their association with L-AA synthesis has been experimentally demonstrated [85,86]. After the reduction, L-galA is converted to L-galA-1,4-lactone, which is also a metabolite in the main biosynthetic L-AA pathway. The lactonisation of L-galA in plants is poorly described – there has been only one L-AA pathway-associated L-galA-1,4-lactone lactonase (ALase) reported in the literature [87]. In the final step, L-galA-1,4-lactone is oxidized to L-AA by mitochondrial L-galA-1,4-lactone dehydrogenase (GALDH), while cytochrome C is used as electron acceptor [86]. This enzyme has been extensively described in the literature.

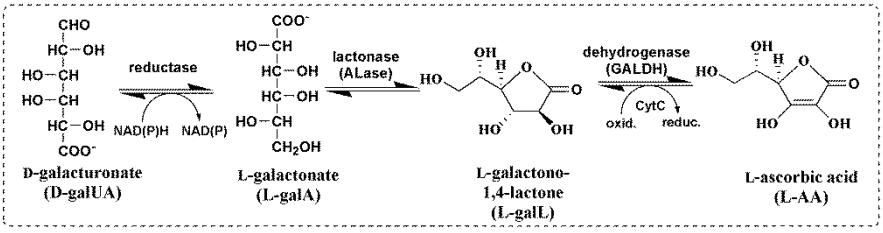

Figure 4. Pathway for conversion of D-galUA to L-AA in plants.

1.5 Pectin-rich biomass as a feedstock for fungal cell factories

1.5.1 Current use of pectin-rich biomass

Citrus processing waste (CPW), sugar beet pulp (SBP), and apple pomace are abundantly available pectin-rich agricultural residues having a pectin content of about 12–35% on a dry mass basis [88]. These biomasses contain only small amounts of lignin, which make them more accesible for enzymatic hydrolysis in bioprocesses. In addition, these residues are commonly stockpiled in the processing site, thus minimising the additional harvesting and transportation costs. Pectin-rich residues are therefore attractive raw materials for biorefineries.

In 2013, global orange production was approximately 50 million tonnes, of which about 20 million tonnes ended up in the orange processsing industry [89]. Juice is the main product from the processed fruits, accounting for about 50% of the wet mass of processed oranges [90]. The other half remaining after the processing makes up the CPW. The orange processing industry is the biggest source of CPW, with about 10 million tonnes of wet CPW generated annually as a residue [90,91]. The chemical composition of CPW varies between different harvests but the dry content is approximately 20%, of which around 25% is pectin on a dry mass basis [92] (Table 1). Thus the total pectin content in the CPW produced annually in the orange processing industry is approximately 500 000 tonnes. Other significant constituents in CPW are soluble sugars, cellulose and hemicellulose [88].

The CPW from the orange juice industry is often dried, pelletised and sold as animal feed. However, the conversion of CPW into feed is energy-intensive, making the process economically unprofitable [93]. In addition to animal feed, the two most typical products from CPW are essential oils, consisting mainly of D-limonene, and pectin. D-Limonene, the main constituent in essential oil, is a cyclic terpene that is obtained from the glands on the outer layer of citrus peel and it is responsible for the typical odour of citrus fruits. It is used, for example, as an odorant and flavouring agent in food and pharmaceutical products and as a solvent in cleaning products. The extraction of D-limonene is well established and is commonly carried out in automated juice extractors prior to juice squeezing, when the outermost layer of oranges, called the flavedo, is released and the essential oil is cold pressed [90]. In alternative methods, D-limonene can be extracted using steam distillation or solvent extraction [90]. About 5 kg of D-limonene can be obtained from 1000 kg of CPW [90]. D-Limonene is known as an inhibitory compound towards microbes, probably due to its membrane-disrupting properties, and thus its removal is beneficial if CPW is intended for use in microbial fermentation [88]. Pectin is the main component in the inner white layer of the peel, also called the albedo [90]. It is used as a gelling agent in the food industry and about 85% of commercially produced pectin is derived from citrus fruit peels [94]. In the extraction, pectin is first solubilised from CPW with acid treatment and then precipitated with organic solvents and recovered from the solution [93,94]. The costs of pectin extraction are relatively high and the use of acids and solvents may cause environmental problems [93]. In addition, the total pectin amount (500 000 tonnes) in the CPW produced annually in orange processing exceeds tenfold the current annual demand (about 40 000 tonnes) of pectin [90,94].

Table 1: Composition of CPW (% of dry mass) as determined in the study of Pourbafrani et al. 2010 [92].

Soluble sugars	**22.90**	**Polysaccharides**	**58.09**	**Other**	**15.77**
D-glucose	8.10	pectin	25.00	protein	6.07
D-fructose	12.00	cellulose	22.00	D-limonene	3.78
sucrose	2.80	hemicellulose	11.09	lignin	2.19
				ash	3.73

In addition to commercially produced D-limonene and pectin, several other by-products and biorefinery concepts have been proposed and investigated for CPW. Other products include ethanol, organic acids, methane, enzymes, prebiotics (functional food), dietary fibres, antioxidants, single cell proteins (microbial proteins for nutrition) and heavy metal adsorption [90,95]. Combined ethanol production with D-limonene and pectin extractions is probably the most studied biorefinery concept for CPW. In this concept, CPW is often physically or chemically pretreated and D-limonene and pectin are extracted [95]. The resulting CPW is then

enzymatically hydrolysed and fermented to ethanol using yeast or bacteria. The leftover from fermentation may be directed to an anaerobic process e.g. for methane production. In addition to CPW, processes producing ethanol from SBP have been investigated. The advantage of SBP as compared to CPW is the lack of inhibitory D-limonene; a simple enzymatic hydrolysis is sufficient to provide a substrate for ethanol fermentation [88]. Sometimes the whole CPW or SBP, still containing the pectin, is fermented to ethanol. The drawback in the pectin fermentation is the inability of ethanologenic yeasts to utilise pentose sugars and D-galUA from pectin [96]. In addition, despite the use of pentose- and D-galUA-utilising bacteria, the high oxidation state of D-galUA may lead to low ethanol yields [88]. Thus it is reasonable to search for other final products than ethanol from the pectin fractions in CPW and SBP.

1.5.2 D-galacturonate – a potential raw material for biotechnological fuel and chemicals production

D-GalUA – the main monomer in pectin – is the uronic acid of D-galactose. It is commercially not widely used as such, although the patent literature reveals some potential applications. One of the applications is the use of oligomeric D-galUA in cosmetic products. These oligomers are considered to have an inhibiting effect on collagen degradation in the skin [97]. In another patent application, D-galUA was proposed to be used as a chelating agent for removing metal ions from waste water [98]. Perhaps a more promising use of D-galUA is its utilisation as a platform compound for other products in biochemical or chemical conversion processes.

Current approaches for biochemical utilisation of D-galUA are focused on its conversion to ethanol. However, this approach lacks efficiency, as a co-substrate providing reducing power is required due to the high oxidation state of D-galUA. The use of biomass hydrolysates containing hexoses and pentoses with D-galUA in ethanol production has been proposed [96]. The most commonly used ethanol producer, the yeast *Saccharomyces cerevisiae*, cannot naturally metabolise D-galUA. Generation of yeast for the fermentation of D-galUA to ethanol would require transfer of the fungal or bacterial D-galUA pathway to the organism. It has been attempted to introduce the isomarese pathway from *E. coli* to yeast, but conversion of D-galUA to ethanol has not yet been achieved [99]. In addition to genes encoding enzymes for D-galUA catabolism, a transporter facilitating the uptake of D-galUA into the cell would be beneficial, even though D-galUA can in fact enter the native yeast cell at low extracellular pH [100]. One D-galUA transporter from *N. crassa* was recently introduced to yeast, enabling D-galUA import at higher extracellular pH [72].

An alternative approach to the biochemical processing of D-galUA is to use an organism, such as filamentous fungi, which is naturally capable of degrading pectic polysaccharides and catabolizing D-galUA. The filamentous fungus *H. jecorina* was engineered to create a disrupted D-galUA pathway (deletion of *gar1*), and uronate dehydrogenase (UDH) from the oxidative bacterial D-galUA pathway was

introduced [101]. The engineered *H. jecorina* strain oxidised the carbonyl group in D-galUA, resulting in mucic acid (galactaric acid) production [101]. The same approach was tested with *A. niger*, although mucic acid or its lactone was most probably metabolised further through an unknown metabolic pathway [101]. Mucic acid is also accessible via chemical oxidation of D-galUA [102,103]. Dicarboxylic acids, such as mucic acid, are potential polymer precursors. Chemical conversion of mucic acid to adipic acid, a precursor of nylon-6,6, has also been reported [104]. In another biotechnological approach, the third enzyme in the D-galUA pathway in *A. niger* and *H. jecorina* (gaaC and *lga1*) was deleted, resulting in strains producing 2-keto-3-deoxy-L-galactonic acid [105]. Keto-deoxy compounds may be useful precursors for chemical syntheses [105]. The third interesting compound from the fungal D-galUA pathway is L-galA, which can be converted further to L-AA. The latter approach is described in more detail in the following sections.

1.6 Aims of study

The aim of this thesis was to identify potential new products from biotechnology utilizing pectin-rich biomass. The fungal catabolic pathway for D-galUA was in the main focus, and chemical compounds that occur in the pathway as metabolites or can be derived from them were evaluated as potential end products.

L-GalA – a rare L-sugar acid – was identified as a potential precursor for L-AA (vitamin C). One of the aims was to generate fungal strains with an engineered D-galUA pathway for L-galA production. Deletion of the dehydratase gene from the D-galUA pathway is crucial for the L-galA producing strains. In addition, an unknown metabolic pathway, possibly including a dehydratase activity, has been considered to be responsible for poor mucic acid production in *A. niger*. Therefore, one of the aims was to investigate all the putative dehydratase-encoding genes in *A. niger* in detail. In addition, an important aim was to identify a simple colorimetric detection method for L-galA to facilitate the detection. Direct biochemical conversion of D-galUA to L-AA is theoretically possible. Thus, another major goal of this thesis was to generate an engineered fungal strain capable of direct L-AA production from D-galUA.

2. Materials and methods

Details of the materials and methods used in the work are presented in the original publications I–V. Only a brief description is provided here.

2.1 Microbial strains

All the *A. niger*, *H. jecorina* (*T. reesei*), *S. cerevisiae* and *E. coli* strains used in this work are listed in Table 2. In addition, the *E. coli* strain TOP10 was used for plasmid production and the *S. cerecisiae* strain ATCC 90845 for homologous recombination.

Table 2: Microbial strains used in this work.

Name	Parental strain	Genetic modifications	Publication
Aspergillus niger			
Wild type, ATCC 1015		none	I
Δ*pyrG*	ATCC 1015	deletion of the orotidine-5'-phosphate decarboxylase (*pyrG*) gene	I
Δ*gaaB*	Δ*pyrG*	deletion of the L-galactonate dehydratase gene (*gaa*B)	I, III, V
Δ*gaaB-gaaA*	Δ*gaaB*	the Δ*gaaB* strain with the D-galacturonate reductase gene (*gaaA*) over-expressed	I, III
Δ*gaaB-Mg*	Δ*gaaB*	the Δ*gaaB* strain with *Malpighia glabra* L-galactonate-1,4-lactone dehydrogenase (*MgGALDH*) over-expressed under the *gpdA* promoter	V
Δ*gaaB-Eg-Mg* (*PgpdA*)	Δ*gaaB*	the Δ*gaaB* strain with *EgALase* and *MgGALDH* over-expressed under the *gpdA* promoter	V

ΔgaaB-Smp30-Mg	*ΔgaaB*	the Δ*gaaB* strain with *Smp30ALase* and *MgGALDH* over-expressed under the *gpdA* promoter	V
ΔgaaB-Eg-Mg (*PgaaA/C*)	*ΔgaaB*	the Δ*gaaB* strain with *EgALase* and *MgGALDH* over-expressed under the bidirectional and D-galacturonate-inducible *gaaA/C* promoter	V
***Hypocrea jecorina* (*Trichoderma reesei*)**			
Δlgd1	Rut-C30	deletion of the L-galactonate dehydratase gene (*lgd1*)	I
Saccharomyces cerevisiae			
gaaB	CEN.PK2-1D	CEN.PK2-1D with over-expression of the *A. niger* L-galactonate dehydratase gene (*gaaB*, GenBank EHA22098.1) in a pYX212-derived p2159 multicopy vector	II
dgdA	CEN.PK2-1D	CEN.PK2-1D with over-expression of the putative *A. niger* dehydratase gene (*dgdA*, GenBank EHA19069.1) in a pYX212-derived p2159 multi-copy vector	II
dgdB	CEN.PK2-1D	CEN.PK2-1D with overexpression of the putative *A. niger* dehydra-tase gene (*dgdB*, GenBank EHA20544.1) in pYX212 derived p2159 multi copy vector	II
sodA	CEN.PK2-1D	CEN.PK2-1D with over-expression of the putative *A. niger* dehydratase gene (*sodA*, GenBank EHA18083.1) in a pYX212-derived p2159 multi-copy vector	II
lraC	CEN.PK2-1D	CEN.PK2-1D with over-expression of the putative *A. niger* dehydratase gene (*lraC*, GenBank EHA27292.1) in a pYX212-derived p2159 multi-copy vector	II
Escherichia coli			
yjjN	BL21 (DH3)	BL21 (DH3) with the *E. coli* L-galactonate dehydrogenase (*yjjN* or *lgoD, UniProt: Q8XB60*) gene expressed in a pBAT4 expression vector	IV

2.2 Media and culture conditions

A. niger spores were produced on potato dextrose (PD) agar plates and collected as suspensions containing 20% glycerol, 0.8% NaCl and 0.025% Tween. For *A. niger* pre-cultures, from 50 to 250 ml of medium containing yeast peptone (YP) supplemented with 30 g l^{-1} of gelatin was inoculated with the spore suspension. Cultures were incubated overnight. The resulting mycelia were collected by vacuum filtration and washed with sterile water. Minimal medium (MM) for *A. nidulans* [106] supplemented with suitable carbon sources (e.g. D-galacturonate, D-xylose or CPW) was used for submerged fermentations. Alternatively, the defined Vogels medium [101] was used instead of MM. Submerged fermentations were inoculated with pre-grown mycelia and incubated for several days. For solid state fermentations, CPW moisturised with water or with a nutritional solution containing K_2HPO_3, $(NH_4)_2SO_4$, $MgSO_4*7H_2O$, KCl and $FeSO_4*7H_2O$ was used as substrate and inoculated with spore suspension. All the *A. niger* cultures were incubated at 28 °C in shake flasks at 200 rpm.

Synthetic complete medium supplemeted with 20 g l^{-1} of D-glucose (SCD) was used for *S. cerevisia* cultures. SCD medium lacking uracil was used for the uracil autotrophic strains. Yeast cultures were grown in 250-ml Erlenmeyer flasks in 50 ml medium and incubated at 30 °C and 250 rpm.

E. coli cultures were carried out in Luria Broth (LB) medium containing 100 µg ml^{-1} of ampicillin and cultures were induced with1 mM of IPTG for protein production. Cultures were incubated at 37 °C and 250 rpm.

2.3 Chemical analysis

Sugars and sugar acids were analysed with HPLC consisting of fast acid analysis (100 by 7.8 mm, Bio-Rad Laboratories) and Animex HPX-87H organic acid analysis (300 by 7.8 mm, Bio-Rad Laboratories) columns. The columns were maintained at 55 °C, and 2.5 or 5 mM H_2SO_4 was used as eluent with a flow rate of 0.5 ml min^{-1}. For the detection, either a Waters 2487 dual-wavelength UV detector or a Waters 410 differential refractometer was used. Concentrations of L-ascorbic acid were detected using Ascorbic Acid Assay Kit II (Sigma-Aldrich). Identification of D-tagaturonic acid was performed with GC/MS (7890A GC and 5975C MS, Agilent) from derivatized samples.

2.4 Transcriptional analysis

Mycelium samples for transcriptional analysis were harvested by vacuum filtration and frozen with liquid nitrogen. RNA extraction was carried out using the RNeasy Plant Mini Kit (Qiagen) and cDNA synthesis using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The analysis was carried out with the LightCycler II

apparatus (Roche) and SYBR Green I Master Mix (Roche). All the transcript levels were normalised to the level of actin (ATCC1015 200483-mRNA).

2.5 Enzymatic activities

For the dehydratase activity, the thiobarbituric acid (TBA) method was applied, detecting all the formed 2-keto-3-deoxy sugar acids. Detection of L-galA dehydrogenase activity was carried out by following the reduction of NAD^+ to NADH at 340 nm using a spectrophotometer. The assay for lactonase activity was based on a pH indicator (p-nitrophenol) detecting released protons from the lactone hydrolysation. The method for detecting the oxidation of L-galactono-1,4-lactone to L-ascorbic acid by the action of a cytochrome c-dependent dehydrogenase was based on the spectrophotometric measurement of cytochrome c reduction at 550 nm.

3. Results and discussion

The fungal catabolic D-galUA pathway was first discovered in *H. jecorina* and the enzymes in the pathway were characterized by Hilditch (Kuorelahti) et al. and Liepins et al. [65,67,68,69]. Later, the corresponding pathway was identified in *A. niger* [66], which is also known as an efficient organism in pectin degradation.

In this thesis, the fungal D-galUA pathway was engineered with the aim of redirecting it to the production of useful chemical compounds. The filamentous fungus *A. niger* was chosen due to its high capacity to degrade pectin-rich biomass. Two compounds that can be derived from the D-galUA pathway, L-galactonic acid (L-galA) and L-ascorbic acid (L-AA), were selected as target products. The production was also tested as a consolidated bioprocess from pectin-rich biomass. In order to deepen understanding of the D-galUA pathway and to discover possible unwanted enzymatic reactions that might disrupt redirection of the D-galUA pathway, all the putative dehydratase-encoding genes were characterized. In addition, another enzyme from *E. coli* with activity against L-galA – L-galA-5-dehydrogenase – was characterized and applied in a specific enzymatic method for L-galA quantification.

3.1 Production of L-galactonic acid with engineered fungal strains

L-GalA is a rare L-sugar acid which could potentially be used in applications similar to those currently assigned to D-gluconic acid (e.g. as an acidifier or chelator). In addition, it can be converted to L-AA through chemical [107] or biochemical [108] conversion. Currently, L-galA is an expensive speciality chemical and not widely used. However, L-galA could have the potential to be used more widely if it were available at lower price.

L-GalA is the first intermediate in the fungal catabolic D-galUA pathway. In earlier studies, deletion of the L-galA-active dehydratase (*lgd1*) in *H. jecorina* resulted in a strain with disrupted gowth on D-galUA [67]. Furthermore, deletion of the corresponding gene in *A. niger* (*gaaB*) resulted in a strain with no growth on D-galUA. Instead, both of these strains, *H. jecorina* Δ*lgd1* and *A. niger* Δ*gaaB*, produced L-galA when D-galUA and D-xylose were provided as carbon sources. D-

Xylose was used as a co-substrate, providing the essential energy and reducing power to maintain the reduction reaction from D-galUA to L-galA. D-Glucose was not used, due to the glucose repression that could prevent activation of the D-galUA pathway, even though it would be a better source of NADPH than D-xylose (first steps in PPP, Fig. 2).

Both of the engineered strains, *H. jecorina* Δ*lgd1* and *A. niger* Δ*gaaB*, were tested in bioreactor cultivations at pH 5.5 with an initial D-galUA concentration of 10 g l^{-1} and using different initial D-xylose concentrations from 2.5 to 11 g l^{-1} (Fig. 5). In these conditions *H. jecorina* Δ*lgd1* was a better L-galA producer, achieving a final titre of 7.2 g l^{-1} when the highest concentration of D-xylose (11 g l^{-1}) was used (Fig. 5A). The production decreased with decreasing D-xylose concentrations. Furthermore, L-galA production started only when the D-xylose was consumed (Fig. 5D). In the case of *A. niger* Δ*gaaB*, the production was lower than in *H. jecorina*, achieving final titres of around 2 g l^{-1}. As in *H. jecorina*, D-xylose was consumed prior to L-galA production; however, the effects of different D-xylose concentrations on production were very small. Approximately 50% of the consumed D-xylose was used for biomass production in all of the *H. jecorina* and *A. niger* cultivations (Figs. 5B and D). In all the cultivations, biomass was decreasing over the course of the time (Fig. 5B). This is probably due to the cell death in the cultivations. In all *A. niger* cultivations as well as in *H. jecorina* cultivations with the lowest amounts of added D-xylose, a major part of D-galUA remained unused in the medium, whereas in the *H. jecorina* cultivation with the highest concentration of supplemented D-xylose all of the D-galUA was utilised at the end of the fermentation (Fig. 5C). Intracellular L-galA concentrations were generally around 50 to 70 mg g biomass^{-1} in both of the strains, corresponding to a volumetric concentration of about 20 g l^{-1} (Fig. 6A) [65]. In *H. jecorina*, L-galA yield and initial specific production rate were not dependent on D-xylose concentrations in the growth medium, but the higher final titre in the high D-xylose cultivations resulted rather from the higher biomass amount in the cultivation (Figs. 6B and 5B).

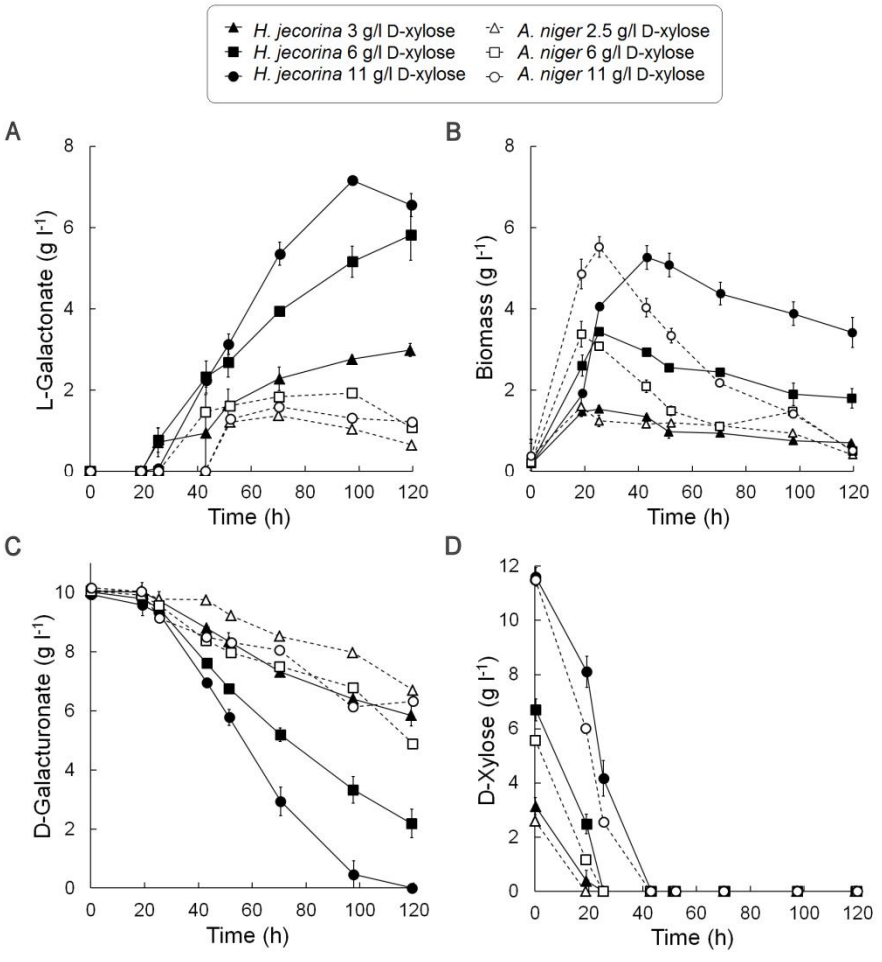

Figure 5. Bioreactor cultivations with *H. jecorina* Δ*lgd1* (solid symbols) and *A. niger* Δ*gaaB* (open symbols) strains. L-GalA (A), biomass (B), D-galUA (C) and D-xylose (D) concentrations were measured. The cultivations were started with an initial D-galUA concentration of 10 g l^{-1}. Different initial D-xylose concentrations (triangle, square or circle) were tested. Error bars represent ± the standard error of the mean (SEM, n=2).

In *H. jecorina* Δ*lgd1*, production of L-galA was more dependent on the supplemeted D-xylose when compared to production of 2-keto-3-deoxy-L-galactonic acid by the engineered *H. jecorina* Δ*lga1* strain [105]. In the production pathway for 2-keto-3-deoxy-L-galactonic acid, D-galUA is first reduced to L-galA which is then converted to its 2-keto-3-deoxy derivative by removing a water molecule by the action of Lgd1 dehydratase. From a redox and energy balance point of view, the pathways to L-galA and 2-keto-3-deoxy-L-galactonic acid are similar and thus the

higher D-xylose dependence in L-galA production may result from the more energy-requiring export process of the product. This is also supported by the fact that higher intracellular (~20 g l^{-1}) than extracellular (~2 to 7 g l^{-1}) concentrations of L-galA were observed. The specific production rates in *H. jecorina* decreased slightly with increasing D-xylose concentration, thus providing evidence against the hyphothesis of energy-dependent export. On the other hand, the final intracellular L-galA concentration was clearly the lowest when the highest D-xylose concentration was provided. Thus, it is difficult to conclude the dominant cause for higher D-xylose dependence in L-galA production when compared to 2-keto-3-deoxy-L-galactonic acid production.

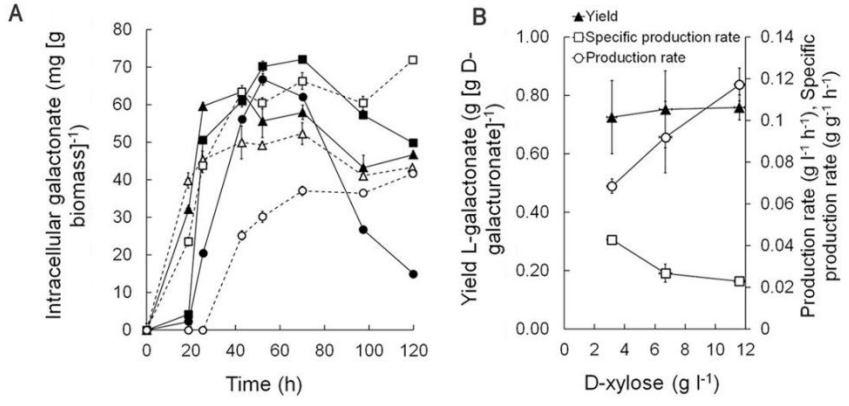

Figure 6. Intracellular L-GalA concentrations in bioreactor cultivations of *H. jecorina* Δ*lgd1* and *A. niger* Δ*gaaB* (A) and yields (triangle; L-galA / consumed D-galUA), initial specific production rates (square; g L-galA / g biomass / h) and initial production rates (circle; g L-galA / l / h) in bioreactor cultivations with *H. jecorina* Δ*lgd1* (B) on different initial D-xylose concentrations. Error bars represent ± the standard error of the mean (SEM, n=2).

The yields (g L-gal / g consumed D-galUA) in *H. jecorina* cultivations were around 0.7, clearly below the theoretical maximum of 1. This was also the case in the *A. niger* cultivations. The facts that extracellular L-galA did not significantly decrease, and that the intracellular L-galA concentrations were at a high level, speak against L-galA consumption by dehydratation to 2-keto-3-deoxy-L-galactonic acid and further catabolisation through the fungal D-galUA pathway. In addition, it will be demonstrated later on in this thesis that none of the four additional putative dehydratases in the *A. niger* genome have affinity for L-galA. Thus, it is more likely that D-galUA is slowly catabolized by some other unknown metabolic pathway.

3.1.1 L-Galactonic acid production is pH sensitive in the engineered *A. niger*

In the first bioreactor cultivations, the *A. niger* Δ*gaaB* strain produced only around 2 g l^{-1} of L-galA, and most of the D-galUA was not utilised (Figs. 5A and C). A possible explanation for the low L-galA concentrations at pH 5.5 is that the production is influenced by pH. Different initial pH values were tested in flask cultivations with *A. niger* Δ*gaaB* (Fig. 7). pH values below 5 did indeed improve the production, and similar final titres of around 6 to 7 g l^{-1} were achieved to those observed in the bioreactor cultivations with *H. jecorina* Δ*lgd1*.

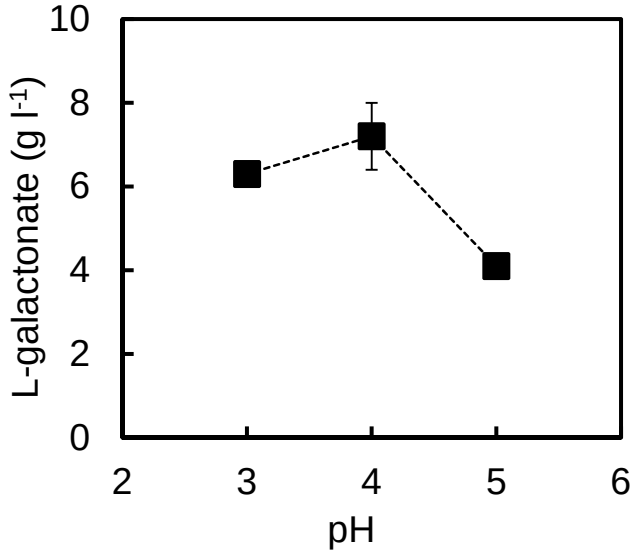

Figure 7. Effect of pH on L-galA production in flask cultivations with *A. niger* Δ*gaaB* after 144 h. A buffered minimal medium containing 20 g l^{-1} of D-galUA and 5 g l^{-1} of D-xylose was used. Error bars represent ± the standard error of the mean (SEM, n=3).

The improved production at pH 4 could be explained by improved D-galUA import. With its pKa of 3.51, D-galUA starts to be present more in its protonated form at lower pH and is possibly more easily transported into the cell. However, in the earlier study with the *A. niger* strain producing 2-keto-3-deoxy-L-galactonic acid, the D-galUA consumption rates at pH 5.5 were from 0.12 to 0.56 g l^{-1} h^{-1} depending on biomass and culture conditions [105], whereas the highest value observed in the low pH cultivations was only 0.15 g l^{-1} h^{-1} (data not shown). In order to obtain more information on D-galUA transport, expression levels of 3 putative D-galUA transporter genes were investigated in *A. niger* wild type and Δ*gaaB* strains

at pH 3 (Fig. 8). This study revealed a difference between the strains: the mRNA level of the putative transporter An14g04280 was clearly at a lower level in Δ*gaaB* compared to wild type. This may be one of the explanations for the lower D-galUA import in the L-galA production strain compared to the strain producing 2-keto-3-deoxy-L-galactonic acid. The transporter protein An14g04280 was later shown to be indeed a functional D-galUA transporter in *A. niger* [109], and the homologous transporter in *Neurospora crassa* was concluded to be most probably a proton/D-galUA symporter [72]. Even though the mRNA level of An14g04280 is lower in Δ*gaaB*, low pH could basically improve the import if even a low level of the An14g04280 gene is transcibed and translated to a functional proton symporter protein. However, on the basis of these observations it is still possible that the improved L-galA production in *A. niger* Δ*gaaB* at low pH is due to improved L-galA export from the cell.

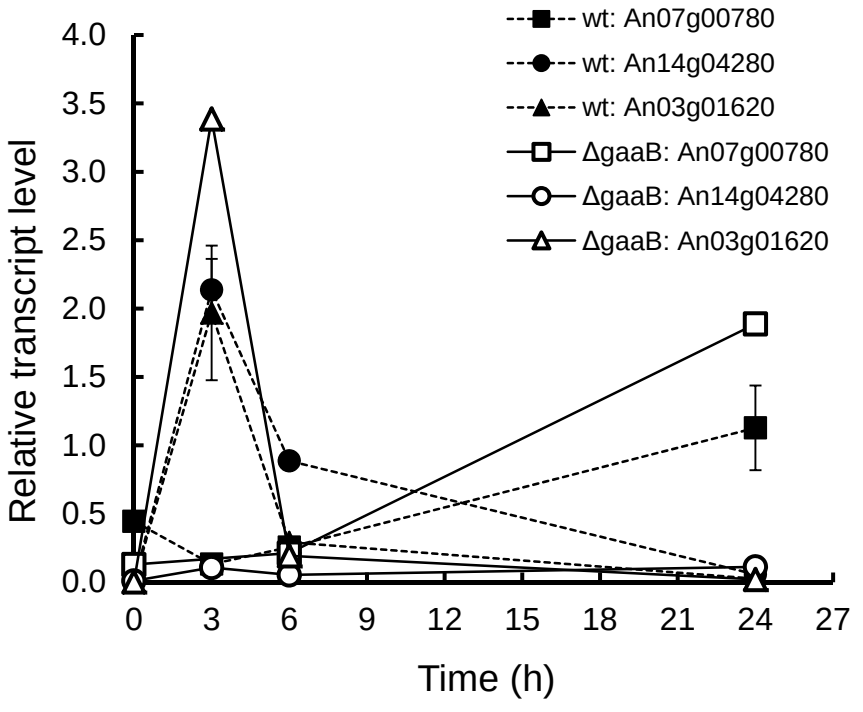

Figure 8. Relative transcript levels of the 3 putative D-galUA transporters in *A. niger* strains in flask cultivations at pH 3 in the medium containing 20 g l^{-1} D-galUA and 5 g l^{-1} D-xylose. Error bars represent ± the standard error of the mean (SEM, n=3).

The pKa of L-galA is around 3.5, leading to an increased fraction of extracellular L-galA being present in its protonated form with decreasing pH. Since the intracellular pH is close to neutral, most of the L-galA in the cell is in its dissociated form. Thus, the increased gradient between the negatively charged dissociated intracellular L-galA and extracellular protons may function as a driving force for L-galA export. In the study of Burgstaller, a model predicted that low extracellular pH is the driving force for the product export in the case of *A. niger* citric acid fermentations [110]. This might also be the case the in L-galA production studied here.

3.1.2 Overexpression of the D-galUA reductase *gaaA* in *A. niger*

In order to improve L-galA production in *A. niger* Δ*gaaB*, the gene coding for D-galUA reductase (*gaaA*) was overexpressed in the strain under the constitutive *gpdA* promoter. Transcript levels of *gaaA* were studied and initial production rates were compared between the strains (Fig. 9). In addition, L-galA production by the resulting strain Δ*gaaB-gaaA* was compared to Δ*gaaB* in flask cultures at different initial pH values (Table 3). As seen in the preliminary studies, the *gaaA* transcription was lagging in the Δ*gaaB* strain compared to the wild type strain. In the Δ*gaaB-gaaA* strain it was confirmed that *gaaA* was initially highly expressed. This was as expected, but the expression returned to the same level as *gaaA* expression in the Δ*gaaB* strain after 24 h (Fig. 9A). Initial L-galA productivities were significantly higher in Δ*gaaB-gaaA* during the first 48 h when compared to the Δ*gaaB* strain (Fig. 9B). Furthermore, the final titres and yields were higher in the Δ*gaaB-gaaA* cultivations at pH 3 and 4, whereas the cultivations at pH 5 resulted in similar L-galA titres in Δ*gaaB* and Δ*gaaB-gaaA* strains.

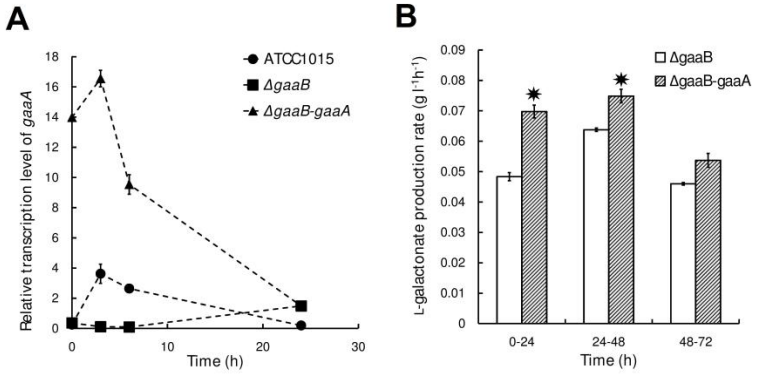

Figure 9. The relative transcript levels of *gaaA* in wild type (ATCC 1015), Δ*gaaB* and Δ*gaaB-gaaA* strains (A) and initial L-galA productivities in Δ*gaaB* and Δ*gaaB-gaaA* strains (B) in flask cultures at pH 3 in the medium containing 20 g l^{-1} D-galUA and 5 g l^{-1} D-xylose. The initial productivities for Δ*gaaB-gaaA* which differed significantly ($p<0.05$) from those obtained with Δ*gaaB* are indicated with an asterisk. Error bars represent ± the standard error of the mean (SEM, n=3).

The overexpression of *gaaA* in *A. niger* improved the initial L-galA productivities in the Δ*gaaB-gaaA* strain compared to Δ*gaaB*. However, a significant fraction of the initial D-galUA remained unutilized in the medium even at low pH. As concluded earlier, poor L-galA export is a possible explanation for the impaired production pathway. Another explanation could be that all the D-xylose is consumed prior to L-galA production, which may cause an insufficiency of available reducing power in the cell. For this reason a complex carbon source containing sugars that are released slowly from their polysaccharides or are catabolized simultaneously with D-galUA could favour the production.

Table 3: L-GalA production by *A. niger* Δ*gaaB* and Δ*gaaB-gaaA* strains at 144 h.

***Aspergillus* MM 2% D-galUA + 0.5% xylose**	**Strain**	**Final titre gl^{-1}**	**Conversion L-galA [D-galUA$_{initial}$]$^{-1}$**	**Yield L-galA [D-galUA$_{consumed}$]$^{-1}$**
pH 5	Δ*gaaB*	4.1 ± 0.2	0.20	0.82
	Δ*gaaB-gaaA*	4.1 ± 0.3	0.20	0.97
pH 4	Δ*gaaB*	7.2 ± 0.8	0.35	0.95
	Δ*gaaB-gaaA*	7.8 ± 0.4	0.38	0.97
pH 3	Δ*gaaB*	6.3 ± 0.1	0.31	0.86
	Δ*gaaB-gaaA*	8.7 ± 0.2	0.43	1.00
Vogel's MM 1% D-GalUA + 0.2% xylose				
pH 4	Δ*gaaB*	4.2 ± 0.1	0.41	0.70
	Δ*gaaB-gaaA*	5.0 ± 0.1	0.49	0.75
pH 3	Δ*gaaB*	4.9 ± 0.1	0.47	0.70
	Δ*gaaB-gaaA*	6.2 ± 0.3	0.59	0.82

3.1.3 Conversion of citrus processing waste to L-galactonic acid in a consolidated bioprocess

Consolidated bioprocessing is a concept for biotechnological one-step conversion of untreated biomass to desired products. *A. niger* is known for its high capacity to secrete pectic enzymes and, for that reason, is a suitable organism for a consolidated bioprocess converting pectin-rich biomass into a product. The engineered *A. niger* strains Δ*gaaB* and Δ*gaaB-gaaA* were investigated for L-galA production using CPW (citrus processing waste) as raw material. Both SmF (submerged fermentation) and SSF (solid state fermentation) were tested for the production, with and without nutritional supplementation.

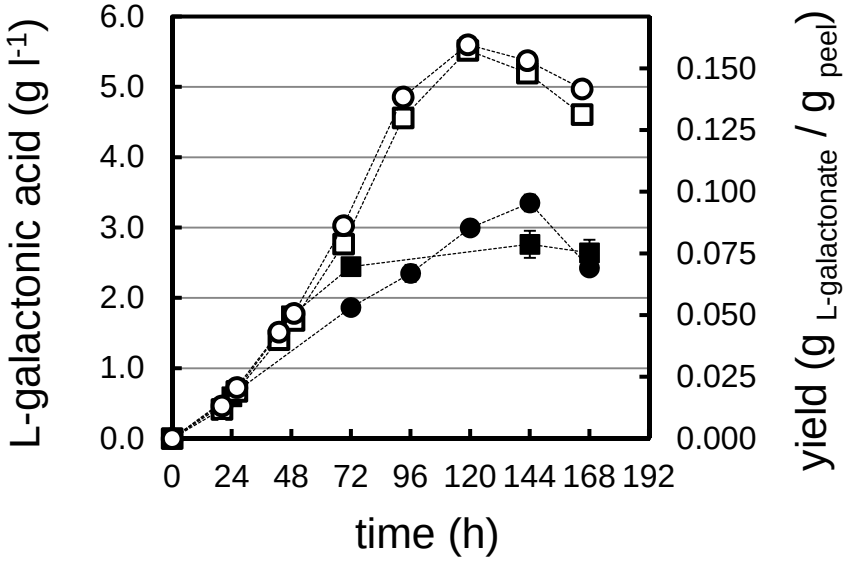

Figure 10. L-GalA production in SmFs on CPW with the engineered strains Δ*gaaB* (squares) and Δ*gaaB-gaaA* (circles) with (open symbols) or without (solid symbols) nutritional supplementation at pH 3. Error bars represent the standard error of the mean (SEM, n=3); if not visible they are smaller than the symbol.

For the SmFs, 1.76 g (DW) of CPW was used as substrate in the final volume of 50 ml in flask cultivations (= 35.1 g l^{-1}). The liquid used was either sterile water or the minimal medium (=nutritional supplementation) and the cultivations were inoculated with pre-grown mycelia. The D-galUA content in the CPW was 27%, and thus around 9.5 g l^{-1} of D-galUA was available in the cultivations as pectic polymers. Both of the engineered strains Δ*gaaB* and Δ*gaaB-gaaA* produced L-galA in the SmFs with approximately similar efficiencies (Fig. 10). The nutritional supplementation containing e.g. a nitrogen source improved the production significantly. In addition, it had a buffering function: the pH in the SmF without the supplementation dropped below 2, whereas in the supplemeted SmFs it remained at a value between 2.5 and 3.5. Even though low pH favours L-galA production, values below 2 may have a negative effect. Without the nutritional supplementation the final titres were around 3 g l^{-1}, whereas titres above 5 g l^{-1} were achieved with the supplementation. In terms of yields, the highest achieved values were around 0.16 g (g CPW)$^{-1}$ and 0.6 g (g D-galUA)$^{-1}$ (Table 4). The overexpression of *gaaA* did not result in improved production in the SmFs.

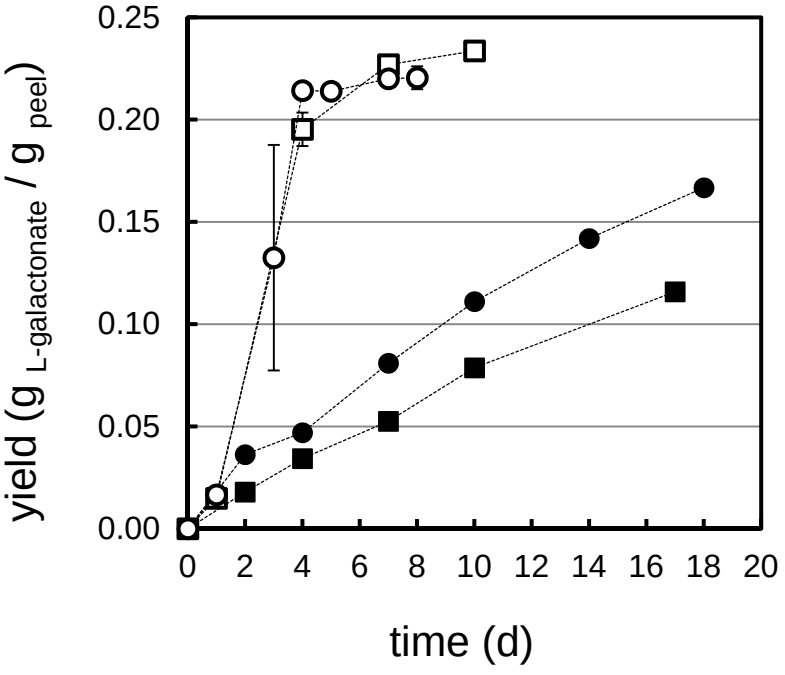

Figure 11. L-GalA production in SSFs on CPW with the engineered strains Δ*gaaB* (squares) and Δ*gaaB-gaaA* (circles) with (open symbols) or without (solid symbols) nutritional supplementation. Error bars represent the standard error of the mean (SEM, n=3), if not visible they are smaller than the symbol.

For the SSF, 1.76 g (DW) of CPW was added to each flask (a similar amount to that in the SmFs), but instead of 50 ml only 6 ml of liquid was added. The CPW was moisturised with either sterile water or nutritional supplementation and inoculated with spore suspension. The L-galA production rate in the SSFs without nutritional supplementation was slow, although achieving final yields that were clearly higher compared to the SmFs without supplementation (Fig. 11 and Table 4). In addition, the overexpression of *gaaA* in the strain Δ*gaaB-gaaA* appeared to have a positive effect on the production. The addition of the nutritional supplementation improved the production drastically, and final yields around 0.23 g (g CPW)$^{-1}$ and 0.87 g (g D-galUA)$^{-1}$ were achieved. The overexpression of *gaaA* did not improve the production in the SFFs with nutritional supplementation.

Table 4: Initial productivities, absolute product yields and product yields as percentages of the theoretical maximum from SmFs and SSFs on a dry mass basis.

Strain	Initial productivity $mg_{L\text{-galactonate}} / g_{peel} / h$		Product yield, $Y_{p/s}$ $mg_{L\text{-galactonate}} / g_{peel}$		Product yield (%) of theoretical maximum $g_{L\text{-galactonate}} / g_{D\text{-galacturonate}}$	
	SmF 0-70.5±1.5 h	SSF 0-96 h	SmF	SSF	SmF	SSF
Δ*gaaB* (without suppl.)	1.01±0.04[a]	0.35±0.01	79±5	116±2[a]	23%	43%
Δ*gaaB-gaaA* (without suppl.)	0.74±0.03[a]	0.49±0.02	95±3	167±2[a]	35%	62%
Δ*gaaB* (with suppl.)	1.16±0.01	[b]2.14±0.09	157±3	[b]233±2[a]	58%	87%
Δ*gaaB-gaaA* (with suppl.)	1.26±0.02	[b]2.35±0.03[a]	159±3	[b]221±6[a]	59%	82%

Errors represent the standard error of the mean (SEM, n=3). [a] The process type (SmF or SFF) was significantly ($p<0.05$) better than the other in the same nutritional conditions. [b] Errors represent ±SEM, n=2.

In summary, the performance of L-galA production from CPW in the SmF process was similar to the production from pure D-galUA in terms of product yields against available D-galUA (compare Tables 3 and 4). In both cases the highest product yield was 59% of the theoretical maximum [g L-gal (g D-galUA)$^{-1}$]. When comparing L-galA production from CPW between the SmFs and SSFs, the yields were clearly higher in the SSFs. The production rates were also higher in the SSFs with nutritional supplementation when compared to the SmFs with nutritional supplementation. In SSFs, sugars resulting from hydrolysis of pectin, cellulose and hemicellulose are not freely diffusible in the solid substrate due to the lack of free water. For this reason it is possible that the most favourable sugars, such as D-glucose, are rapidly utilised in the vicinity of the hyphae and thus do not cause catabolite repression [111]. This may allow more efficient pectin degradation and D-galUA utilisation in SSF. In fact, it has been shown that *A. niger* produces pectic enzymes more efficiently in SSF compared to SmF [112]. Thus, yields close to 90% of the theoretical maximum were achieved in SSF.

3.2 Dehydratases in *Aspergillus niger* and their relevance in the engineered D-galacturonate pathway

Sugar acid dehydratases are a group of enzymes which catalyse the removal of oxygen and hydrogen from a sugar acid, forming water as by-product. Several dehydratase activities for sugar acids, resulting in their 2-keto-3-deoxy compounds, have been described in fungi. However, the gene sequences that code for

these dehydratases are often not known and, in most cases, the characterisation of these enzymes is incomplete.

In the fungal D-galUA catabolism, the second enzyme in the pathway, encoded by the gene *gaaB*, is a dehydratase splitting off a water molecule from L-galA and forming 2-keto-3-deoxy-L-galactonic acid. The deletion of this gene resulted in the strain (Δ*gaaB*) reducing D-galUA to L-galA, which accumulates in the medium. However, the yields of L-galA per consumed D-galUA were below the theoretical maximum, indicating either that there is still some L-galA dehydratase activity left in the cell or that a fraction of D-galUA or L-galA was catabolized through an unknown pathway. Furthermore, the expression of a bacterial uronate dehydrogenase (UDH) in the *A. niger* strain with deleted D-galUA reductase encoding the gene *gaaA* (Δ*gaaA-UDH*) resulted in a strain capable of D-galUA catabolization, although the strain was constructed for mucic acid (galactaric acid) production [101]. In this case as well, a dehydratase activity towards mucic acid or its lactone (galactarolactone) could explain the D-galUA utilisation. The enzyme GaaB (JGI ID 53563) had already been shown to be an L-galA dehydratase [113]. However, it may have dehydratase activity towards other substrates as well, which could explain D-galUA consumption in the case of the *A. niger* Δ*gaaA-UDH* strain. For these reasons, all of the putative sugar acid dehydratases in *A. niger* were investigated in more detail.

In addition to *gaaB*, four genes encoding putative sugar acid dehydratases were identified in this study from the *A. niger* genome. Due to the fact that histidine-tagged and purified *H. jecorina* Lgd1 dehydratase, a close homologue of *A. niger* GaaB, lost its activity [67], it was not attempted to purify his-tagged proteins but all of the putative *A. niger* sugar acid dehydratases were expressed in yeast from a multi-copy plasmid and the enzyme activities were measured from crude extracts (Table 5).

The putative dehydratase with the JGI ID 191792 has recently been shown to be upregulated in the presence of L-rhamnose [73] and it has sequence homology to the L-rhamnonate dehydratase Lra3 from *Scheffersomyces stipitis* [114]. In the present study, the enzyme was named LraC in *A. niger* and it did indeed show activity against L-rhamnonate. The putative dehydratases with the JGI IDs 49896 and 50500 were revealed to be D-galactonic acid dehydratases and were named accordingly DgdA and DgdB, respectively. The fifth of the putative dehydratases, with the JGI ID 38317, did not show activity for any of tested sugar acids and, for that reason was named SodA (some other dehydratase). In addition, the conserved residues among dehydratases, histidine and aspartic acid [115], were missing from the SodA sequence, indicating that SodA may in fact not have dehydratase activity for any substrate.

None of the dehydratases, except GaaB, had activity against L-galA. Thus, it can be concluded that L-galA is unlikely to be dehydratated to 2-keto-3-deoxy-L-galactonic acid and catabolised further through the D-galUA pathway in the Δ*gaaB* strain. This observation supports the hypothesis concerning the presence of an unknown metabolic pathway in the Δ*gaaB* strain that may consume L-galA or D-galUA that can be re-oxidised from L-galA in the reverse reaction by GaaA. In the

case of the strain Δ*gaaA-UDH* for mucic acid production [101], it is unlikely that GaaB is responsible for the catabolism of mucic acid or its lactone. Even though GaaB had the most relaxed substrate specifity of the tested dehydratases, no activity was found against either mucic acid or galactarolactone. The activity against galactarolactone was tested in a reaction mixture in which D-galUA and purified UDH enzyme (oxidizing D-galUA to galactarolactone) were added together with GaaB crude extract (data not shown).

Table 5: Dehydratase activities of the five putative *A. niger* dehydratases against different sugar acids. The dehydratase genes were expressed in *S. cerevisiae* and the activities from crude extracts are given as nkat per mg of total protein.

Substrate	*gaaB*	*lraC*	*dgdA*	*dgdB*	*sodA*
L-Rhamnonate	–	0.07	–	–	–
L-Galactonate	0.05	–	–	–	–
D-Gulonate	–	–	–	–	
D-Gluconate	0.03	–	–	–	–
L-Gulonate	–	–	–	–	–
D-Arabonate	–	–	–	–	–
D-Lyxonate	–	–	–	–	–
L-Lyxonate	–	–	–	–	–
D-Xylonate	–	–	–	–	–
Mucic acid	–	–	–	–	–
L-Fuconate	0.52	–	–	–	–
D-Mannonate	0.02	–	–	–	–
L-Mannonate	0.02	0.05	–	–	–
D-Riborate	–	–	–	–	–
L-Arabonate	–	–	–	–	–
D-Galactonate	–	–	0.82	0.23	–
D-Galacturonate	–	–	–	–	–
D-Glucuronate	0.02	–	–	–	–

The characterisation of *A. niger* sugar acid dehydratases did not explain the D-galUA catabolization in the strains Δ*gaaB* and Δ*gaaA-UDH,* in which the catabolic D-galUA pathway is disrupted. Consequently, it is possible that *A. niger* is capable of utilising D-glaUA via an unknown pathway that does not include a dehydratation step. However, the study showed the dehydratase specificities of the *A. niger* dehydratase enzymes for selected sugar acids. In addition GaaB, which is known to be part of the catabolic D-galUA pathway, had tenfold higher activity against L-

fuconate compared to L-galA. L-Fuconate occurs as a metabolite in the fungal catabolic L-fucose pathway, in which it is dehydrated to 2-keto-3-deoxy-L-fuconate [116]. The data suggests that GaaB may have a function in the L-fucose pathway, even though this was not confirmed in the present study.

3.3 L-Galactonate dehydrogenase: characterisation and a novel enzymatic method for L-galactonate quantification

As in general among sugars and sugar acids, detection and quantification of L-galA rely on chromatographic detection methods, such as high performance liquid chromatography (HPLC). HPLC is a versatile analytical tool for sugars and sugar acids, although its sensitivity is often relatively low and analysis times are long. Colorimetric analysis is an alternative option when a specific chemical compound is to be quantified from the mixture. In this approach, a colour reagent is used to react with the analyte of interest, resulting in a colour change that can be measured using spectrophotometry. A subclass of colorimetric analyses includes enzymatic methods in which the colorimetric change is based on an enzymatic reaction. Enzymatic methods can also be based on coupled reactions, such as in the case of diaphorase assay. In the diaphorase assay, NAD^+ is reduced to NADH in the first enzymatic reaction that is specific for the analyte. In the second reaction NADH is re-oxidised to NAD^+ by diaphorase enzyme, simultaneously converting its substrate p-iodonitrotetrazolium (INT) to formazan, a coloured (red) compound absorbing light at 492 nm [117]. Basically, diaphorase reaction can be coupled with any oxidoreductase enzyme that reduces NAD^+ to NADH. In this work, the aim was to develop a sensitive and specific method for quantification of L-galA based on the diaphorase reaction.

D-GalUA reductases, such as GaaA, are the only characterised oxidoreductases that can oxidise L-galA (back to D-galUA in the reverse reaction). However, D-galUA reductases typically prefer NADPH instead of NADH, and thus are not optimal enzymes to couple with diaphorase. *E. coli* is also capable of catabolizing L-galA, and exhibited oxidoreductase activity against L-galA when the L-galA-grown crude extracts were analysed [118]. The reaction product was suggested to be D-tagaturonic acid, which occurs as a metabolite in the catabolic D-galUA pathway in *E. coli* (Fig. 3). In addition, transcription of the *yjjN* gene (NCBI GeneID 913585), encoding a putative oxidoreductase, was upregulated in the presence of L-galA and the gene deletion resulted in a strain with no growth on L-galA [119]. However, the activity of the protein YjjN was never characterised.

In order to develop a sensitive and rapid colorimetric diaphorase assay for L-galA, the enzyme YjjN was histidine-tagged, purified and characterized. Several different sugars and sugar acids were tested with YjjN in the presence of NAD^+. Oxidoreductase activity was found only against L-galA and L-gulonic acid (Fig. 12). The K_m and k_{cat} for L-galA were 19.5 mM and 0.51 s^{-1}, respectively. The activity against L-gulonic acid was substantially lower. The reaction product from L-galA by YjjN was confirmed to be D-tagaturonic acid using GC-MS analysis. Thus, YjjN

is indeed an L-galA-5-dehydrogenase. YjjN oxidoreductase activity against L-galA was strictly NAD^+ dependent, since no activity was found with $NADP^+$. Addition of EDTA into the reaction mixture inactivated the enzyme, supporting the hypothesis of its dependence on zinc ions.

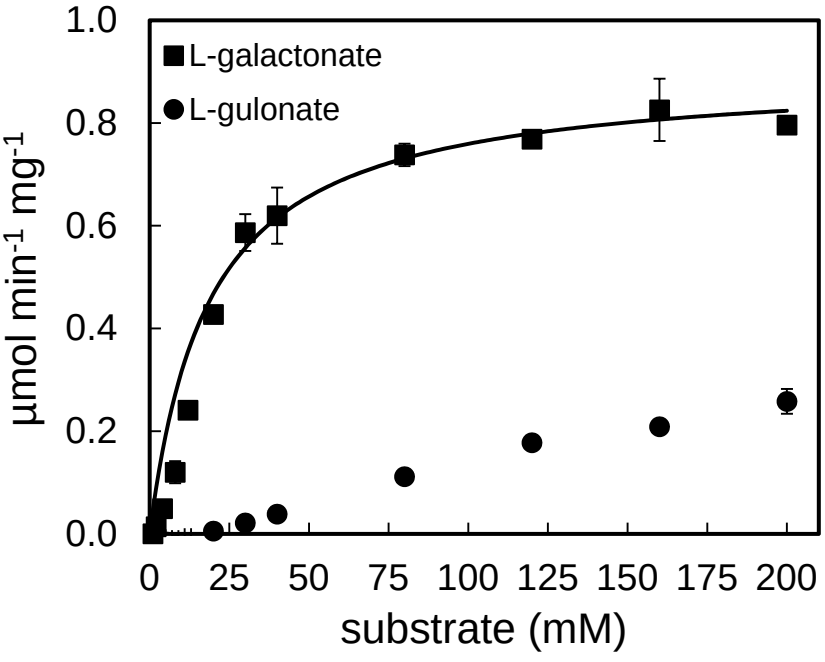

Figure 12. YjjN dehydrogenase activity from purified protein against L-galA (squares) and L-gulonic acid (circles) in the presence of NAD^+. Errors represent ±SD, n=3.

YjjN was tested in a coupled reaction together with diaphorase, NAD^+, INT and L-galA or L-gulonic acid (Fig. 13). The resulting assay was superior to HPLC analysis: the detection limit for L-galA was 1.65 µM and that for L-gulonic acid was 10 µM. A drawback in the YjjN/diaphorase assay is however the poor stability of YjjN enzyme. Even short-term storage of purified YjjN at +4 decreased the activity dramatically. Stabilisation of YjjN or use of a more stable homologue would be required for a practical colorimetric assay for L-galA and L-gulonate. However, this study confirms that YjjN is the L-galA-5-dehydrogenase that is a component of the catabolic L-galA pathway in *E. coli*.

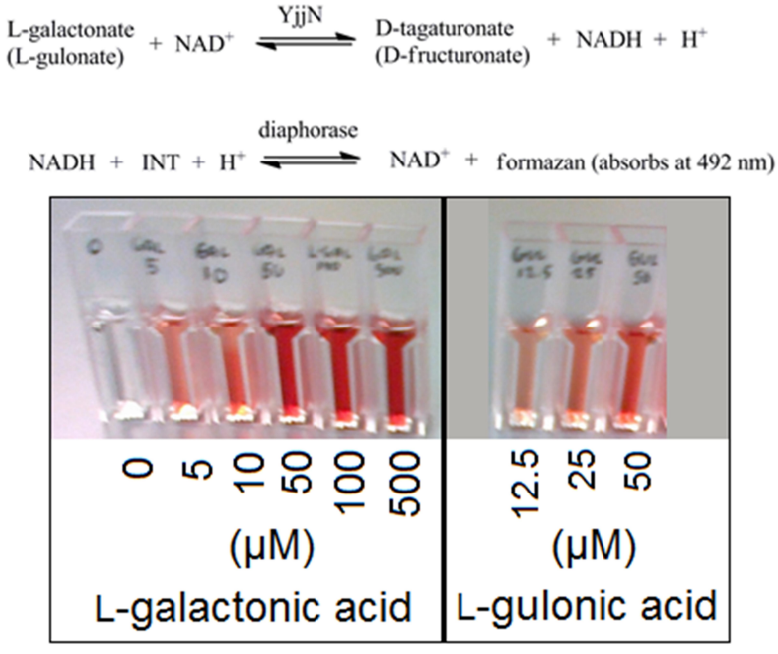

Figure 13. The coupled YjjN/diaphorase assay for L-galA and L-gulonic acid quantification.

3.4 Metabolic engineering of *Aspergillus niger* for production of L-ascorbic acid

Synthetic L-ascorbic acid (L-AA) is widely used as a supplement in pharmaceuticals and as an antioxidant in the beverage, food and feed industries. Currently, synthetic L-AA is predominantly produced from D-glucose in a process including several chemical and biochemical steps. However, a one-step manufacturing process from a cheaper raw-material, such as a single fermentation from D-glaUA, may possibly offer an alternative for the current manufacturing process.

L-GalA is found as an intermediate from one of the alternative biosynthetic L-AA pathways in plants. The pathway originates from D-glaUA, which is converted via L-galA and L-galactono-1,4-lactone (L-galL) to L-AA, and the reactions are catalysed by the enzymes D-galUA reductase, ALase and GALDH, respectively (Fig. 4). In order to generate a one-step bioprocess for L-AA production, the two last steps from the plant pathway (ALase and GALDH) were introduced into the *A. niger* strain Δ*gaaB* that is capable of producing L-galA from D-galUA. The tested ALase genes were *EgALase* from *Euglena gracilis* and *smp3*0 from rat, whereas the GALDH-encoding gene was from *Malpighia glabra*. The genes were expressed either under the constitutive *gpdA* promoter or under the bidirectional and D-galUA-inducible *A. niger gaaA/C* promoter. All of the gene expression cassettes

were transformed into the *A. niger* Δ*gaaB* strain, where the cassettes were randomly integrated into the genome. Several transformants from each transformation were screened for L-AA production and the best strains were selected for further studies.

The transcription of ALase- and GALDH-encoding genes that were expressed under the *gpdA* promoter in *A. niger* Δ*gaaB* strain was confirmed using qPCR (data not shown). In addition, the functionality of the D-galA inducible gene expression in the Δ*gaaB-Eg-Mg** strain was verified in flask cultures (Fig. 14). After transfer to a medium containing D-galUA, expression of the heterologous genes was induced. The patterns of *gaaA* and *MgGALDH* transcript levels were as expected, due to the fact that the orientation of *MgGALDH* compared to the bidirectional *gaaA/C* promoter was similar to the orientation of homologous *gaaA*. The transcript level of *EgALase* was higher when compared to *MgGALDH*. The orientation of *EgALase* compared to the *gaaA/C* promoter was similar to that of the homologous *gaaC* gene. The transcript level of *gaaC* has been observed to be higher than that of *gaaA* in *A. niger* wild type strain in the presence of D-galUA [101].

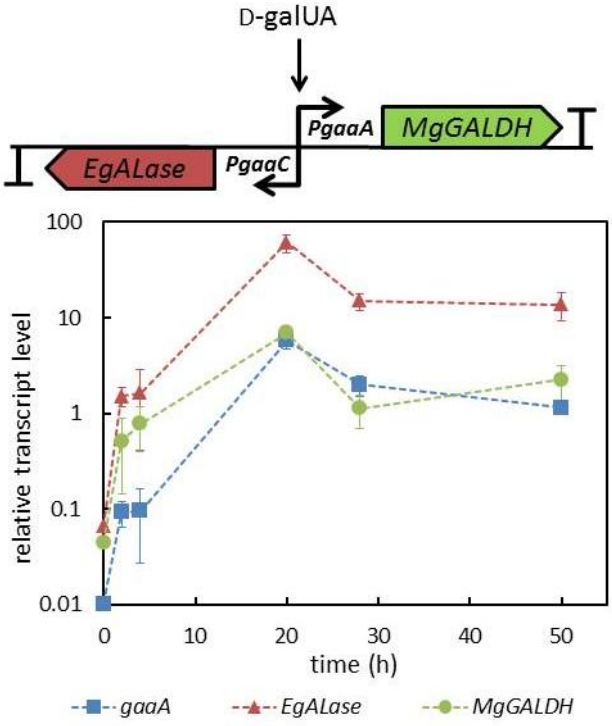

Figure 14. The D-galUA-inducible expression cassette for *EgALase* and *MgGALDH* and relative transcript levels of *gaaA* (blue squares), *EgALase* (red

triangles) and *MgGALDH* (green circles) in the strain Δ*gaaB-Eg-Mg* (*PgaaA/C*) after the transfer to medium containing D-galUA. Errors represent ±SEM, n=3.

In addition to the transcriptional analysis, enzymatic activities of the introduced ALase and GALDH genes were tested from the resulting strains. No lactonase activity of Smp30 or EgALase towards L-galL or linear L-galA was detected in any of the strains. In addition, the expression of Smp30 and EgALase proteins was tested in yeast and *E. coli* expression systems. However, lactonase activity was also not detectable in yeast or *E. coli*. Thus, it is likely that expression of the lactonases in heterologous hosts results in an inactive protein, or that the protein is inactivated during the cell disruption. In the case of GALDH activity towards L-galL, all the *A. niger* strains with the introduced *MgGALDH* gene showed significant GALDH activity (Fig. 15). In the strain Δ*gaaB-Eg-Mg* (*PgaaA/C*) the activity was lower when compared to the strains in which the gene was under the constitutive *gpdA* promoter.

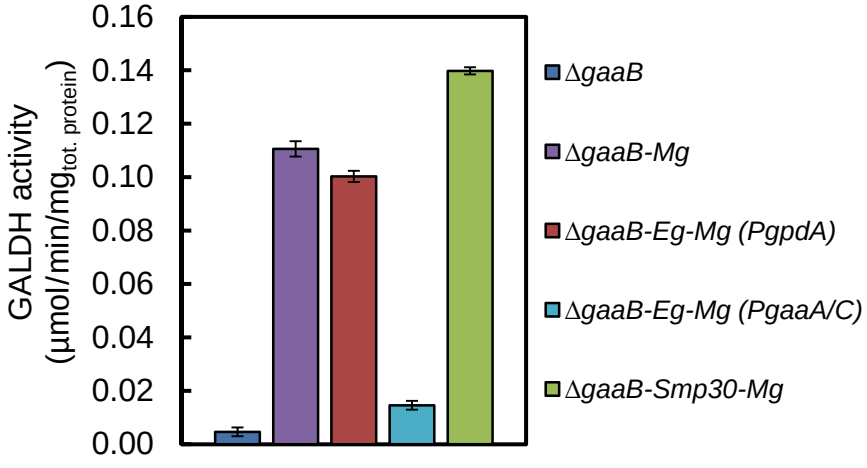

Figure 15. GALDH activity for L-galL in the crude extracts of Δ*gaaB*, Δ*gaaB-Mg* Δ*gaaB-Eg-Mg* (*PgpdA*), Δ*gaaB-Eg-Mg* (*PgaaA/C*) and Δ*gaaB-Smp30-Mg* strains cultured in minimal medium supplemented with D-galUA and D-xylose for 20 h. GALDH activity of the strains expressing *MgGALDH* differed significantly from the activity of the Δ*gaaB* strain ($P<0.05$, Student's t-test). Error bars represent ± SEM (n=3).

The parental strain Δ*gaaB* and the engineered strains were tested for L-AA production in flask cultivations on D-galUA medium at an initial pH of 3.0 (Fig. 16). L-AA concentrations reached 55–83 mg l^{-1} in the engineered strains, whereas concentrations below 3.5 mg l^{-1} were observed from the parental strain. The production of L-AA started earlier in the strains expressing *smp30* or *EgALase* when compared to the strain Δ*gaaB-Mg* with no lactonase (Fig. 16 at 24 h). However, in

the strains in which *MgGALDH* was expressed under the *gpdA* promoter, the highest observed L-AA titre was slightly higher in Δ*gaaB-Mg* when compared to the *ΔgaaB-Eg-Mg* strain. In fact, the highest L-AA titre among the strains with *MgGALDH* under the *gpdA* promoter correlates with the GALDH activity measurement (compare Figs. 15 and 16). The strain Δ*gaaB-Eg-Mg* (*PgaaA/C*) with the D-galUA-inducible production pathway produced similar concentrations of L-AA to the strains with a constitutively expressed production pathway. However, the highest titre with Δ*gaaB-Eg-Mg* (*PgaaA/C*) was observed after 96 hours, whereas in the other strains the production peak was at 48 hours. L-AA concentrations decreased steadily throughout all the cultivations. This may occur due to the exposure to air, which is known to cause oxidation of L-AA.

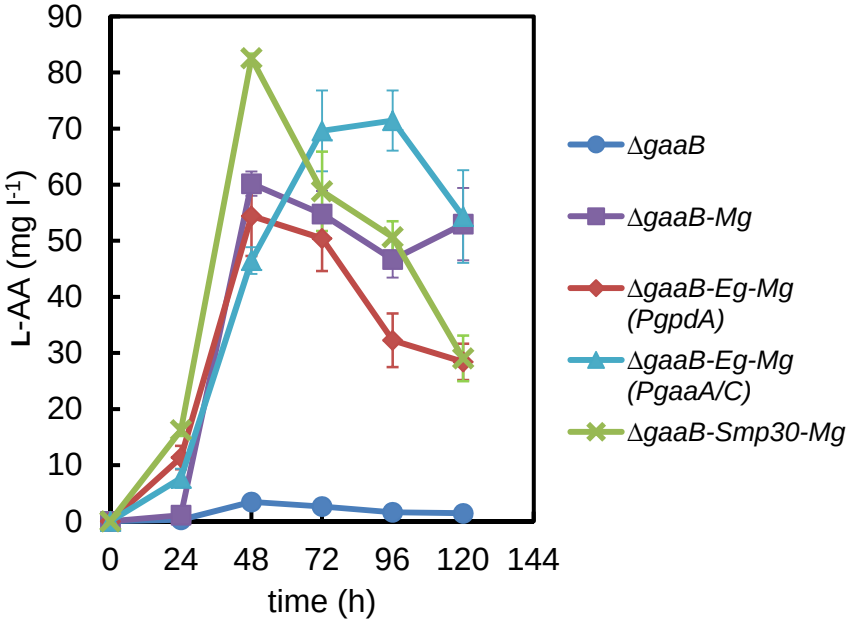

Figure 16. L-AA production by engineered *A. niger* strains in minimal medium supplemented with D-galUA and D-xylose. Error bars represent ± SEM (n=3).

In order to test a consolidated bioprocess for L-AA production, a pectin-rich biomass, CPW, was used as substrate in flask cultivations (SmFs). Due to the fact that D-galUA availability prior to pectin hydrolysis is limited, and we wanted to avoid the unnecessary and energy-wasting L-AA pathway expression at the beginning of cultivation, we decided to use the strain Δ*gaaB-Eg-Mg* (*PgaaA/C*). L-AA production from CPW was observed after 72 hours, which is later than in the case of the cultivations on pure D-galUA (Fig. 17). The observed lag phase was probably due to pectin hydrolysis and utilization of the more preferred carbon sources.

The highest titre of around 170 mg l^{-1} was observed after 96 hours. The highest L-AA levels with Δ*gaaB-Eg-Mg* (*PgaaA/C*) strains on pure D-galUA and CPW were observed after 96 hours in both cases; however, the highest titre was almost 2.5-fold higher on CPW. This might be due to the more available co-substrates in CPW.

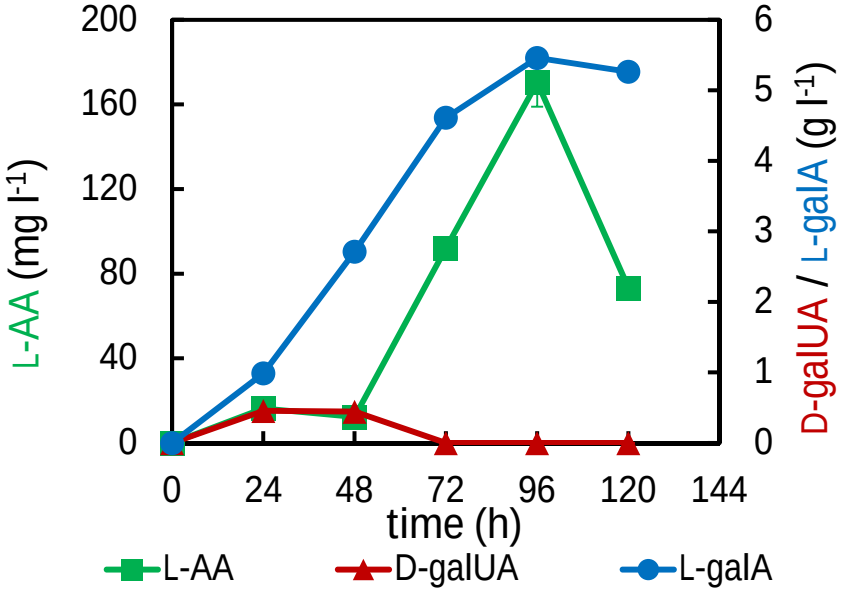

Figure 17. Concentrations of extracellular L-AA (green squares), D-galUA (red triangles) and L-galA (blue circles) in a culture of the strain Δ*gaaB-Eg-Mg* (*PgaaA/C*) in minimal medium supplemented with CPW. Error bars represent ± SEM (n=3) and where not visible, are smaller than the symbol.

Based on the experimental data, the production of L-AA in the engineered *A. niger* strains was most probably limited by the lactonisation reaction between L-galA and L-galL. Despite several attempts, no ALase activity was observed in any of the engineered strains. In addition to *A. niger* strains, *EgALase* and *smp30* expression also failed in yeast and *E. coli*. Thus the expression of EgALase and Smp30 as active proteins in a heterologous host may be challenging. This conclusion is also supported by the literature in which the successful production of these proteins required additional chaperone proteins expressed in the production hosts [87,120,121]. The strain Δ*gaaB-Mg* with no introduced ALase gene was capable of producing L-AA. This observation indicates that the lactonisation reaction required for the conversion of D-galUA to L-AA may be spontaneous. Low pH drives the spontaneous reaction towards the lactone and the reaction may take place extracellularly, after which the lactone is possibly imported back into the cell and oxidised to L-AA. This hypothesis is supported by the fact that no L-AA was observed

in cultures at higher initial pH (5) on D-galUA with the engineered *A. niger* strains (data not shown).

4. Conclusions and future prospects

The fungal catabolic D-galUA pathway was engineered and redirected, resulting in the production of two interesting chemical compounds: L-galA and L-AA. The production was obtained from pure monomeric D-galUA and from untreated pectin-rich biomass. The possible obstacles for engineering the D-galUA pathway in *A. niger* deriving from dehydratase enzyme activities were investigated in more detail. In addition, a bacterial L-galA dehydrogenase was characterised and applied in colorimetric L-galA quantification.

4.1 L-Galactonic acid production

The engineered strains of *H. jecorina* Δ*lgd1* and *A. niger* Δ*gaaB* were capable of producing L-galA from D-galUA in SmFs. The obtained yields below the theoretical maximum putatively resulted from an impaired production pathway due to poor L-galA export from the cells. In *A. niger* Δ*gaaB*, the production was pH-dependent and increased at low pH. A possible explanation for this is the improved L-galA export that may be driven by low extracellular pH. The engineered *A. niger* strain was also capable of L-galA production from CPW in a consolidated bioprocess.

The metabolic push for the L-galA production pathway obtained by overexpressing *gaaA* in the *A. niger* Δ*gaaB* strain improved the initial productivity when compared to the parental strain (Δ*gaaB*). However, yields remained below the theoretical maximum. When a complex carbon source (CPW) was used as substrate in SmFs the product yields remained at approximately the same levels as were obtained from pure D-galUA. In the SSF processes from CPW, yields closer to the theoretical maximum were obtained.

The characterisation of all the putative sugar acid dehydratases from the *A. niger* genome indicated that L-galA is not consumed by another dehydratase enzyme in the engineered strain. Most probably the main bottleneck for the production is the inefficient L-galA export machinery in the cells. For this reason, an approach of metabolic pull by boosting the export process could result in improved production. This is however difficult at present due to the fact that the transporter protein responsible for L-galA export is not known. Nevertheless, the highest product yields, close to 90% of the theoretical maximum, that were achieved in the

consolidated bioprocess from CPW are promising and support the view that *A. niger* is a suitable production host for the utilisation of pectin-rich biomass and production of new organic acids that are not produced by the wild type strains.

4.2 L-Galactonate dehydrogenase

Bacteria have different metabolic pathways for D-galUA catabolisation when compared to fungi. In addition, some bacteria, such as *E. coli,* can utilise L-galA as sole carbon source. L-GalA is the first metabolite in the fungal D-galUA pathway. In this work, the first enzyme in the bacterial L-galA pathway – L-galA-5-dehydrogenase – was confirmed to be encoded by the gene *YjjN*. In addition, the YjjN protein was applied in a colorimetric quantification method for detection of L-galA and L-gulonic acid. The resulting method was superior to HPLC detection in terms of analysis time and sensitivity. If the stability of the enzyme can be improved, the method could be applied e.g. in quantification kits for rapid and reliable L-galA or L-gulonic acid detection.

4.3 L-Ascorbic acid production

Synthetic L-AA is a widely used nutrient and preservative that is currently produced industrially from D-glucose in a multi-step process. On the other hand, several pectin-rich residual biomasses, such as citrus peel and sugar beet pulp, are abundantly available and currently inefficiently used. In this work we used the fungus *A. niger* that is naturally capable of utilising pectin-rich biomass, in a consolidated bioprocess and generated engineered strains with redirected D-galUA metabolism producing L-AA.

The product yields and titres remained low with the resulting strains. The most probable bottleneck for the production was the lactonisation reaction from L-galA to L-galL. However, this is the first demonstration and proof-of-principle on introducing the metabolic pathway converting D-galUA to L-AA in a microbial host. In addition, a practical pectin-rich biomass was successfully tested for L-AA production in the consolidated bioprocess.

4.4 Future prospects

The use of renewables for fuels and power production has been one of the central societal trends in recent years. Production of renewable fuels and energy has been supported by different political decisions and actions, such as tax privileges and subsidies. Among other technologies, industrial biotechnology has benefitted from this situation and the field has developed rapidly during the past decade. However, truly economically feasible industrial biotechnology processes outcompeting petroleum-based fuels are still rare. Consequently, integrated production of fuels and more value-added products, such as chemicals, may provide more com-

petitive biotechnological processes for biorefining. With only few exceptions, production of chemicals in biotechnology requires genetic engineering of the host organism, and the development of efficient production hosts is challenging. However, the potential that is hidden in cellular metabolism is tremendous. Synthetic biology is a new emerging field offering genetic tools that can be used for metabolic engineering. One of the goals in synthetic biology is to generate a well-established and characterized tool box for genetic engineering. Thus, it will be possible to generate even more complex and well controlled metabolic pathways that are not found in nature, and to introduce them into host organisms and establish pathways for new chemicals.

Pectin-rich biomass residues, such as CPW and sugar beet pulp, are attractive raw materials for biorefineries. Due to the high oxidation state of the main component D-galUA and the inability of ethanologenic yeasts to catabolise D-galUA, it may be challenging to develop efficient processes converting D-galUA to ethanol. Thus, alternative products including the compounds presented in this thesis could provide more realistic options for the use of the D-galUA fraction in pectin-rich biomass. The processes developed in this work were carried out in laboratory scale without extensive process optimisation. Despite this limitation, the yields in L-galA production were at least promising. With careful process design and optimisation, it would be possible to increase L-galA productivities and yields significantly. The process for L-AA production would need significant improvement in terms of yield and productivity before being industrially realistic. This would require an efficient lactonisation reaction of L-galA in the production host. However, this thesis is a proof of concept for production of L-galA and L-AA from pure D-galUA and from pectin-rich biomass in a consolidated bioprocess using engineered strains of filamentous fungi.

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PUBLICATION I

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Engineering Filamentous Fungi for Conversion of D-Galacturonic Acid to L-Galactonic Acid

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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 hydrolyzed. 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* strains produced L-galactonate at yields of 0.6 to 0.9 g per g of substrate consumed. Although *T. reesei* Δ*lgd1* could produce L-galactonate at pH 5.5, a lower pH was necessary for *A. niger* Δ*gaaB*. Provision of a cosubstrate improved the production rate and titer 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.

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 250×10^6 and 115×10^6 metric tons, respectively. After beet processing, 5 to 10% of the sugar beet remains as dried sugar beet pulp. This pulp contains ca. 25% pectin (6). Citrus peel contains ca. 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 the pulp and peel are dried since; otherwise, they rot rapidly. Disposal of the material is problematic because of the bad odor 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 (6). This process is only economical when done on a 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 flavor 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 (5, 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 valorize 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 catabolized through a pathway (Fig. 1) that includes reactions catalyzed 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 can induce pectinolytic and D-galacturonic acid catabolic genes in *A. niger*, regardless of whether D-galacturonic acid is metabolized or not (4, 14).

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

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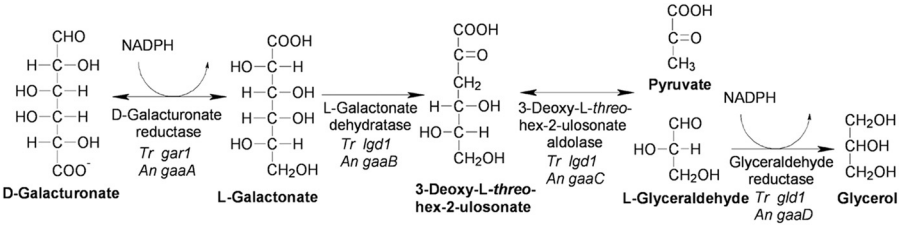

FIG 1 Fungal D-galacturonic acid pathway. The genes encoding the enzymes in *T. reesei* (*Tr*) and *A. niger* (*An*) are indicated. The deletion of *lgd1* in *T. reesei* and *gaaB* in *A. niger* disrupted the pathway and generated strains that accumulated L-galactonate.

convert D-galacturonic acid to L-galactonic acid, which is excreted into the medium.

L-Galactonic acid is currently expensive and not widely used, but has the potential to be used more widely once it is available at a low price. The physicochemical properties are similar to those of D-gluconic acid, which is widely used as a chelator, in the pharmaceutical, cosmetic, and other industrial (e.g., dyes, detergents, solvents, and paints) sectors and as an acidifier in food. L-Galactonic acid is also a precursor for L-ascorbic acid (vitamin C) synthesis. The L-galactono-1,4-lactone that forms from L-galactonic acid at acidic pH can be oxidized 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 (i.e., the *pyrG* gene) deleted (14), was used to construct the *gaaB* deletion strain. The cassette for deletion of *gaaB* contained 1,550 bp from the *A. niger gaaB* promoter, 1,533 bp from the *A. niger gaaB* terminator, and a 1,920-bp fragment containing the *pyrG* gene flanked with its native promoter and terminator. These fragments were obtained by PCR of *A. niger* ATCC 1015 genomic DNA using the 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 EcoRI and PvuII (both NEB) and the terminator fragment (*gaaB-3*) with EcoRI to produce an intermediary construct by ligation using T4 DNA ligase (NEB). This intermediary construct was digested with XhoI (NEB) and Ecl136II (Fermentas) and ligated to the XhoI-digested promoter fragment (*gaaB-5*). The resulting vector was digested with Ecl136II and treated with phosphatase. The *pyrG* DNA fragment, after digestion with SmaI, was inserted between the two *gaaB* flanking regions. The deletion cassette, 5,006 bp containing the *gaaB* flanking regions and the *pyrG* gene, was released by EcoRI+XhoI digestion and introduced into the *A. niger* ATCC 1015 Δ*pyrG* strain 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.

TABLE 1 Primers used to generate vectors for the deletion of *gaaB* and the incorporation of *gaaA* in *A. niger* ATCC 1015 Δ*pyrG* in order to confirm integration and for quantitative PCR

Primer	Sequence (5′–3′)
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

The cassette for the overexpression of *A. niger* D-galacturonate reductase (*gaaA*) contained the native *gaaA* gene between the *gpdA* promoter and *trpC* terminator from *A. nidulans*, following the hygromycin B phosphotransferase (*hph*) gene under the *gpdA* promoter. The *gaaA* fragment was obtained by PCR from ATCC 1015 genomic DNA using the primers gaaA-exp-F and gaaA-exp-R (Table 1). The plasmid (JKp1-*hph*) containing the *gpdA-trpC-hph* fragment was derived from pRS426 (ATCC). Both JKp1-*hph* and the PCR-amplified *gaaA* fragment were digested with SacI and XmaI (both NEB), followed by ligation using T4 DNA ligase to generate the intermediary construct JKp1-*hph-gaaA*. JKp1-*hph-gaaA* was digested with BspHI and PsiI (both NEB), and the fragment containing the *gpdA-gaaA-trpC-hph* cassette was introduced into the *A. niger* ATCC 1015 *gaaB*Δ strain by transformation. Transformants were screened for integration of the *gpdA-gaaA-trpC-hph* cassette by growth in the presence of 400 μg of hygromycin B (Calbiochem) ml^{-1}. Integration of the transformed cassette into the genome was confirmed by PCR with the primers gpdA-F and gaaA-exp-R (Table 1).

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

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

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

For larger-scale cultures, mycelium was grown in bioreactors in 500 ml (Multifors; maximum working volume, 500 ml; Infors HT, Switzerland). Cultures were maintained at 30°C and 800 rpm, with a 1.6 volume of gas volume $culture^{-1}$ min^{-1} (vvm). The culture pH was kept constant at pH 4.5, 4.9, or 5.5 by the addition of sterile 1 M KOH or 1 M H_3PO_4. Polypropylene glycol (mixed molecular weight [21]) was added to control foam production. The initial biomass concentration in *T. reesei* cultures was 0.3 g $liter^{-1}$, and in *A. niger* cultures the concentrations were 0.4 g $liter^{-1}$ in bioreactors and 0.7 to 1.4 g $liter^{-1}$ in flasks.

Chemical analyses. Samples (1 to 60 ml, depending on the culture scale and density of biomass) were removed at intervals, and the mycelium was separated from the supernatant by filtration through cloth. For analysis of intracellular L-galactonate concentrations, biomass that had been washed first with an equal volume of 9 g of NaCl $liter^{-1}$ and then with distilled water was frozen at −20°C and subjected to freeze-drying. After the sample was weighed, the L-galactonate in the dried biomass was extracted in 5 mM H_2SO_4 as described previously for the extraction of intracellular 2-keto-3-deoxy-L-galactonate (20). Intracellular amounts are given as mg per g of dry biomass, but the concentration may be estimated by assuming that the volume (in ml) of cytoplasm per g of dry biomass would be similar to that of *Penicillium chrysogenum*, which has been determined to be 2.86 ml per g of dry biomass (15).

The concentrations of D-xylose, D-galacturonate, and L-galactonate were determined by HPLC using a fast acid analysis column (100 by 7.8 mm; Bio-Rad Laboratories, Hercules, CA) linked to an Aminex HPX-87H organic acid analysis column (300 by 7.8 mm; Bio-Rad Laboratories) with 2.5 or 5.0 mM H_2SO_4 as the eluant and a flow rate of 0.5 ml min^{-1}. The column was maintained at 55°C. Peaks were detected using a Waters 410 differential refractometer and a Waters 2487 dual-wavelength UV (210-nm) detector.

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 minikit (Qiagen), and 1 μg of total RNA was used for cDNA synthesis with a Transcriptor high-fidelity cDNA synthesis kit (Roche) according to 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 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 the corresponding primers listed in Table 1. The level of expression of *gaaA* and the genes encoding the putative transporters was normalized to actin by using the accompanying software (Advance Relative Quantification tool).

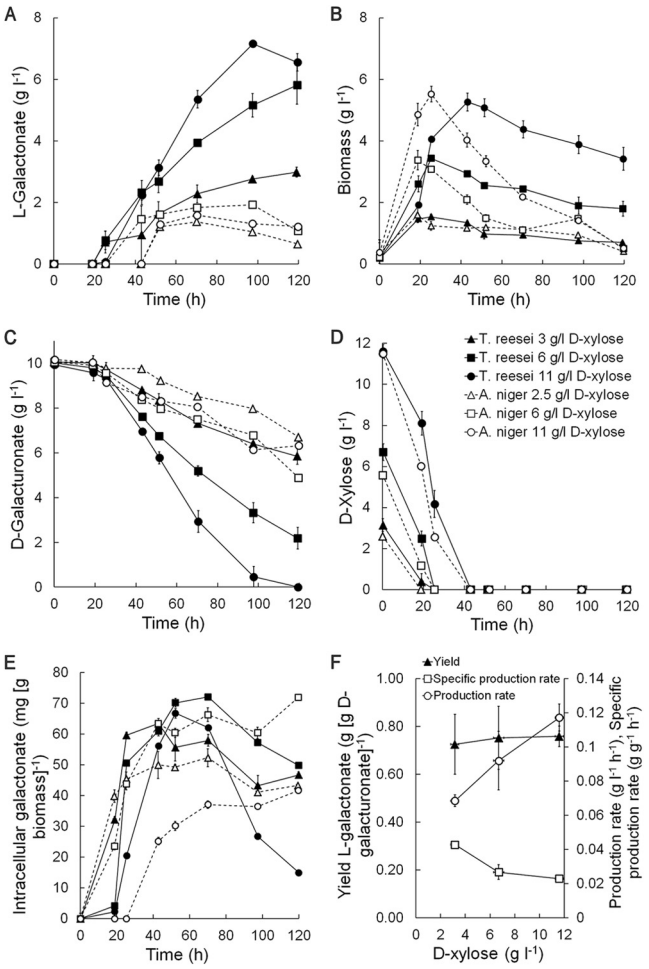

FIG 2 (A to E) Concentrations of extracellular L-galactonate (A), biomass (B), D-galacturonate (C), and D-xylose (D) and intracellular L-galactonate from *T. reesei* Δ*lgd1* (solid symbols) and *A. niger* Δ*gaaB* (open symbols) (E) in modified Vogel medium initially containing 10 g of D-galacturonate $liter^{-1}$ and 2.5, 3, 6, or 11 g of D-xylose $liter^{-1}$, as indicated, at pH 5.5, 800 rpm, 1.6-vvm aeration, and 30°C. (F) Effect of D-xylose concentration on yield of L-galactonate on D-galacturonate consumed, and volumetric production and specific production rates of L-galactonate for *T. reesei* Δ*lgd1*. Error bars represent ± the standard error of the mean (SEM; $n = 2$).

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 $liter^{-1}$) and *A. niger* Δ*gaaB* (5.9 ± 0.1 g $liter^{-1}$) produced L-galactonate when incubated for 120 h in flasks initially containing 10 g of D-galacturonate $liter^{-1}$ and 2 g of D-xylose $liter^{-1}$ as cosubstrates (initial pH of 5.1). Less L-galactonate (2.0 ± 0.1 g $liter^{-1}$) was produced by *A. niger* Δ*gaaB* when no D-xylose was provided, and thus D-xylose was included as a cosubstrate in all further experiments.

When *T. reesei* Δ*lgd1* was cultivated in a bioreactor, L-galactonate production and D-galacturonate utilization increased with the provision of increasing concentrations of D-xylose as cosubstrate (Fig. 2). Up to 7.2 g of L-galactonate $liter^{-1}$ was produced in

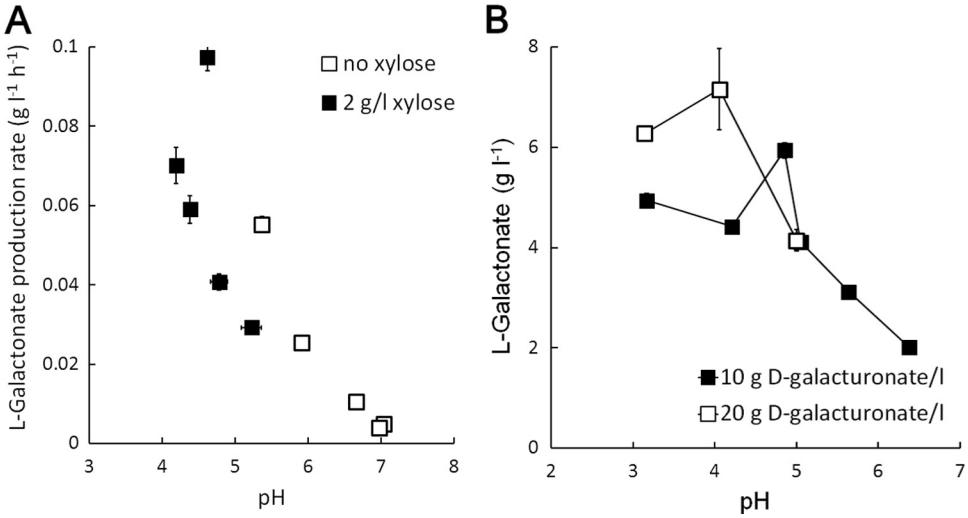

FIG 3 L-Galactonate production by *A. niger* Δ*gaaB* in flasks. (A) L-Galactonate production rate as a function of pH for unbuffered cultures provided 10 g of D-galacturonate liter^{-1} at an initial pH of 5.2 with no D-xylose (open symbols) or 2 g of D-xylose liter^{-1} (solid symbols) provided for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

the culture provided 11 g of D-xylose liter^{-1}. The initial production rate was 0.07 to 0.12 g of L-galactonate liter^{-1} h^{-1}, and the final yields were 0.60 to 0.85 g of L-galactonate per g of D-galacturonate consumed (Fig. 2). Although initial yields of 0.9 to 1.0 g of L-galactonate per g of D-galacturonate were observed, the yield decreased during the production phase. The biomass concentration also increased with increasing provision of D-xylose (yield of 0.5 g biomass g D-xylose^{-1}), and the specific L-galactonate production rate was lower when 11 g of D-xylose liter^{-1} was provided than with 3 g liter^{-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 of intracellular L-galactonate g biomass^{-1}. Intracellular D-galacturonate remained <2 mg g biomass^{-1} (data not shown).

A. niger Δ*gaaB* produced only 1.4 to 1.9 g of L-galactonate liter^{-1} when cultivated in bioreactors at pH 5.5 (Fig. 2), although 5.9 g liter^{-1} had been produced in the preliminary flask experiment. The biomass concentrations were similar to those of *T. reesei* Δ*lgd1* (yield of 0.56 g biomass g D-xylose^{-1}), as were the intracellular concentrations of L-galactonate (Fig. 2). D-Galacturonate (10 to 30 mg g biomass^{-1}) was also detectable in mycelia from the cultures which received 6 or 11 g of D-xylose liter^{-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 medium used here is not well buffered, and thus the 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. The 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. niger*. Indeed, L-galactonate production decreased with increasing pH above 5.0 in flask cultures but was generally high (5 to 6 g liter^{-1}) at pH values below 5 (Fig. 3). L-Galactonate production was further improved at pH 3 to 4 by cultivating the strain in buffered medium with 20 g of D-galacturonate liter^{-1} and 5 g of D-xylose liter^{-1} (Fig. 3).

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

Bioconversion of polygalacturonate to L-galactonate. *A. niger* Δ*gaaB* converted polygalacturonate to L-galactonate at a similar rate (initial rate of 0.04 g liter^{-1} h^{-1}, increasing to 0.07 g liter^{-1} h^{-1} after the addition of extra D-xylose) and titer (2.5 g of L-galactonate liter^{-1} within 72 h) as it converted the monomer D-galacturonate (Fig. 4). L-Galactonate (1.2 g liter^{-1}) was present in the culture supernatant after 26 h but did not accumulate above 2.8 g liter^{-1} at any time during the cultivation. The addition of D-xylose after 127 h resulted in a total of 6.5 g of L-galactonate liter^{-1} (yield of 0.85 g of L-galactonate per g of D-galacturonate consumed^{-1}) after 171 h, increasing to 7.6 g liter^{-1} after 195 h. The intracellular concentration of L-galactonate (52 ± 4 mg g biomass^{-1}) was similar to that observed in other L-galactonate-producing cultures and also decreased after the addition of D-xylose (Fig. 4). Low concentrations of D-galacturonate (0.2 to 4.3

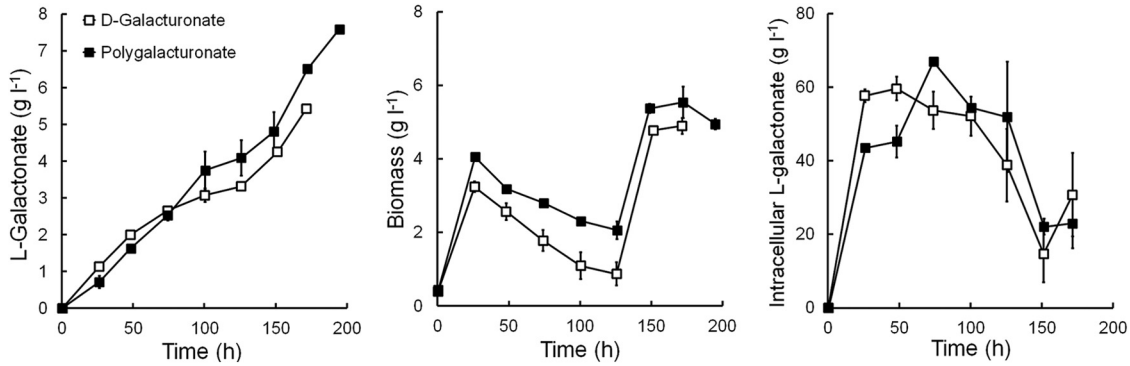

FIG 4 Concentrations of L-galactonate, biomass, and intracellular L-galactonate in *A. niger* Δ*gaaB* cultures in modified Vogel medium with 5 g of D-xylose liter^{-1} and containing 10 g of D-galacturonate liter^{-1} (open symbols, pH 4.8) or 15 g of polygalacturonate liter^{-1} (solid symbols pH 4.5). The cultures were maintained at 30°C, 800 rpm, and 1.6-vvm aeration and were given an additional 9 g of D-xylose liter^{-1} at 127.8 h. Error bars represent ± the SEM ($n = 2$) and, where not visible, are smaller than the symbol.

mg g biomass^{-1}) were also extracted from mycelia incubated in polygalacturonate (data not shown).

Overexpression of *A. niger gaaA*. Since *gaaA* expression appeared to be low in the Δ*gaaB* strain, the galacturonate reductase coding gene, *gaaA*, was overexpressed in *A. niger* Δ*gaaB*. *A. niger* ATCC 1015, the Δ*gaaB* strain, and the overexpression strain (Δ*gaaB-gaaA*) were grown in modified Vogel medium with 10 g of D-galacturonate liter^{-1} and 2 g of D-xylose liter^{-1} at an initial pH of 3 in flasks. The expression of *gaaA* in *A. niger* Δ*gaaB* was considerably lower compared to the wild type after 6 h (Table 2). In contrast, in *A. niger* Δ*gaaB-gaaA* expression of *gaaA* was much higher at 0 and 6 h, as expected (Table 2). After 24 h, *gaaA* expression levels in *A. niger* Δ*gaaB* and *A. niger* Δ*gaaB-gaaA* were similar, whereas its expression in the wild type had decreased (Table 2), probably due to D-galacturonate depletion.

Approximate L-galactonate production rates were determined for the flask cultures. During the first 24 h after inoculation, *A. niger* Δ*gaaB-gaaA* produced L-galactonate at a significantly ($P < 0.05$) higher rate (0.070 g of L-galactonate liter^{-1} h^{-1}) than *A. niger* Δ*gaaB* (0.048 g of L-galactonate liter^{-1} h^{-1}; Table 3). After 24 h, the difference in the production rates of the Δ*gaaB* and Δ*gaaB-gaaA* strains decreased, and after 48 h, when L-galactonate production by both strains was decreasing, their production rates were similar ($P > 0.05$; 0.046 and 0.054 g of L-galactonate liter^{-1} h^{-1}, respectively; Table 3).

The final L-galactonate titers of the Δ*gaaB* and Δ*gaaB-gaaA* strains were compared in both modified Vogel medium and *A. nidulans* minimal medium in flasks (Table 4). Both the L-galactonate titer and the yield were generally higher for *A. niger* Δ*gaaB-gaaA* than for *A. niger* Δ*gaaB* when grown at pH 3 or 4 in either medium (Table 4). At pH 5 in *A. nidulans* minimal medium, the final L-galactonate titer 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 4).

Transcription of putative transporter genes in *A. niger* Δ*gaaB*. The relative transcript levels of three genes which have been identified as possible transporters of D-galacturonate (An07g00780, An14g04280, and An03g01620, [12]) were assessed in *A. niger* ATCC 1015 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 ATCC 1015 within 3 h of the transfer, whereas the 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 (Fig. 2 to 4). This confirmed that D-galacturonate was still taken up in the deletion strains, as was also the case when either the D-galacturonate reductase genes (*gar1* or *gaaA* in *T. reesei* and *A. niger*, respectively [14]) or the 2-keto-3-deoxy-L-

TABLE 2 Relative expression of *gaaA* in *A. niger* ATCC 1015, Δ*gaaB*, and Δ*gaaB-gaaA* strains[a]

Time (h)	Relative expression of *gaaA* (avg ± SEM [$n = 3$])		
	ATCC 1015	Δ*gaaB* strain	Δ*gaaB-gaaA* strain
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

[a] The relative expressions of *gaaA* in *A. niger* ATCC 1015, Δ*gaaB*, and Δ*gaaB-gaaA* strains grown in flasks in modified Vogel medium with 10 g of D-galacturonate liter^{-1} and 2 g of D-xylose liter^{-1} at an initial pH 3.0 are presented.

TABLE 3 L-Galactonate production rates for *A. niger* Δ*gaaB* and Δ*gaaB-gaaA* strains[a]

Time interval (h)	Avg L-galactonate production rate (g liter^{-1} h^{-1}) ± SEM ($n = 3$)[b]	
	Δ*gaaB* strain	Δ*gaaB-gaaA* strain
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}

[a] The L-galactonate production rates were determined for *A. niger* Δ*gaaB* and Δ*gaaB-gaaA* strains grown in flasks in modified Vogel medium with 10 g of D-galacturonate liter^{-1} and 2 g of D-xylose liter^{-1} at an initial pH of 3.0.
[b] Values in the same row with different superscript letters differed significantly ($P < 0.05$).

TABLE 4 L-Galactonate production at 144 h by *A. niger* Δ*gaaB* and the Δ*gaaB* strain overexpressing *gaaA* (Δ*gaaB-gaaA*)[a]

Medium	Initial pH	Strain	Mean amt of L-GalA (g liter^{-1}) ± SEM	Conversion (g g^{-1}) of L-GalA/D-GalUA$_{initial}$	Yield (g g^{-1}) of L-GalA/D-GalUA$_{consumed}$
A. nidulans MM	5	Δ*gaaB*	4.1 ± 0.2	0.20	0.82
	5	Δ*gaaB-gaaA*	4.1 ± 0.3	0.20	0.97
	4	Δ*gaaB*	7.2 ± 0.8	0.35	0.95
	4	Δ*gaaB-gaaA*	7.8 ± 0.4	0.38	0.97
	3	Δ*gaaB*	6.3 ± 0.1	0.31	0.86
	3	Δ*gaaB-gaaA*	8.7 ± 0.2	0.43	1.00
Modified Vogel medium	4	Δ*gaaB*	4.2 ± 0.1	0.41	0.70
	4	Δ*gaaB-gaaA*	5.0 ± 0.1	0.49	0.75
	3	Δ*gaaB*	4.9 ± 0.1	0.47	0.70
	3	Δ*gaaB-gaaA*	6.2 ± 0.3	0.59	0.82

[a] L-Galactonate (L-GalA) production at 144 h by *A. niger* Δ*gaaB* and the Δ*gaaB* strain overexpressing *gaaA* (Δ*gaaB-gaaA*) was determined in buffered *A. nidulans* minimal medium (MM) with 20 g of D-galacturonate liter^{-1} and 5 g of D-xylose liter^{-1} and in modified Vogel medium with 10 g of D-galacturonate liter^{-1} and 2 g of D-xylose liter^{-1} in flasks at an initial pH of 3, 4, or 5. Mean values are shown ($n = 3$). The conversion and yield on D-galacturonate (D-GalUA) are also shown.

galactonate aldolase genes, *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 of L-galactonate liter^{-1} h^{-1}) as previously reported for the conversion to keto-deoxy-L-galactonate (0.10 to 0.14 g liter^{-1} h^{-1} [20]) but was faster than the conversion to galactarate (0.024 to 0.046 g liter^{-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 of L-galactonate liter^{-1} h^{-1}) was much slower than the conversion to keto-deoxy-L-galactonate (0.27 to 0.33 g liter^{-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 g g^{-1} for *A. niger* Δ*gaaB*. Thus, the yields were only slightly lower than the theoretical yield (1.0 g of L-galactonate g of D-galacturonate^{-1}) but still indicated that some of either the D-galacturonate or the produced L-galactonate was 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 measurable production of biomass from D-galacturonate in these strains.

TABLE 5 Relative expression of putative transporters An07g00780, An14g04280, and An03g01620 in *A. niger* ATCC 1015 and Δ*gaaB* strains[a]

Putative transporter	Time (h)	Avg relative expression ± SEM ($n = 3$)	
		ATCC 1015	Δ*gaaB* strain
An07g00780	0	0.4 ± 0.0	0.1 ± 0.0
	3	0.1 ± 0.0	ND
	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

[a] The relative expression of putative transporters An07g00780, An14g04280, and An03g01620 in *A. niger* ATCC 1015 and the Δ*gaaB* strain grown in flasks in modified Vogel medium with 10 g of D-galacturonate liter^{-1} and 2 g of D-xylose liter^{-1} at an initial pH of 3. ND, no data.

Although the production of both L-galactonate and keto-deoxy-L-galactonate require NADPH as a cofactor for the D-galacturonate reductase, L-galactonate production was more dependent on the addition of D-xylose as a cosubstrate (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 of 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 of L-galactonate g biomass^{-1}) in the corresponding strains (20). After provision of additional cosubstrate 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 ~2.86 times the dry biomass (10), the average intracellular concentration of L-galactonate was ~20 g liter^{-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-galactonate may limit the rate of D-galacturonate conversion by feedback inhibition and/or providing substrate for the reverse reaction, which has been shown to occur with both the *T. reesei gar1* (10) and the *A. niger gaaA* (13) D-galacturonate reductases. The K_m for L-galactonate of *T. reesei* gar1 is 4 mM (0.8 g liter^{-1}) (10), which is much lower than the intracellular L-galactonate concentrations observed. Thus, the accumulation of L-galactonate may limit the reaction more than accumulation of keto-deoxy-L-galactonate, since the action of the L-galactonate dehydratase is irreversible (9). Generation of intracellular D-galacturonate may also have affected the uptake of the substrate, about which little is known in filamentous fungi. Intracellular D-galacturonate was, however, only observed in *A. niger* and not in *T. reesei*.

In contrast to keto-deoxy-L-galactonate production (20), L-galactonate production was more efficient in *T. reesei* than in *A.*

niger at pH 5.5, producing higher titers at higher rates (Fig. 2). *T. reesei* was also found to be more effective than *A. niger* in the production of galactarate (14), and these results confirm that *T. reesei* is an interesting and useful host for organic acid production, even though it is not known as a high producer of organic acids, nor is it tolerant to a very low culture pH.

Low galactarate production by *A. niger* Δ*gaaA-udh* was attributed to subsequent metabolism of the galactarate (14). The metabolism of L-galactonate appeared negligible (Fig. 4) or limited (Fig. 2) in *A. niger* Δ*gaaB*; instead, L-galactonate production by *A. niger* was found to be pH dependent, with the highest production rates and titers observed at pH values below 5.0 and no reduction in production even at pH 3.0 (Fig. 3). At pH 4.5 to 4.8, the production of L-galactonate by *A. niger* Δ*gaaB* was as good as that of *T. reesei* Δ*lgd1* at pH 5.5. At a low extracellular pH, more of the product is protonated to L-galactonic acid (pKa ~3.5), creating a greater difference in concentration between the dissociated intra- and extracellular L-galactonate pools. If the protonated organic acid is not reimported into 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 liter^{-1} h^{-1} [20]) or galactarate at pH 5.0 (0.21 to 0.46 g liter^{-1} h^{-1} [14]) than were observed during L-galactonate production at any pH (0.04 to 0.15 g liter^{-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 the *A. niger* ATCC 1015, CBS120.49, and Δ*gaaA* strains (12, 14). In ATCC 1015, the transcription of these three genes was induced simultaneously within 4 h of transfer to D-galacturonate, and the induction of *gaaB* and *gaaC* remained similar in *A. niger* Δ*gaaA* compared to ATCC 1015 (14). In the present study, we observed that *gaaA* was not induced in *A. niger* Δ*gaaB* even 6 h after exposure to D-galacturonate (Table 2), although transcription had increased after 24 h. In ATCC 1015, *gaaA* expression was already reduced after 24 h of 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 responses of *gaaA* and *gaaC* might be expected since these genes share a 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 mechanism, the delayed induction of *gaaA* in the Δ*gaaB* strain would account for low initial rates of D-galacturonate conversion.

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

D-Xylose was previously found to be a good cosubstrate in the production of keto-deoxy-L-galactonate (20), but D-galacturonate did not appear to be taken up while D-xylose was being consumed (Fig. 2). Limited D-galacturonate uptake while *gaaA* expression was high in *A. niger* Δ*gaaB-gaaA* probably limited the improvement in L-galactonate production that could be achieved by this strain. In addition, only two of the three putative D-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 does *T. reesei*, which is unable to degrade pectin (20). Using the current *A. niger* Δ*gaaB* strain, the 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 cosubstrates (e.g., D-galactose, D-xylose, and L-arabinose) for the initial production of biomass and NADPH. A more gradual provision of cosubstrate 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 cosubstrate 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 enhancement in production by both strain engineering and process development may provide an efficient source of L-galactonate for, e.g., microbial ascorbic acid production and other applications.

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PUBLICATION II

Conversion of orange peel to L-galactonic acid in a consolidated process using engineered strains of *Aspergillus niger*

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Conversion of orange peel to L-galactonic acid in a consolidated process using engineered strains of *Aspergillus niger*

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Abstract

Citrus processing waste is a leftover from the citrus processing industry and is available in large amounts. Typically, this waste is dried to produce animal feed, but sometimes it is just dumped. Its main component is the peel, which consists mostly of pectin, with D-galacturonic acid as the main monomer. *Aspergillus niger* is a filamentous fungus that efficiently produces pectinases for the hydrolysis of pectin and uses the resulting D-galacturonic acid and most of the other components of citrus peel for growth. We used engineered *A. niger* strains that were not able to catabolise D-galacturonic acid, but instead converted it to L-galactonic acid. These strains also produced pectinases for the hydrolysis of pectin and were used for the conversion of pectin in orange peel to L-galactonic acid in a consolidated process. The D-galacturonic acid in the orange peel was converted to L-galactonic acid with a yield close to 90%. Submerged and solid-state fermentation processes were compared.

Keywords: Citrus processing waste; Orange peel; Consolidated bioprocessing; L-galactonic acid; D-galacturonic acid; *Aspergillus niger*

Introduction

Utilization of agricultural wastes for the production of fuels and chemicals using microbial fermentations is an attractive concept, due to the low costs of the raw material and beneficial environmental aspects. However, the profitability of utilizing agricultural residues can be reduced by logistical costs, if the feedstock is dispersed over a wide area and by the need for pre-treatments before the fermentation. Consequently, the ideal process would utilize an existing logistic chain and a feedstock that does not need extensive pre-treatment. Citrus processing waste (CPW) is an example of such a feedstock: its generation is centralized within a citrus processing plant and, due to low lignin content (Edwards and Doran-Peterson 2012), it does not require extensive pre-treatment.

The current annual world production of citrus fruits is greater than 80 million tonnes, of which oranges constitute about 50 million tonnes (USDA 2013). About 20 million tonnes of these oranges are used by the orange processing industry (USDA 2013). The production of orange juice generates a large quantity of CPW, with around 44–60% of the mass of processed orange fruit ending up as CPW (Widmer et al. 2010, López et al. 2010). Thus, approximately 10 million tonnes of wet CPW is generated annually in the world by the orange processing industry alone.

CPW, in turn, contains mainly peels. These peels are rich in pectin and also contain a significant quantity of D-limonene. The pectin content in CPW is around 25% on a dry mass basis, corresponding to about 5% on a wet mass basis (Pourbafrani et al. 2010). Currently, the markets for pectin and D-limonene consume only a relatively small fraction of the CPW. For example, the world market for pectin, which is used as a gelling agent in the food industry, was estimated as 42 000 tonnes per year in 2009 (Staunstrup 2009), whereas the CPW generated annually by the orange processing industry could produce approximately 500 000 tonnes of pectin. In other words, over 90% of the CPW produced by the orange processing industry is in excess of the world pectin market. This excess CPW

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could be used for animal feed. However, this is seldom economic, due to the high energy requirements for the processes of drying and milling that are necessary to transform the CPW into a meal that is appropriate for adding to animal feed (Grohman et al. 2013). As a result, the CPW is sometimes simply discarded. It is therefore highly desirable to find new ways of converting pectin-rich residues into useful products, following the worldwide trend of using renewable resources to substitute petroleum derivatives.

So far, most attempts to produce higher-value products from CPW have involved extracting compounds such as pectin and D-limonene and fermenting the remaining matter to produce ethanol (Edwards and Doran-Peterson 2012). However, due to the limited size of the world pectin market and the inability of ethanologenic yeasts to ferment D-galacturonic acid (Van Maris et al. 2006), the main constituent in pectin, it is possible to utilize only a part of CPW in these processes. Other proposed products from CPW include methane (López et al. 2010), citric acid (Rivas et al. 2008), succinic acid (Li et al. 2010a) and enzymes (López et al. 2010). In the current work, we open a new route to increase the value of CPW using engineered *Aspergillus niger* strains to convert the pectin to L-galactonic acid in a consolidated process.

L-Galactonic acid and its lactone (L-galactono-1,4-lactone) are currently expensive speciality chemicals and are not widely used. However, these compounds would have the potential to be used more widely if they were available at a lower price. L-Galactonic acid is also a precursor for L-ascorbic acid (vitamin C): L-galactono-1,4-lactone, which is formed from L-galactonic acid upon acidification, can be directly converted to L-ascorbic acid through a fermentative process (Onofri et al. 1997, Roland et al. 1986) or through a chemical process (Csiba et al. 1993). The annual production of synthetic L-ascorbic acid is about 100 000 tonnes (Pappenberger and Hohmann 2013). The 500 000 tonnes of pectin in the annually produced CPW contains around 375 000 tonnes of D-galacturonic acid. In turn, equimolar conversion of this D-galacturonic acid to L-galactonic acid and, subsequently, to L-ascorbic acid could be theoretically achieved. Therefore, even after factoring in conversion efficiencies below 100%, CPW could easily supply the raw material for world L-ascorbic acid production. The production of polymers derived from L-galactonic acid has also been studied (Romero Zaliz and Varela 2003, Romero Zaliz and Varela 2005).

L-Galactonic acid is an intermediate of the reductive pathway for catabolism of D-galacturonate that is found in moulds such as *Aspergillus niger* (Figure 1) (Richard and Hilditch 2009). In *A. niger*, D-galacturonate is first reduced to L-galactonate by the D-galacturonate reductase, GAAA (Martens-Uzunova and Schaap 2008). L-Galactonate is subsequently converted to 2-keto-3-deoxy-L-galactonate (3-deoxy-L-threo-hex-2-ulosonate) by the L-galactonate dehydratase, GAAB, which is then further metabolized to pyruvate and glycerol by the action of GAAC and GAAD (Martens-Uzunova and Schaap 2008).

The deletion of the gene coding for the L-galactonate dehydratase, *gaaB*, resulted in a strain of *A. niger* that does not grow on D-galacturonic acid but instead converts D-galacturonate to L-galactonate (Kuivanen et al. 2012). Since this strain produces pectinases, it was able to produce L-galactonate from polygalacturonate in a submerged fermentation (Kuivanen et al. 2012). The aim of the current work was to determine whether this strain could be used to hydrolyse the pectin in the CPW and convert the resulting D-galacturonic acid to L-galactonic acid in a single fermentation process.

Materials and methods

Strains

The *Aspergillus niger* strain ATCC 1015 (CBS 113.46) was used as a wild type. Engineered strains ATCC 1015 Δ*gaaB* (L-galactonate dehydratase deletion strain) and Δ*gaaB-gaaA* (L-galactonate dehydratase strain with overexpression of the D-galacturonate reductase *gaaA*) were described previously (Kuivanen et al. 2012). The strains Δ*gaaB* and Δ*gaaB-gaaA* are deposited in VTT Culture Collection as D-121454 and D-121455, respectively.

Preparation of the substrate

Citrus processing waste (CPW), remaining after the preparation of fresh orange juice, was obtained from a local restaurant in Curitiba (Brazil). The CPW was dried at 60°C and milled with a grinder to a particle size of 0.9-2.4 mm. The ground CPW was washed with water in order to remove soluble sugars and dried again at 60°C. Finally, the powder was autoclaved for 40 min at 120°C.

Submerged fermentations

For the submerged fermentations (SmF) with CPW, mycelia were pre-grown in 250-ml Erlenmeyer flasks in a medium containing (in g l^{-1}): yeast extract 10, peptone 20 and gelatin 30. Pre-cultures were inoculated with a spore suspension and incubated overnight at 28°C, 200 rpm. Mycelia were harvested by vacuum filtration and rinsed with sterile water. Submerged cultures contained 40 g l^{-1} of the prepared CPW, which, due to the residual water, represents 35.1 g l^{-1} on a dry mass (DM) basis and were supplemented with *A. nidulans* defined minimal medium (Barratt et al. 1965), containing (in g l^{-1}): $NaNO_3$ 6, KCl 0.52, $MgCl_2$ 0.52 and KH_2PO_4 1.52. In a separate culture, only sterile water with 40 g l^{-1} of the prepared CPW was used. Cultures were carried out in 250-ml Erlenmeyer flasks containing 50 ml final volume and were inoculated

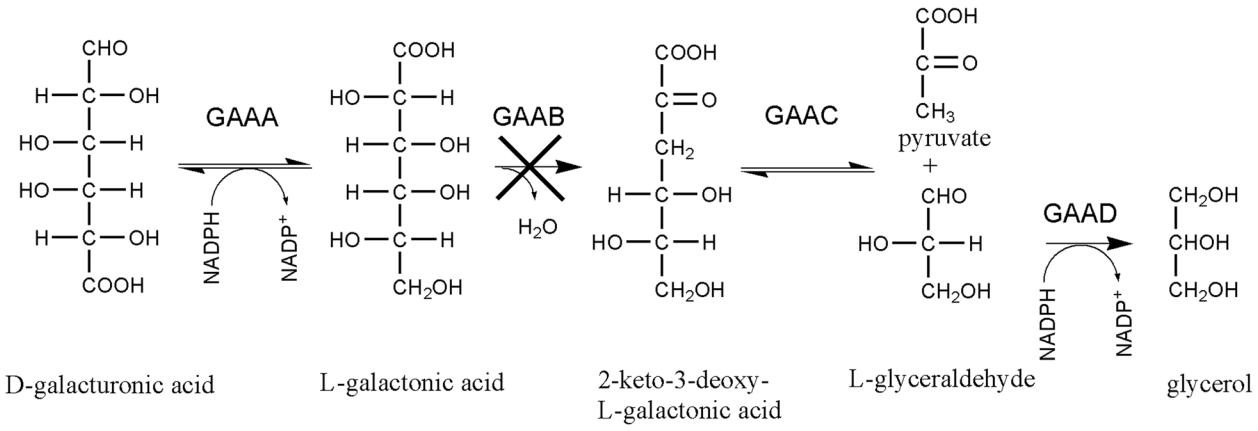

Figure 1 The catabolic D-galacturonic acid pathway in *A. niger*. The enzymes of this pathway are D-galacturonic acid reductase (GAAA), L-galactonate dehydratase (GAAB), 2-keto-3-deoxy-L-galactonate aldolase (GAAC) and L-glyceraldehyde reductase (GAAD). The strains used in the present work were engineered to have no GAAB activity.

with 10 g l^{-1} (2.3 g l^{-1} DM) of pre-grown mycelia and incubated at 28°C and at 200 rpm. Samples were taken for HPLC analysis at intervals and solid matter (mycelia + insoluble substrate) was removed by centrifugation. In the fermentation for purification of the L-galactonic acid, 40 g l^{-1} of the prepared CPW was fermented in 500 ml of water in a 2000 ml flask. The culture was inoculated and incubated as described above.

Solid-state fermentations

For the small scale solid-state fermentation (SSF) 2 g (1.76 g DM) of the CPW, prepared as described above, was added to each 100-ml Erlenmeyer flask. Sterile water (6 ml) containing inoculum of 2×10^7 spores was added. The flasks were incubated at 28°C and a relative humidity of 96%. The effect of nutritional supplementation in SSF was studied by adding either 6 ml of pure water or a solution containing (in g l^{-1}): K_2HPO_4 3, $(NH_4)_2SO_4$ 13, $MgSO_4.7H_2O$ 5, KCl 10 and $FeSO_4.7H_2O$ 0.1.

In order to investigate the potential for scaling up the process, packed-bed bioreactors (28 cm by 3.5 cm, ~270 ml) were used. For each column, 10 g (8.78 g DM) of the prepared CPW was wetted with 30 ml of the nutritional supplementation (as above) containing an inoculum (10^7 spores per g prepared CPW). Columns were incubated in a water bath maintained at 28°C. Each column received a flow rate of air (saturated with water at 28°C) of 150 ml min^{-1}.

For the extraction of the fermentation products, 15 ml of sterile water were added per 1 g (0.88 g DM) of fermented solids and the suspension was incubated at 28°C and 200 rpm for an hour. The liquid was collected by vacuum filtration and analysed by HPLC.

D-galacturonic acid content of the CPW

In order to estimate the total D-galacturonic acid content in the substrate, 1 g (0.88 g DM) of the prepared CPW was hydrolysed with 127 PGNU (polygalacturonase unit, as defined by Novozymes) per ml of a commercial pectinase (Pectinex Ultra SP-L, Novozymes) in a final volume of 30 ml. The reaction mixture was incubated overnight at 30°C and at 100 rpm. The hydrolysed orange peel suspension was centrifuged and the supernatant was analysed by HPLC. The water content in the dried CPW was determined by heating the CPW in oven at 100°C overnight and weighing to determine the loss of mass.

Transcriptional analysis

For the transcriptional analysis in the submerged fermentations, 1 ml samples were collected and the mycelia were harvested by vacuum filtration, frozen with liquid nitrogen and stored at −80°C. RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

cDNA was prepared using the First Strand cDNA Synthesis Kit (Roche) following the manufacturer's instructions. Quantitative PCR was carried out using the LightCycler SYBR green I master mix and the LightCycler 96 System. The transcription of putative D-galacturonate transporters An14g04280 and An03g01620 (Martens-Uzunova and Schaap 2008) was quantified with the primers listed previously (Kuivanen et al. 2012). The transcription levels were normalized to actin using the accompanying software (Advanced Relative Quantification Tool).

L-galactonic acid purification

After the 500-ml SmF, the broth was separated by vacuum filtration and loaded onto an ion-exchange resin

(Dowex 1X8, formate form). The bound L-galactonic acid was eluted with a linear formic acid gradient from 0 to 3 M. Fractions were collected and analysed by HPLC. The fractions with the highest L-galactonic acid content were combined and lyophilized. The resulting powder was dissolved in water and the proportions of linear L-galactonic acid and L-galactono-1,4-lactone were quantified using the colorimetric lactone assay (Hestrin 1949). In the assay, 200 μl of the dissolved product was mixed with 400 μl of solution containing 4 M hydroxylamine and 4 M NaOH, followed by acidification with 200 μl of 4 M HCl and addition of 200 μl of 100 g l^{-1} $FeCl_3$ in 0.1 M HCl. The absorbance was measured at 540 nm. Commercial L-galactono-1,4-lactone (Sigma) was used for the standard curve.

In an alternative procedure, the suitability of calcium precipitation for the purification was studied, using a method similar to that described for succinate (Li et al. 2010b). In order to remove some of the impurities and clarify the broth, filtered final broth was stirred with activated carbon (12.5% w/v) for an hour. The activated carbon was then removed by filtration and the resulting clear liquid was collected. In order to precipitate the product as calcium L-galactonate, the liquid was stirred and solid $Ca(OH)_2$ was added until the pH rose to 12.6. The resulting precipitate was collected by filtration and rinsed with cold water. L-Galactonic acid was released from the calcium salt by adding 0.5 M H_2SO_4 until pH 2.0. The precipitate ($CaSO_4$) was removed by filtration and the filtrate was analysed by HPLC.

HPLC analysis and calculations

The concentrations of sugars and sugar acids were determined by HPLC, using a fast acid analysis column (100 by 7.8 mm, Bio-Rad laboratories, Hercules, CA) linked to an Animex HPX-87H organic acid analysis column (300 by 7.8 mm, Bio-Rad Laboratories), with 5 mM H_2SO_4 as the eluent and a flow rate of 0.5 ml min^{-1}. The column was maintained at 55°C. Peaks were detected with a Waters 410 differential refractometer.

All the sugar and sugar acid contents, the product yields and production rates were calculated on the basis of the DM of CPW used. Margins of error are presented as standard errors of the means (SEM). All the statistical tests were carried out using Student's t-test. In the Table 1, the "product yield" is calculated from the highest L-galactonic acid concentration during the fermentation (Figures 2A and 3A). The "product yield of theoretical maximum" represents the ratio of the highest L-galactonic acid content (in the fermentation) to the D-galacturonic acid content in the CPW (determined as described above).

Results

In order to investigate the conversion of CPW to L-galactonic acid, we inoculated ground CPW with wild type and engineered *A. niger* strains (*ΔgaaB* and *ΔgaaB-gaaA*) and tested solid-state (SSF) and submerged (SmF) fermentations. A key value-added by-product from CPW is D-limonene, which is inhibitory for many microbes (Pourbafrani et al. 2010), including *A. niger* (Sharma and Tripathi 2008). In our process, it was not specifically extracted, although the washing of CPW possibly decreased its D-limonene content. The inhibitory effect of D-limonene was not studied in the process. After the washing, the insoluble material, containing mainly pectin, cellulose and hemicellulose, was fermented in SmF and SSF processes. The D-galacturonic acid resulting from the pectin hydrolysis was reduced to L-galactonic acid by the Δ*gaaB* and Δ*gaaB-gaaA* strains. L-Galactonic acid production was not observed in either process using the wild type strain.

Composition of CPW

The content of D-galacturonic acid in the substrate was analysed by hydrolysing the prepared CPW with pectinases. The D-galacturonic acid was quantified by HPLC. This allowed calculation of the maximal theoretical yield of L-galactonic acid that can be obtained from CPW (1 mol of L-galactonic acid per 1 mol of D-galacturonic acid). The water content in the prepared dry CPW substrate was 12.2 ± 0.4%. In the remaining matter, the content of free D-galacturonic acid was 270 ± 7 mg per g CPW (on a DM basis) after pectinase hydrolysis. The other main soluble components in the hydrolysed CPW were, in mg per g CPW, D-glucose 55 ± 1, D-galactose 62 ± 1, L-arabinose 67 ± 2 and L-rhamnose 15 ± 1. The soluble sugars and sugar acids released in the pectinase hydrolysis accounted for 46.6% of the dry mass of the CPW.

Consolidated conversion of orange peel to L-galactonic acid in submerged fermentation

In SmF, supplementation with the mineral salt solution improved both the initial productivity and the final yield of L-galactonic acid for both *ΔgaaB* and *ΔgaaB-gaaA* (Table 1 and Figure 2A). The initial productivity increased from values of 0.74-1.01 mg g^{-1} h^{-1} to around 1.2 mg g^{-1} h^{-1} and the product yield increased from values of 79–95 mg g^{-1} to around 160 mg g^{-1}. The highest L-galactonic acid titer among SmFs, 5.60 ± 0.09 g l^{-1} (after 119 h), was obtained with *ΔgaaB-gaaA* in the supplemented process (Figure 2A). The pH during the fermentation was lower in SmFs without supplementation (Figure 2B). Although low pH increased L-galactonic acid production in SmF in a previous study (Kuivanen et al. 2012), the value below 2 that was reached in the current study might have had a negative effect.

In order to investigate the transport of the substrate into the cell, the transcription of two putative D-galacturonic acid transporters, An14g04280 and An03g01620, was

Table 1 Initial productivities, product yields and product yields as a percentage of the theoretical maximum from SmFs and SSFs on a DM basis

Strain	Initial productivity $mg_{L\text{-galactonate}}/g_{peel}/h$		Product yield, $Y_{p/s}$ $mg_{L\text{-galactonate}}/g_{peel}$		Product yield (%) of theoretical maximum $g_{L\text{-galactonate}}/g_{D\text{-galacturonate}}$	
	SmF	SSF	SmF	SSF	SmF	SSF
	0-70.5 ± 1.5 h	0-96 h				
Δ*gaaB* (without suppl.)	1.01 ± 0.04[c]	0.35 ± 0.01	79 ± 5	116 ± 2[c]	23%	43%
Δ*gaaB-gaaA* (without suppl.)	0.74 ± 0.03[c]	0.49 ± 0.02	95 ± 3	167 ± 2[c]	35%	62%
Δ*gaaB* (with suppl.)	1.16 ± 0.01	[d]2.14 ± 0.09	157 ± 3	[d]233 ± 2[c]	58%	87%
Δ*gaaB-gaaA* (with suppl.)	1.26 ± 0.02	[d]2.35 ± 0.03[c]	159 ± 3	[d]221 ± 6[c]	59%	82%

[c]The process type (SmF or SFF) significantly ($p < 0.05$) better than the other in the same nutritional conditions.
[d]Errors present ± SEM, n = 2.
Errors represent ± SEM, n = 3.

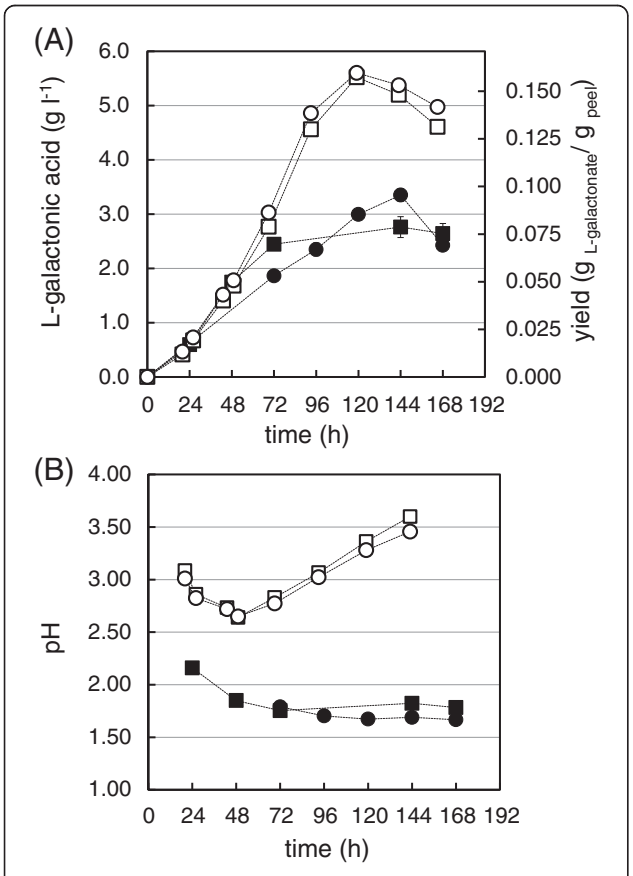

Figure 2 The engineered strains *ΔgaaB* (squares) and *ΔgaaB-gaaA* (circles) grown on CPW in SmFs with (open symbols) and without (solid symbols) nutritional supplementation. (A) L-Galactonic acid production, **(B)** the pH over the course of the fermentation. The production presented on a DM basis, error bars represents ± SEM, n = 3, if not visible then they are smaller than the symbol.

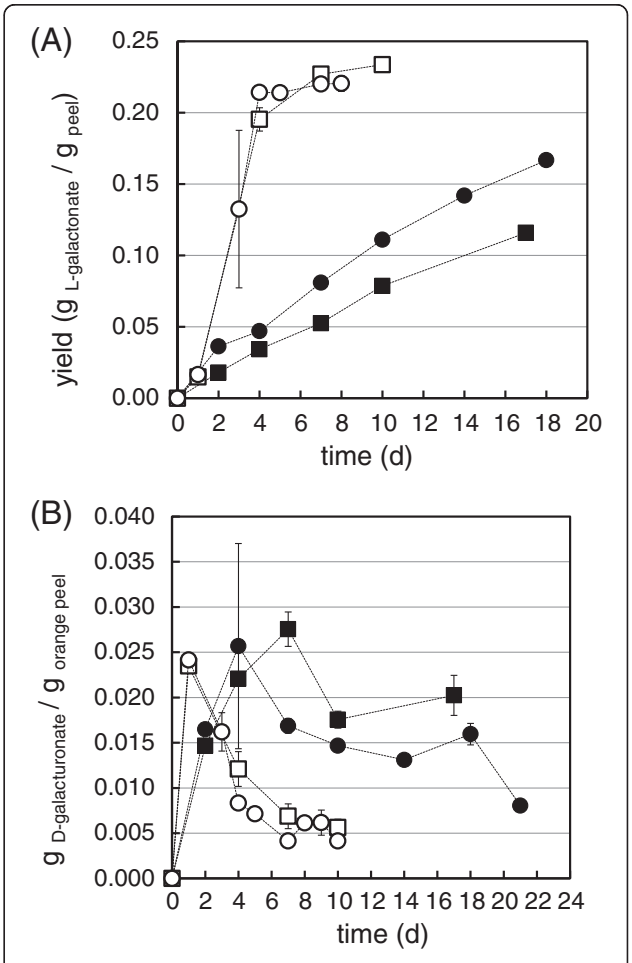

Figure 3 The engineered strains *ΔgaaB* (squares) and *ΔgaaB-gaaA* (circles) grown on CPW in SSFs with (open symbols) and without (solid symbols) nutritional supplementation. (A) L-Galactonic acid production, **(B)** the released D-galacturonic acid over the course of the fermentation. The production presented on a DM basis, error bars represents ± SEM, n = 3 without the salt supplementation and n = 2 with the salt supplementation, if not visible then they are smaller than the symbol.

followed by qPCR in the SmFs carried out with *ΔgaaB* without the nutritional supplementation (Figure 4). The transcription of An14g04280 in *ΔgaaB* did not show any induction on CPW, whereas the transcription of An03g01620 was clearly induced.

Consolidated conversion of orange peel to L-galactonic acid in solid-state fermentation

As with SmF, in SSF supplementation with the mineral salts solution improved both the initial productivity and the final yield of L-galactonic acid for both *ΔgaaB* and Δ*gaaB-gaaA* (Table 1 and Figure 3A). The initial productivity increased from values of 0.35-0.49 mg g^{-1} h^{-1} to values of 2.14-2.35 mg g^{-1} h^{-1} and the product yield increased from values of 116–167 mg g^{-1} to values 220–230 mg g^{-1}. In the SSFs, the D-galacturonic acid profiles were also determined (Figure 3B). The rates of release and consumption of D-galacturonic acid were both faster in the supplemented SSF. In addition, both of the engineered strains produced lower D-galacturonic acid concentrations during the fermentation when compared to the wild type (wild type data not shown).

In large-scale SSF processes, packed-bed bioreactors are often used since they are less labour-intensive than tray-type processes (such as that undertaken in the current work in the Erlenmeyer flasks). In order to assess the performance of the process in this type of bioreactor, glass columns were loaded with 10 g (8.78 g DM) of CPW and the nutritional supplementation was used. The product yields were slightly lower than those in flask-scale SSFs: after 4 days, the L-galactonic acid yields obtained with *ΔgaaB* and Δ*gaaB-gaaA* were 166 and 153 mg g^{-1}, respectively, corresponding to 61 and 57%, respectively, of the theoretical maximum yields.

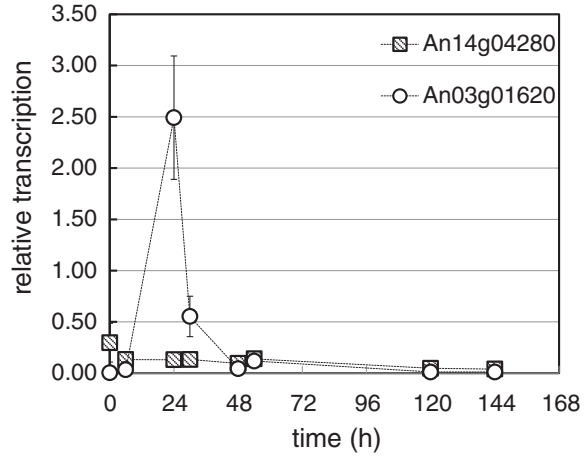

Figure 4 Transcription of the putative D-galacturonate transporters An14g04280 and An03g01620 in *ΔgaaB* strain over the course of the SmF on CPW. The transcription levels are presented as relative to the transcription of actin. Error bars represents ± SEM, n = 3, at 24 h for An03g01620 n = 2, if not visible then they are smaller than the symbol.

Purification and applications of L-galactonic acid

L-Galactonic acid produced from CPW in a 500-ml SmF was purified using a Dowex resin and lyophilisation. Seventeen percent of the L-galactonic acid in the fermentation broth was recovered. In this purified product, 69% of the L-galactonic acid was in the linear form, whereas 31% was present as L-galactono-1,4-lactone. The alternative method for L-galactonic acid purification involved treatment of the fermentation broth with activated carbon then precipitation of L-galactonic acid as a calcium carboxylate salt. After treatment of the salt with sulphuric acid, the final liquid contained 20% of the initial L-galactonic acid.

Discussion

There are several previous reports that describe processes converting pectin-rich biomass to ethanol using *Saccharomyces cerevisiae* or *Escherichia coli* (Edwards and Doran-Peterson 2012). Due to the inability of these organisms to hydrolyse pectin and cellulose, additional pectinolytic and cellulolytic enzymes are required. From that perspective, it is an advantage to use a production organism, such as *A. niger*, that is naturally capable of hydrolysing pectin and cellulose: this enables a consolidated bioprocess. In the current work, we established a new consolidated bioprocess converting CPW to L-galactonic acid.

In the present study, the volumetric titers of L-galactonic acid after 72 h in the supplemented SmFs with Δ*gaaB* and Δ*gaaB-gaaA* were around 3 g l^{-1} resulting in an approximate volumetric productivity of 42 mg l^{-1} h^{-1} (Figure 2A). This value is relatively close to the initial productivities of 46–64 and 54–70 mg l^{-1} h^{-1} that were obtained with Δ*gaaB* and Δ*gaaB-gaaA*, respectively, in SmF of a defined medium supplemented with 10 g l^{-1} of D-galacturonic acid and 2 g l^{-1} of D-xylose (Kuivanen et al. 2012). The product yields (% of theoretical maximum, i.e. g L-galactonic acid per g initial D-galacturonic acid) obtained in the defined conditions were 47 and 59% with Δ*gaaB* and Δ*gaaB-gaaA*, respectively (Kuivanen et al. 2012). The corresponding values in this study were similar, being 58 and 59% respectively (Table 1). However, the L-galactonic acid yields, 87 and 82% of the theoretical maximum, obtained in SSF with Δ*gaaB* and Δ*gaaB-gaaA*, respectively, in the current study, were clearly higher when compared to those of the SmFs (Table 1). The SSFs in packed-bed column bioreactors resulted in yields close to those of achieved in SmF. However, the longest bioreactor fermentation was extracted after 4 days, which may be too short a time for the optimal yield.

The higher product yields in the SSFs might be due to a difference in pectin hydrolysis under submerged and solid-state conditions. In fact, the presence of free sugars, such as D-glucose or sucrose, strongly decreases the

production of endo- and exo-pectinases by *A. niger* in SmF, but not in SSF, which indicates a possible difference in the regulation of pectin metabolism between submerged and solid-state conditions (Solis-Pereira et al. 1993). This is most likely due to the concentration gradients that arise, with the concentrations of soluble sugars remaining low in the vicinity of the hyphae and therefore not causing catabolite repression (Viniegra-González and Favela-Torres 2006).

Small amounts of D-galacturonic acid were still present in the SSFs after the L-galactonic acid production had slowed significantly (compare profiles in Figure 3A and B over the period of 72–216 h). In contrast, no D-galacturonic acid was found at the end of the SmFs (i.e. after 120 h, data not shown), even though the yields of L-galactonic acid were only 23–59% of the theoretical maximum values (Table 1). There are two possible explanations for the final product yields being below 100%: pectin hydrolysis may have been incomplete or some of the L-galactonic acid that was produced may have been consumed. The strain *ΔgaaB* was unable to catabolize L-galactonic acid in submerged cultures when pure D-galacturonic acid or polygalacturonate were used (Kuivanen et al. 2012). However, several additional carbon sources are present in the CPW and might induce expression of unspecific dehydratases, allowing some L-galactonic acid consumption, since the genome of *A. niger* contains at least four putative dehydratase coding genes in addition to *gaaB*. A similar kind of phenomenon was described in an engineered *A. niger* strain with a disturbed D-galacturonate pathway (a deletion of D-galacturonate reductase, *gaaA*) and an introduced uronic acid dehydrogenase: The resulting strain was able to utilize D-galacturonate through an unknown pathway (Mojzita et al. 2010).

The transcription of two putative D-galacturonate transporters An14g04280 and An03g01620 was followed during the SmF without the nutritional supplementation (Figure 4). These transporters have a strong similarity to hexose transporters and their transcription is induced in wild type *A. niger* when cultured on D-galacturonate, polygalacturonate and sugar beet pectin (Kuivanen et al. 2012, Martens-Uzunova and Schaap 2008). In the present study, only induction of An03g01620 was observed. This transcriptional pattern is similar to that obtained when *A. niger ΔgaaB* was cultivated on a mixture of pure D-galacturonic acid and D-xylose (Kuivanen et al. 2012). The reason for altered expression of An14g04280 in *ΔgaaB* remains unclear, however, it may have a negative effect on the D-galacturonic acid transport into the cell. The peak of the An03g01620 induction during growth on CPW occurred in the current work at 24 h whereas the corresponding peak on pure D-galacturonic acid was at 3 h (Kuivanen et al. 2012). The time difference is most likely due to the inhibitory effect of other sugars of the CPW on pectin hydrolysis and D-galacturonic acid catabolism at the beginning of the SmF.

In the context of L-ascorbic acid (vitamin C) synthesis, it is advantageous to know whether the product from the consolidated process is linear L-galactonic acid or L-galactono-1,4-lactone: a single reduction step is required to form L-ascorbic acid from the lactone, whereas the linear form would need to be first lactonized. The L-galactonic acid recovered from the Dowex resin was a mixture of linear and lactonized form. The lactonization of L-galactonic acid is thermodynamically more favourable at low pH, thus the elution with formic acid probably caused the partial lactonization. We also tested the suitability of calcium precipitation for the L-galactonic acid recovery because it is widely used in the industry. The purification yield remained relatively low (20%) since the process was not optimized for L-galactonic acid. However, this method, once optimized, could potentially be used for L-galactonic acid recovery.

Currently, industrial production of L-ascorbic acid begins with D-sorbitol, which is produced by the hydrogenation of D-glucose derived from corn or wheat (Bremus et al. 2006). The predominant manufacturing methods are still based on Reichstein process, which is a chemical process (Reichstein and Grüssner 1934). Current processes, that also involve fermentation steps, are still energy intensive (Bremus et al. 2006) and use valuable D-glucose as a raw material. From that perspective, a production method utilizing a single fermentation step and low value CPW as the raw material would be highly desirable.

In the present work, L-galactonic acid was produced from CPW in a consolidated process by engineered *A. niger* strains. Close to 90% product yield was achieved, based on the D-galacturonic acid contained in the pectin of the CPW. To the best of our knowledge, this is the first report on a consolidated bioprocess in which a pectin-rich residue is converted to a high value biochemical using an engineered microorganism.

Competing interests

The authors declare that they have no competing interests.

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PUBLICATION III

Categorisation of sugar acid dehydratases in *Aspergillus niger*

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Categorisation of sugar acid dehydratases in *Aspergillus niger*

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ABSTRACT

In the genome of *Aspergillus niger* five genes were identified coding for proteins with homologies to sugar acid dehydratases. The open reading frames were expressed in *Saccharomyces cerevisiae* and the activities tested with a library of sugar acids. Four genes were identified to code for proteins with activities with sugar acids: an L-galactonate dehydratase (*gaaB*), two D-galactonate dehydratases (*dgdA*, *dgdB*) and an L-rhamnonate dehydratase (*lraC*). The specificities of the proteins were characterised. The L-galactonate dehydratase had highest activity with L-fuconate, however it is unclear whether the enzyme is involved in L-fuconate catabolism. None of the proteins showed activity with galactaric acid or galactarolactone.

1. Introduction

In fungi, several sugar acid dehydratases are known that split off a water molecule from a sugar acid to generate the 2-keto-3-deoxy derivative of the sugar acid. Only in a few cases the gene sequences are known that code for the enzymes.

In filamentous fungi, an L-galactonate dehydratase was identified in *Hypocrea jecorina* (Kuorelahti et al., 2006), *Aspergillus niger* (Martens-Uzunova and Schaap, 2008) and *Botrytis cinerea* (Zhang et al., 2011). The enzymes in these species are close homologues and convert L-galactonate to 3-deoxy-L-threo-hex-2-ulosonate (2-keto-3-deoxy-L-galactonate). This enzyme is part of the eukaryotic D-galacturonic acid pathway. In the pathway, D-galacturonate is reduced to L-galactonate and 3-deoxy-L-threo-hex-2-ulosonate, the reaction product of the L-galactonate dehydratase, is split by an aldolase to pyruvate and L-glyceraldehyde. The latter is then reduced further to glycerol (Richard and Hilditch, 2009).

In the literature, also other eukaryotic pathways are described that use a sugar acid dehydratase. For example, L-rhamnose is oxidised to L-rhamnonate which is then reacting with a dehydratase. The resulting 3,6-dideoxy-L-erythro-hexulosonic acid (2-keto-3-deoxy rhamnonate) is subsequently split by and aldolase to pyruvate and L-lactaldehyde. The gene for the L-rhamnonate dehydratase was identified in *Scheffersomyces stipitis* as part of a cluster that contained all the genes coding for the enzymes for L-rhamnose catabolism (Watanabe et al., 2008; Koivistoinen et al., 2012a).

Similar to the L-rhamnose pathway is the fungal L-fucose pathway. L-Fucose is oxidised to L-fuconate which is then reacted with a dehydratase and the reaction product split by an aldolase to pyruvate and L-lactaldehyde (Guimarães and Veiga, 1990). For this pathway only the enzyme activities were described.

There are also reports that D-galactose can be catabolised by a similar pathway in filamentous fungi. Many fungal microrganisms use the Leloir pathway for D-galactose catabolism. In filamentous fungi such as *A. niger* and *H. jecorina* also an oxidoreductive pathway exist for D-galactose catabolism, which is actually the main pathway for this sugar in *A. niger* (Mojzita et al., 2012a). A third pathway for D-galactose catabolism was described in an *A. niger* strain that could be described as a non-phosphorylated De Ley–Doudoroff pathway (De Ley and Doudoroff, 1957). In this pathway D-galactose is oxidised by an NAD-utilising dehydrogenase to D-galactono-lactone which is then hydrolysed. The resulting D-galactonate is converted by a dehydratase to 3-deoxy-D-threo-hex-2-ulosonate (2-keto-3-deoxy D-galactonate) which is then split by an aldolase to pyruvate and D-glyceraldehyde (Elshafei and Abdel-Fatah, 2001). The genes coding for enzymes with such enzyme activities have not been described.

In this study we cloned all open reading frames of the homologues of sugar acid dehydrates that are present in the genome of the *Aspergillus strain* ATCC 1015 and expressed them in the yeast

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Saccharomyces cerevisiae. After the expression we analysed the dehydratase activities with a library of sugar acids.

2. Materials and methods

2.1. Cloning the open reading frames

The open reading frame for the protein with the Protein ID 191792 in the genome of the *A. niger* strain ATCC 1015 (http://genome.jgi-psf.org/) (GenBank accession number: EHA27292.1) was cloned by amplifying the 3 exons of the gene by PCR and recombining them with the yeast expression vector by homologues recombination in yeast. The exons were amplified by PCR from genomic DNA using the primers lraC_ORF_A_F/lraC_ORF_A_R, lraC_ORF_B_F/lraC_ORF_B_R and lraC_ORF_C_F/lraC_ORF_C_R (Table 1). The *S. cerevisae* strain CEN.PK2-1D was transformed with the three resulting PCR products and the EcoRI and BamHI digested plasmid p2159 according to the method of Gietz and Woods (Gietz and Woods, 2002). The plasmid p2159 was derived from the pYX212 (R&D systems) by modifying the multiple cloning site (Verho et al., 2004). The transformants were selected for uracil prototrophy. The resulting plasmid was verified by sequencing.

The gene for the Protein ID 38317 (GenBank accession number: EHA18083.1) had no intron and was amplified by PCR with the primers sodA_ORF_F and sodA_ORF_R (Table 1). The PCR product was digested with EcoRI and NheI and ligated to the corresponding sites of p2159.

The two exons of the gene for the Protein ID 49896 (GenBank accession number: EHA19069.1) were amplified by PCR using the primer pairs dgdA_ORF_A_F/dgdA_ORF_A_R and dgdA_ORF_B_F/dgdA_ORF_B_R. The yeast expression vector was then made by homologous recombination in yeast with the XmaI/NheI digested p2159.

The gene for the Protein ID 50500 (GenBank accession number: EHA20544.1) consisted of 4 exons that were amplified with the primers dgdB_ORF_A_F, dgdB_ORF_A_R, dgdB_ORF_B_F, dgdB_ORF_B_R, dgdB_ORF_C_F, dgdB_ORF_C_R, dgdB_ORF_D_F, dgdB_ORF_D_R. The four resulting fragments and the EcoRI and NheI digested pYX212 were ligated through homologous recombination in yeast.

The gene for the Protein ID 53563 (GenBank accession number: EHA22098.1) was amplified by PCR from cDNA of *A. niger* grown on D-galacturonic acid using primers gaaB_ORF_F and gaaB_ORF_R (Table 1). The PCR product was digested with XmaI and BamHI and ligated to the corresponding sites of p2159. All constructs were verified by sequencing.

The expression vectors with the different open reading frames were then used to transform the yeast strain CEN.PK2-1D (European *S. cerevisiae* Archive for Functional Analysis) and the resulting strains cultivated in selective glucose medium in the absence of uracil.

2.2. Cell extract and activity assay

Overnight cultures in 50 ml of synthetic complete medium with 2% D-glucose lacking uracil were harvested by centrifugation, washed once and resuspended in 1 ml of 10 mM phosphate buffer. To make a cell extract the resuspended cells were added to 2 ml vials containing approximately 1 ml of glass beads (0.5 mm diameter Sigma) and 80 μl of protease inhibitors (Complete, Roche). The cells in the vials were disrupted in two rounds of three cycles of 30 s each in the Precellys 24 machine (Bertin Technologies). The cell extracts were centrifuged for 20 min, at +4 °C in 14,000 rpm in a table-top centrifuge and the supernatants were used for the enzyme activity assays. The protein concentration was estimated using the Protein Assay kit (BioRad) using BSA as standard. For the dehydratase activity the cell extract was added to medium containing 10 mM of a sugar acid from our sugar acid library and 20 mM sodium phosphate buffer pH 7.0. The sugar acid library contained the following sugar acids salts: L-rhamnonate, L-galactonate, D-gulonate, D-gluconate, L-gulonate, D-arabonate, D-lyxonate, L-lyxonate, D-xylonate, meso-galactarate (muconate), L-fuconate, D-mannonate, L-mannonate, D-ribonate, L-arabonate, D-galactonate, D-galacturonate, D-glucuronate, galactarolactone and glucarolactone. Galactarolactone and glucarolactone were produced from D-galacturonic acid and D-glucuronic acid respectively using the purified uronate dehydrogenase (*udh*) from *Agrobacterium tumefaciens* and NAD as described previously (Boer et al., 2010). The reaction was stopped by mixing 45 μl of this mixture with 5 μl of 12% (w/v) trichloroacetic acid (TCA). The production of 2-keto-3-deoxy sugar was measured with the thiobarbituric acid assay as described by

Table 1
List of the primers used to amplify all the gene sequences of the characterised proteins: gaaB (GenBank: EHA22098.1), dgdA (GenBank: EHA19069.1), (dgdB GenBank: EHA20544.1), sodA (GenBank: EHA18083.1) and lraC (GenBank: EHA27292.1).

Primer	Sequence
gaaB_ORF_F	TCCCCCCGGGACCATGGTCAAGATCACAAGT
gaaB_ORF_R	TTAGGATCCTTTACCTCCCCACTATACTATC
lraC_ORF_A_F	TAAATCTATAACTACAAAAAACACATACAGGAATTAAAATGGGTTCTACCGGACAATT
lraC_ORF_A_R	ATGTTACTGTCAATCAACCAGTGACCTCCTTTGACATTATGATAATCACCTCCGG
lraC_ORF_B_F	TTGATGGTGTGGGATCCGGAGGTGATTATCATAATGTCAAAGGAGGTCACTGGTT
lraC_ORF_B_R	CATCTTCTCGAACAGGTCATTGGTATCTCTGGGGTCAGCACCAAGCAGGAAGCGC
lraC_ORF_C_F	CCATCAACACTTGAGCGCTTCCTGCTTGGTGCTGACCCCAGAGATACCAATGAC
lraC_ORF_C_R	TTCAGTTAGCTAGCTGAGCTCGACTCTAGAGGATCTTAAGTAGACTCCTGCTGGTTCT
dgdB_ORF_A_F	TAAATCTATAACTACAAAAAACACATACAGGAATTAAAATGGCTCCCATCAAGTCCAT
dgdB_ORF_A_R	GTCTCCAAATTGTCTGCCAGACATGCTCGATGTCATCAGCCTCATAACCAACAAT
dgdB_ORF_B_F	GAGATTATTGGGAGGATTGTTGGTTATGAGGCTGATGACATCGAGCATGTCTGGC
dgdB_ORF_B_R	TAACGCACTTGAGGCCCTGCGCGATACGGGCCTTGGCAGCTACCTCAACATCACT
dgdB_ORF_C_F	GGAGGTGACCGGCCAAGTGATGTTGAGGTAGCTGCCAAGGCCCGTATCGCGCAGG
dgdB_ORF_C_R	AGCAACTTGCATTGAAGCTGCAAGGGCAATTGGACCGAGGGGACAGTGCGGCGCA
dgdB_ORF_D_F	GTATGACGTGGCGATTGCGCCGCACTGTCCCCTCGGTCCAATTGCCCTTGCAGCT
dgdB_ORF_D_R	TTCAGTTAGCTAGCTGAGCTCGACTCTAGAGGATCTTACTACCACTCTCGGATTCCCC
sodA_ORF_F	TTACCTGAATTCAAAATGACCGAACTAAGAATCAC
sodA_ORF_R	TCCACCGCTAGCTCAATCATAACTAGCAACCG
dgdA_ORF_A_F	ACATACAGGAATTCGAGCTCGGTACCCGGGATCATGGTCAAGATCAAGTCTATCG
dgdA_ORF_A_R	CAGGCCATTTGCCATATGTGCTCAATGTCATCTGCTTCGTAGCCTTGAAATC
dgdA_ORF_B_F	TGACATTGAGCACATATGGC
dgdA_ORF_B_R	CGTTCATTGTTCCTTATTCAGTTAGCTAGCCTACCACTCCCGGATCCCTC

Buchanan et al. (1999). For each sugar acid a control was made with a yeast strain with the same expression vector but not expressing any additional gene. With none of the sugar acids any activity to form a 2-keto-3-deoxy sugar acid was detected. The sugar acids that were not commercially available were prepared from the corresponding sugars as described previously (Yew et al., 2006). Care was taken that in the thiobarbituric acid assay the sugar acid concentrations did not exceed 10 mM. For that reason the reaction mixture was diluted prior the thiobarbituric acid assay for the estimation of the Michaelis–Menten constants.

3. Results

Protein ID 191792: The protein from the *A. niger* strain ATCC1015 (LraC) with the Protein ID 191792 was expressed in *S. cerevisiae* and the enzyme activities tested with crude cell extracts and the sugar acids as detailed in Table 2. High activity was observed with L-rhamnonate and some with L-mannonate. L-Rhamnonate and L-mannonate have a similar chemical structure (Fig. 1); the only difference between them is at the C6 where the L-rhamnonate has no hydroxy group. The Lra3 of *S. stipitis* is a homologue enzyme. Also with this enzyme, activities with L-rhamnonate and L-mannonate were observed (Koivistoinen et al., 2012a). The Michaelis–Menten constants were estimated with L-rhamnonate as substrate (Vmax = 0.095 nkat per mg of extracted protein and Km = 2.99 mM, Fig. 2).

Protein ID 38317: The protein with the ID 38317 (SodA) was expressed in yeast *S. cerevisiae* and the activity tested with our library of sugars. With none of the sugars we tested dehydrates activity was observed with the crude cell extract (Table 2).

Protein ID 49896: The protein with the ID 49896 (DgdA) was expressed in *S. cerevisae* and its crude cell extract was tested as described above (Table 2). It showed a high activity with D-galactonate as substrate. Michaelis–Menten constants were estimated with D-galactonate as substrate. The Michaelis Menten constants are Vmax = 8.52 nkat per mg of extracted protein and Km = 8.9 mM (Fig. 3).

Protein ID 50500: The protein with the ID 50500 (DgdB) was expressed in *S. cerevisiae* and tested as described above (Table 2).

Table 2
Dehydratase activities of the five sugar acid dehydratases after expression in *S. cerevisiae* with different sugar acids. The activities are given in nkat/mg of extracted protein. The detection limit was estimated 0.01. The (–) indicates that no activity was observed or that the activity was close to the detection limit.

Substrate	gaaB	lraC	dgdA	dgdB	sodA
L-Rhamnonate	–	0.07	–	–	–
L-Galactonate	0.05	–	–	–	–
D-Gulonate	–	–	–	–	–
D-Gluconate	0.03	–	–	–	–
L-Gulonate	–	–	–	–	–
D-Arabonate	–	–	–	–	–
D-Lyxonate	–	–	–	–	–
L-Lyxonate	–	–	–	–	–
D-Xylonate	–	–	–	–	–
Mucic acid	–	–	–	–	–
L-Fuconate	0.52	–	–	–	–
D-Mannonate	0.02	–	–	–	–
L-Mannonate	0.02	0.05	–	–	–
D-Riborate	–	–	–	–	–
L-Arabonate	–	–	–	–	–
D-Galactonate	–	–	0.82	0.23	–
D-Galacturonate	–	–	–	–	–
D-Glucuronate	0.02	–	–	–	–

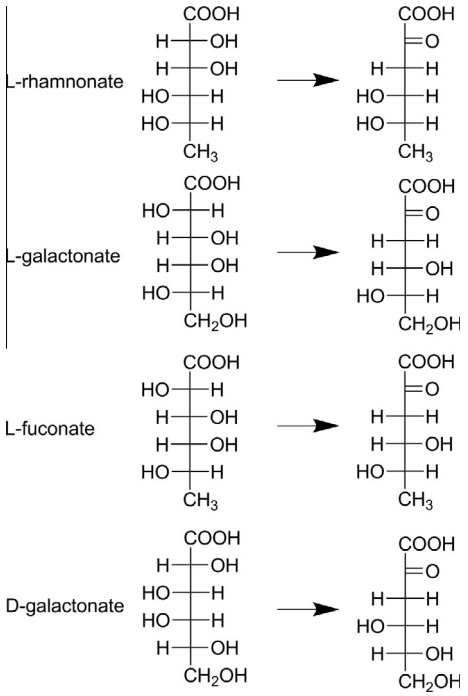

Fig. 1. Fischer projection of the sugar acids that show activity with the dehydratases present in *A. niger* and their reaction products. The LraC shows activity with L-rhamnonate and L-mannonate, the GaaB activity with L-galactonate and L-fuconate and the DgdA and DgdB have activity with D-galactonate.

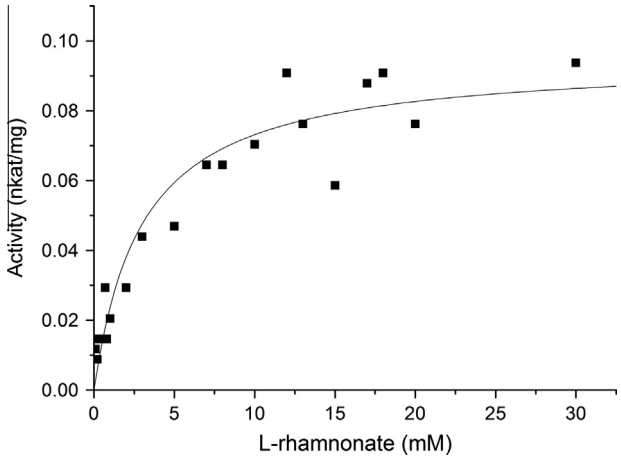

Fig. 2. Kinetic properties of the protein with the Protein ID 191792 (LraC), in a crude cell extract, using L-rhamnonate as substrate. The curve was calculated assuming Michaelis–Menten kinetics and using the constants Vmax = 0.095 nkat/mg and Km = 3 mM.

We also tested the DgdB with 6 histidines added at the N-terminal end. This his-tagged protein however did not show activity with any sugar acid. The non-tagged protein showed high activity with D-galactonate suggesting that it is a D-galactonate dehydratase. For the Michaelis–Menten constants we estimated a Vmax of 2.21 nkat per mg of extracted protein and a Km of 21.74 mM (Fig. 3).

Protein ID 53563: The protein with the ID 53563 (GaaB) was expressed in *S. cerevisiae* and the crude cell extract was tested against the sugar acids (Table 2). It showed a weak activity with several sugars: D-gluconate, D- and L-mannonate, and D-glucuronate. Considerable activity was observed with L-galactonate and an even higher activity was observed with L-fuconate. Both sugars have the

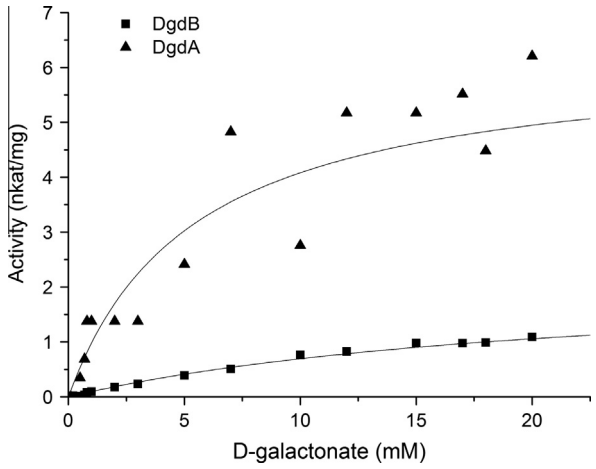

Fig. 3. Comparison of kinetic properties between the protein with the Protein ID 49896 (DgdA) and with the Protein ID 50500 (DgdB) using D-galactonate as substrate. The Michaelis–Menten curve for DgdA was calculated using Vmax = 8.52 nkat/mg and a Km = 8.9 mM. The Michaelis–Menten curve for DgdB was calculated with Vmax = 2.21 nkat/mg and a Km = 21.74 mM.

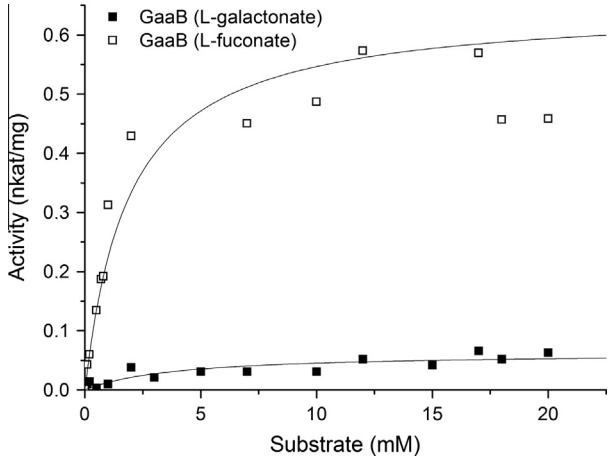

Fig. 4. Comparison of kinetic properties of the protein with the ID 53563 (GaaB) in crude cell extracts, using either L-galactonate or L-fuconate as substrate. The Michaelis–Menten curve with L-galactonate was calculated using the constants Vmax = 0.063 nkat/mg and Km = 3.92 mM as with L-fuconate, using the constants Vmax = 0.65 nkat/mg and Km = 1.89 mM.

same configuration except that L-fuconate is lacking the hydroxyl group at the C6 (Fig. 1). We tested the activities with these two sugars in order to estimate the Michaelis–Menten constants. The constants were estimated as Vmax = 0.65 nkat per mg of extracted protein and Km = 1.89 mM, for L-fuconate; for L-galactonate, Vmax = 0.063 nkat per mg of extracted protein and Km = 3.92 mM (Fig. 4). All proteins were also tested for activity with galactarolactone and glucarolactone that was produced from D-galacturonate and D-glucuronate immediately before use using an uronate dehydrogenase. None of the proteins showed activity with these lactones.

4. Discussion

The *A. niger* strain ATCC 1015 is a wild type strain that was used in the first patented citric acid process in the beginning of the last century. It has a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocellulose and pectin and can utilise a large variety of carbon sources. It is also one of the *A. niger* strains where the genome sequence is available which made it suitable for this study.

The genome of the *A. niger* strain ATCC 1015 contains five genes that code for proteins with homology to sugar acid dehydratases. The open reading frames of these genes were expressed in the yeast *S. cerevisiae* from a multi copy vector with a strong promoter. The yeast cells were then lysed and the crude cell extract tested for dehydratase activities with about 20 different sugar acids. We did not purify the enzymes. For purification enzymes are often tagged with an affinity tag such a histidine tag. For the eukaryotic dehydratases this tagging had however a detrimental effect on the enzyme activities. In the case of the *S. stipitis* L-rhamnonate dehydratase the enzyme was inactivated (Koivistoinen et al., 2012a) and in the case of the *H. jecorina* L-galactonate dehydratase the enzyme activity was lost when C-terminally tagged and activity was reduced when the enzyme was tagged N-terminally (Kuorelahti et al., 2006). To have comparable results we expressed the open reading frames of the different dehydratases from the same yeast expression vector and normalised the activity to the total protein of the extract. The dehydratases are producing a 2-keto-3-deoxy sugar acid that can be detected with the thiobarbituric acid assay which is essentially detecting deoxy sugars. Without the overexpression of any dehydratase no activity with any sugar acid could be detected indicating that this expression system is suitable for testing this class of enzymes.

Of the five proteins, the protein with the ID 191792 (LraC) had the highest homology with the L-rhamnonate dehydratase Lra3 from *S. stipitis* (Watanabe et al., 2008). The corresponding gene is also part of a cluster that was previously identified as a potential cluster of genes of the L-rhamnose catabolic pathway (Koivistoinen et al., 2012a). The protein had indeed L-rhamnonate dehydratase activity after expression in *S. cerevisiae*. Similar to the *S. stipitis* protein it showed also activity with L-mannonate (Koivistoinen et al., 2012a). Also a procaryotic L-rhamnose dehydratase was described previously. This enzyme had, besides the activity with L-rhamnonate, activity with L-mannonate but also with L-lyxonate and D-gulonate (Rakus et al., 2008).

The protein with the ID53563 (GaaB) is a homologue to the L-galactonate dehydratase that was previously identified in *H. jecorina* (Lgd1) and in the *A. niger* strain CBS 120.49 (ATCC 9029) (GaaB) (Kuorelahti et al., 2006; Martens-Uzunova and Schaap, 2008). The enzyme that was studied here (*A. niger* strain ATCC1015) is also an L-galactonate dehydratase similar to the previously characterised proteins and has a similar Km value. The protein showed also activity with L-fuconate. L-Galactonate and L-fuconate have a similar chemical structure; the only difference is that L-fuconate has no hydroxyl group at the C6 (Fig. 1). The activity with L-fuconate was higher than with L-galactonate and the affinities were similar (Km L-galactonate = 3.92 mM/L-fuconate = 1.89 mM; Fig. 4). This could be an indication that this enzyme is not only functional in the D-galacturonic acid pathway

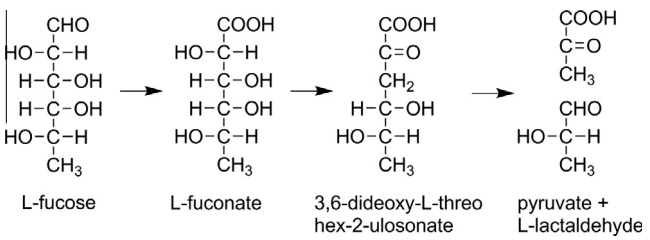

Fig. 5. The eukaryotic L-fucose pathway as suggested by Guimarães and Veiga. L-Fucose is oxidised to L-fuconate in an NAD requiring reaction with L-fuconolactone as an intermediate. L-Fuconate is then converted by the action of a dehydratase and an aldolase to pyruvate and L-lactaldehyde.

but also in the L-fucose pathway. An eukaryotic L-fucose pathway with L-fuconate as an intermediate was described at the level of enzyme activities (Fig. 5) (Guimarães and Veiga, 1990), however no genes were related to this pathway. In order to see if such an L-fucose pathway exists in this *A. niger* strain we tested for growth on L-fucose but did not observe growth in liquid medium. However L-fucose disappeared from the medium. To test whether the disappearance of L-fucose is due to the GaaB activity we tested growth and disappearance of L-fucose from the medium with a strain that had a deletion in the gaaB (*A. niger* ATCC 1015 Δ*gaaB* (Kuivanen et al., 2012)). Also this strain did not grow on L-fucose but L-fucose disappeared from the medium. Since it was not clear whether this strain is catabolising L-fucose it cannot be decided whether this enzyme is functional also in the L-fucose pathway. The next enzyme in the L-fucose pathway would be an aldolase to split the 2-keto-3-deoxy-L-fucose to pyruvate and L-lactaldehyde (Fig. 5). The aldolase from the D-galacturonic acid pathway might catalyse also this reaction. The aldolase from *H. jecorina* D-galacturonic acid pathway, Lga1, a close homologue of the *A. niger* GaaC, was unspecific except for the substrate configuration at the C4 (Hilditch et al., 2007). All this could suggest that the L-galactonate dehydratase or a close homologue might be functional in the L-fucose pathway in related species but in this strain the L-fucose pathway is not active.

The proteins with the ID 49896 and 50500 showed activity with D-galactonate. These D-galactonate dehydratases could be part of an oxidative pathway for D-galactonate catabolism that was previously decribed in an *A. niger* strain (Elshafei and Abdel-Fatah, 2001). In this pathway D-galactose is oxidised by an NAD dependent dehydrogenase to D-galactono-lactone which is either spontaneously or with the aid of a lactonase hydrolysed to the linear D-galactonate. A D-galactonate dehydratase produces then 2-keto-3-deoxy D-galactonate which is then split by an aldolase to pyruvate and D-glyceraldehyde. These enzyme activities have been reported but so far the corresponding genes were elusive. It is also not clear whether this pathway is active in all *A. niger* strains. In the *A. niger* strain ATCC 1015 D-galactose is predominantly catabolised through the oxido-reductive pathway that is similar to the L-arabinose pathway (Seiboth and Metz, 2011). Deletions of the genes of this pathway such as the *ladB* (Mojzita et al., 2012b), *xhrA* (Mojzita et al., 2012a) or *sdhA* (Koivistoinen et al., 2012b) resulted in reduced growth on D-galactose. It might be that the oxidative D-galactose pathway with D-galactonate as intermediate is only active in selected strains or that it is induced only in unusual conditions. This pathway would also require an aldolase splitting the 2-keto-3-deoxy-D-galactonate to pyruvate and D-glyceraldehyde. The aldolase from the D-galacturonic acid pathway is not suitable for this. At least the enzyme from *H. jecorina* is not active with 2-keto-3-deoxy-D-galactonate (Hilditch et al., 2007).

With the protein with the ID38317 (SodA), we did not see any activity with any of the sugar acids tested. This suggests that this enzyme is either not a sugar acid dehydratase or our library did not contain the substrate for this enzyme. It is also possible that the heterologous expression did not result in an active protein. Rakus et al. obtained a crystal structure from the L-rhamnonate dehydratase from *Salmonella typhimurium* and suggested a reaction mechanism for the dehydratation. In this mechanism the His329 which is hydrogen bonded to the Asp302 abstracts a proton from the C2 to generate the enoldiolate intermediate (Rakus et al., 2008). The corresponding amino acid sequences of the *A. niger* enzymes are in Fig. 6. While all proteins with sugar acid dehydratase activity have histidine and aspartic acid in the corresponding positions, the SodA does not. This could be interpreted that this protein is not a sugar acid dehydratase.

It was previously observed that when the gene for the D-galacturonic acid reductase *gaaA* was deleted, growth on D-galacturonic acid stopped. However when in addition to the *gaaA* deletion a

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                *                                *
DgdA      SVDILQPDIAHCGGISELRRIASMAETYDVAIAPHCPLGS
DgdB      SVDVLQPDIAHAGGISETKRIATMAETYDVAIAPHCPLGP
LraC      NLDILQPDVMWVGGMTELLKVSALASAYDLPVVPHASGPY
GaaB      ALTVLQADACRVGGVNEVLAILLLARKFGVPIVPHSGGVG
SodA      AVDGIGLKISKTGGLTRGRRVRDICLAAGYTMSVQDTSGS
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Fig. 6. Alignment of proteins sequences of the *A. niger* proteins with homologies to sugar acid dehydratases. The asterisks at D256 and H283 of the DgdA indicate the amino acids that are in the catalytic moiety functioning as the base that is abstracting the proton from the C2 during the dehydratation. The SodA is lacking the aspartic acid and the histidine in the equivalent positions.

D-galacturonic acid dehydrogenase was expressed, the resulting strain was growing again on D-galacturonic acid (Mojzita et al., 2010). This would suggest that *meso*-galactaric acid (mucic acid) or the galactaro-1,4-lactone that is formed by the dehydrogenase is catabolised. The only pathway that is described for galactaric acid catabolism involves a dehydratase or in the case of galactaro-1,4-lactone an galactarolactone cycloisomerase (Andberg et al., 2012) to produce 2-keto-3-deoxy galactarate. The cycloisomerase is from the same protein family as the sugar acid dehydratases. Since the genome of *A. niger* has no homologue of the bacterial galactarate dehydratase and galactarate has a similar structure as D- or L-galactonate, we tested the dehydratases for activity with galactaric acid galactaro-1,4-lactone, however no activity was detected with any of the enzymes. This suggests that there is either another pathway for galactarate or proteins from another protein family are active.

5. Conclusions

A. niger has one gene for a L-rhamnonate dehydratase, *lraC*, that is part of the pathway for L-rhamnose catabolism and one gene for L-galactonate dehydratase, *gaaB*, that is part of the pathway for D-galacturonic acid catabolism. The LraC hac also activity with L-mannonate. The GaaB has a higher activity with L-fuconate than with L-galactonate, however it is unclear whether this gene is involved in L-fucose catabolism. *A. niger* has also two genes for D-galactonate dehydratase, *dgdA* and *dgdB*. An oxidative pathway for D-galactose catabolism with D-galactonate dehydratase as a part of the pathway had been suggested however the remaining genes of this pathway have not been identified.

Acknowledgments

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PUBLICATION IV

The *yjjN* of *E. coli* codes for an L-galactonate dehydrogenase and can be used for quantification of L-galactonate and L-gulonate

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PUBLICATION V

Metabolic engineering of the fungal D-galacturonate pathway for L-ascorbic acid production

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Metabolic engineering of the fungal D-galacturonate pathway for L-ascorbic acid production

Joosu Kuivanen*, Merja Penttilä and Peter Richard

Abstract

Background: Synthetic L-ascorbic acid (vitamin C) is widely used as a preservative and nutrient in food and pharmaceutical industries. In the current production method, D-glucose is converted to L-ascorbic acid via several biochemical and chemical steps. The main source of L-ascorbic acid in human nutrition is plants. Several alternative metabolic pathways for L-ascorbic acid biosynthesis are known in plants. In one of them, D-galacturonic acid is the precursor. D-Galacturonic acid is also the main monomer in pectin, a plant cell wall polysaccharide. Pectin is abundant in biomass and is readily available from several waste streams from fruit and sugar processing industries.

Results: In the present work, we engineered the filamentous fungus *Aspergillus niger* for the conversion of D-galacturonic acid to L-ascorbic acid. In the generated pathway, the native D-galacturonate reductase activity was utilized while the gene coding for the second enzyme in the fungal D-galacturonic acid pathway, an L-galactonate consuming dehydratase, was deleted. Two heterologous genes coding for enzymes from the plant L-ascorbic acid pathway – L-galactono-1,4-lactone lactonase from *Euglena gracilis* (*EgALase*) and L-galactono-1,4-lactone dehydrogenase from *Malpighia glabra* (*MgGALDH*) – were introduced into the *A. niger* strain. Alternatively, an unspecific L-gulono-1,4-lactone lactonase (*smp30*) from the animal L-ascorbic acid pathway was introduced in the fungal strain instead of the plant L-galactono-1,4-lactone lactonase. In addition, a strain with the production pathway inducible with D-galacturonic acid was generated by using a bidirectional and D-galacturonic acid inducible promoter from the fungus. Even though, the lactonase enzyme activity was not observed in the resulting strains, they were capable of producing L-ascorbic acid from pure D-galacturonic acid or pectin-rich biomass in a consolidated bioprocess. Product titers up to 170 mg/l were achieved.

Conclusions: In the current study, an L-ascorbic acid pathway using D-galacturonic acid as a precursor was introduced to a microorganism for the first time. This is also the first report on an engineered filamentous fungus for L-ascorbic acid production and a proof-of-concept of consolidated bioprocess for the production.

Keywords: L-ascorbic acid, D-galacturonic acid, L-galactonic acid, Citrus peel, *Aspergillus niger*, Metabolic engineering

Background

L-Ascorbic acid (L-AA), also known as vitamin C, is a six-carbon organic compound with reducing agent properties. It occurs naturally in many animal and plant cells having biological functions, such as being an antioxidant and enzyme cofactor [1,2]. Synthetic L-AA is commercially used for several purposes in food, beverage, feed and pharmaceutical industries. The annual production of synthetic L-AA is about 110 000 tonnes with the fluctuating market price of about 10 USD per kg [3].

Industrial L-AA production has been traditionally based on the Reichstein process which is an efficient multi-step and mostly chemical manufacturing method converting D-glucose to L-AA [4]. In the first step, D-glucose is hydrogenated to D-sorbitol followed by the oxidation of D-sorbitol to L-sorbose that is commonly carried out using a bacterial fermentation [3]. The resulting L-sorbose is then chemically oxidized to 2-keto-L-gulonate and lactonized

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to L-AA. Other proposed and reported biotechnological steps have focused on the microbial conversion of D-sorbitol, L-sorbose or D-glucose to 2-keto-L-gulonic acid that is the last intermediate in the Reichstein process [5]. Currently, the predominant industrial process for L-AA production is a so called two-step fermentation process [3]. In the first step, D-sorbitol is oxidized to L-sorbose using *Gluconobacter oxydans*. The second step is a mixed fermentation with *Ketogulonicigenium vulgare* and *Bacillus megaterium*, converting L-sorbose to 2-keto-L-gulonic acid which is then chemically converted to L-AA.

In addition to the partially biotechnological processes, some approaches using wild type or engineered microbes for the direct L-AA production have been reported in the literature. A one-step process from biomass sugar to L-AA would be advantageous when compared to the currently used two-step fermentation process. Yeast have a native biosynthetic pathway for D-erythroascorbic acid (D-EA), a 5-carbon analogue of L-AA, where D-arabinose is converted to D-EA via D-arabino-1,4-lactone [6]. The D-EA pathway seems to be relatively unspecific converting also L-galactose and L-galactono-1,4-lactone (L-galL) to L-AA [7,8]. The reactions are similar to the reactions of L-AA synthesis in plants via the Smirnoff-Wheeler (S-W) pathway. In addition to yeast, a strain of *K. vulgare*, the bacterium that is used in the two-step fermentation process, is capable of converting D-sorbitol, L-sorbose, L-sorbosone and L-gulose directly to L-AA [9]. Also several strains of algae producing small concentrations of extracellular L-AA have been reported in the literature [10].

In the field of metabolic engineering, there are a few attempts to introduce the plant S-W pathway to yeast either by overexpressing the native substrate-unspecific D-EA pathway genes or expressing the S-W pathway genes from *Arabidopsis thaliana*. The resulting strains were capable of converting L-galactose, D-glucose and D-galactose to L-AA [11-13]. The product titers were reported to be about 100 mg l^{-1} from L-galactose [11], 0.1 mg l^{-1} OD^{-1} from D-glucose [12] and 30 mg l^{-1} from D-galactose [13].

In plants, L-AA is considered to be synthesized predominantly through the S-W pathway where D-glucose is converted via GPD-D-mannose and L-galactose to L-galL which is oxidized to L-AA in the final step by the L-galL dehydrogenase (GALDH) [14]. In addition to S-W, a few alternative pathways for L-AA synthesis have been observed in plants. One of them originates from D-galacturonic acid (D-galUA) which is reduced to L-galactonic acid (L-galA) and further lactonized to L-galL, which is the last intermediate in the S-W pathway [1]. Even though the function of a D-galUA reductase for L-AA biosynthesis was shown in higher plants [15], the evidence of the pathway converting D-galUA to L-AA is still incomplete – the gene coding for an L-galL aldonolactonase (ALase) catalysing the lactonization of L-galA to L-galL has been described only in the photosynthetic organism *Euglina gracilis* [16]. This activity was however found in animals as part of the biosynthetic L-AA pathway. In the animal pathway, L-gulonic acid is lactonized to its corresponding 1,4-lactone by an ALase that is encoded by the *smp30* gene. The enzyme is unspecific and can also catalyse the lactonization of L-galA [17].

In the present study, instead of using the plant S-W pathway for L-AA synthesis, we have focused on the alternative pathway converting D-galUA to L-AA. D-GalUA is the main monomer in pectin, a heteropolysaccharide found from plant cell walls, which is especially abundant in some non-woody biomass types, such as citrus fruit peels and sugar beet pulp. Both of these materials are abundantly available as agro-industrial side streams. The enzymes for degradation of pectin to D-galUA the catabolic pathways for its utilization are known in many microbial organisms. In fungi, such as *Aspergillus niger*, the pathway for D-galUA catabolism is reductive having a similar reduction step from D-galUA to L-galA as in the L-AA synthesis pathway originating from D-galUA in plants (Figure 1) [18]. Previously, we reported engineered *A. niger* strains capable of converting pure D-galUA and citrus processing waste (CPW) to L-galA that can be chemically converted to L-AA [19,20]. In the present study, we have engineered *A. niger* strains that are capable of direct production of L-AA from D-galUA and CPW.

Results

Pathway assembly and transcription

The fungal catabolic D-galUA pathway and the proposed D-galUA originating biosynthetic L-AA pathway in plants share the same reduction step yielding L-galA in the first reaction (Figure 1). Thus, the *A. niger* strain Δ*gaaB* with the disrupted L-galA dehydratase gene, which is known to accumulate L-galA, was used as a platform strain. In addition, two heterologous genes from the plant or animal L-AA pathway, an ALase and a GALDH, were introduced to the strain. Several different *A. niger* Δ*gaaB* based strains with an ALase (*EgALase* or *smp30*) and a GALDH (*MgGALDH*) or only the GALDH were generated and tested (Table 1). The *MgGALDH* gene that was used contained its native mitochondrial signal peptide region at the beginning of the ORF. In addition, a constitutive (*PgpdA*) and a D-galUA inducible (*PgaaA/C*) promoter were compared.

To avoid unnecessary and energy wasting expression of the L-AA pathway genes in non-producing conditions, the strain Δ*gaaB-Eg-Mg* (*PgaaA/C*) with D-galUA inducible *EgALase* and *MgGALDH* expression was generated using the bidirectional *gaaA/C* promoter. Since there are no reports on the use of *gaaA/C* promoter in heterologous expression, the functionality of the D-

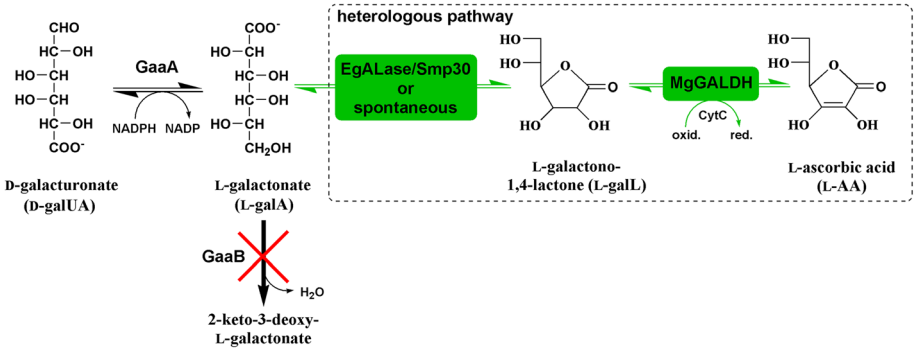

Figure 1 Schematic figure of the disrupted fungal D-galUA pathway and the heterologous pathway introduced in *A. niger* for L-AA biosynthesis: native GaaA (D-galUA reductase), native GaaB (L-galA dehydratase), EgALase (L-galL aldonolactonase from *Euglena gracilis*), Smp30 (aldonolactonase from rat functioning in the animal L-AA pathway) and MgGALDH (L-galL dehydrogenase from Malpighia glabra).

galUA inducible transcription of *EgALase* and *MgGALDH* from the *gaaA/C* promoter was tested using RT-qPCR (Figure 2). The transcription of both of the heterologous genes was clearly induced at 20 hours after the shift to D-galUA containing medium. The pattern of *MgGALDH* transcription was similar to the transcription of the native *gaaA* gene, which was expected since *MgGALDH* was orientated in the same way behind the bidirectional *gaaA/C* promoter as the *gaaA* gene is. The transcription of *EgALase* was higher when compared to *gaaA* and *MgGALDH* after the D-galUA induction. A similar observation was reported earlier in the case of native *gaaC* gene (2-keto-3-deoxy-L-galactonate aldolase) which had a higher transcription when compared to *gaaA* growing in D-galUA medium [21]. *EgALase* had the same orientation at *gaaA/C* promoter as *gaaC* gene in the wild type strain.

Enzyme activities

Enzyme activities of the introduced L-AA pathway genes were measured from crude extracts. The GALDH activity assay was based on the measurement of reduced cytochrome C as a result from oxidation of L-galL. The activity was tested from the parental strain Δ*gaaB* and from the further engineered strains that were cultured 20 h in the medium supplemented with 20 g l^{-1} D-galUA and 5 g l^{-1} D-xylose (Figure 3). All the strains with *MgGALDH* under the constitutive *gpdA* promoter showed the activity for L-galL while only residual activity was found from the parental strain Δ*gaaB*. In the case of Δ*gaaB-Eg-Mg* (*PgaaA/C*) strain, the GALDH activity was lower; however, it was still significantly higher if compared to the values from Δ*gaaB* ($P < 0.05$, Student's *t*-test).

Table 1 Plasmids and *A. niger* strains used in this work

Plasmid	Genes expressed	Promoter
JKp1-EgALase	The codon optimized *Euglina gracilis* Alase (*EgALase*) [GenBank: AB306917]	*gpdA*
JKp1-Smp30	The codon optimized rat *Smp30* [GenBank: CAA48786]	*gpdA*
JKp1-MgGALDH	The codon optimized *Malpighia glabra* GALDH (*MgGALDH*) [GenBank: ACG75919] ORF	*gpdA*
Bidir-EgALase-MgGALDH	*EgALase* and *MgGALDH*	*gaaA/C*
Strain	**Genes expressed**	**Description**
Δ*gaaB*	none	ATCC 1015 with the deleted L-GalA dehydratase gene gaaB
Δ*gaaB-Mg*	*MgGALDH*	Δ*gaaB* with *MgGALDH* expressed under *gpdA* promoter
Δ*gaaB-Eg-Mg* (*PgpdA*)	*EgALase* and *MgGALDH*	Δ*gaaB* with *EgALase* and *MgGALDH*, both expressed under *gpdA* promoter
Δ*gaaB-Smp30-Mg*	*Smp30* and *MgGALDH*	Δ*gaaB* with *Smp30* and *MgGALDH*, both expressed under *gpdA* promoter
Δ*gaaB-Eg-Mg* (*PgaaA/C*)	*EgALase* and *MgGALDH*	Δ*gaaB* with *EgALase* and *MgGALDH*, both expressed under bidirectional *gaaA/C* promoter

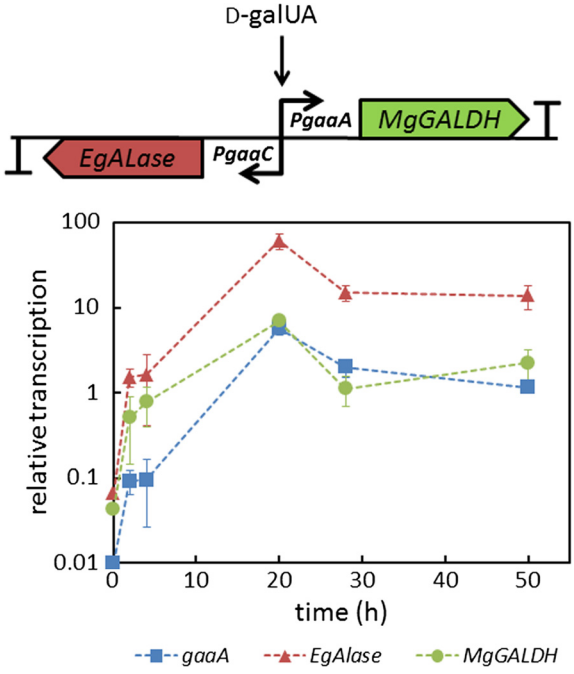

Figure 2 The D-galUA inducible expression cassette and relative transcription of *gaaA* (blue squares), *EgALase* (red triangles) and *MgGALDH* (green circles) in the strain Δ*gaaB-Eg-Mg* (*PgaaA/C*) after the shift from pre-cultures to medium supplemented with D-galUA and D-xylose. Values were normalized to the expression of actin. Error bars represent ± SEM (n = 3).

In the lactonization of L-galA to L-galL, which is catalysed by an ALase, protons are absorbed in an equimolar manner increasing the pH in the reaction. The relation between absorption at 405 nm and proton concentration was quantified being $\Delta A_{405} = 0.003$ per 10 μM of protons which should provide high enough sensitivity for the ALase assay. We tested all the engineered strains for ALase activity in the presence of L-galA; however, we could not detect this activity.

Production of L-AA

The parental strain Δ*gaaB* and the different engineered strains were tested for L-AA production even though the lactonase activities were not detectable. We used flask cultures on defined medium supplemented with D-galUA and D-xylose and with the initial pH adjusted to 3.0. With the strain Δ*gaaB* only L-AA concentrations of below 3.5 mg l^{-1} were observed in the cultures (Figure 4). All the engineered strains having *MgGALDH* under *gpdA* or *gaaA/C* promoter produced L-AA at concentrations of around 55–83 mg l^{-1}. Also the strain Δ*gaaB-Mg* without an ALase produced similar L-AA concentrations as the strain Δ*gaaB-Eg-Mg* (*PgpdA*) with the introduced *EgALase* gene. In fact, the L-AA production corresponds to the GALDH activity in the strains having *MgGALDH* under *PgpdA* (Figure 3). The clearest difference between the engineered strains was the timing of the highest L-AA concentration in the medium – all the strains with *MgGALDH* under *gpdA* promoter had the highest titer at 48 h whereas Δ*gaaB-Eg-Mg* (*PgaaA/C*) had the highest titer at 96 h. The decreasing L-AA concentrations during the time course may be due to the instability of the product causing by the exposure to air. In addition, the production turned out to be pH dependent since L-AA production was not observed when the initial pH was 5.0 (data not shown).

Even though, D-xylose was used as a co-substrate, one of the reasons for limited L-AA production may be the lack of NADPH for the D-galUA reductase *gaaA* in the first reaction in the pathway. In addition, energy and carbon sources are needed for the expression of heterologous L-AA pathway genes. To overcome these possible

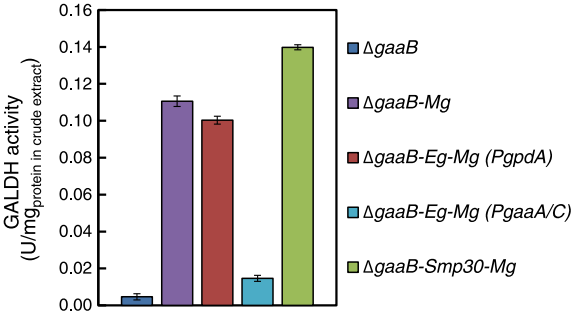

Figure 3 GALDH activity for L-galL from the crude extracts of Δ*gaaB*, Δ*gaaB-Mg* Δ*gaaB-Eg-Mg* (*PgpdA*), Δ*gaaB-Eg-Mg* (*PgaaA/C*) and Δ*gaaB-Smp30-Mg* cultured in minimal medium supplemented with D-galUA and D-xylose for 20 h. All of the values from the strains with introduced *MgGALDH* differed significantly from the value from Δ*gaaB* ($P < 0.05$, Student's *t*-test). Error bars represent ± SEM (n = 3).

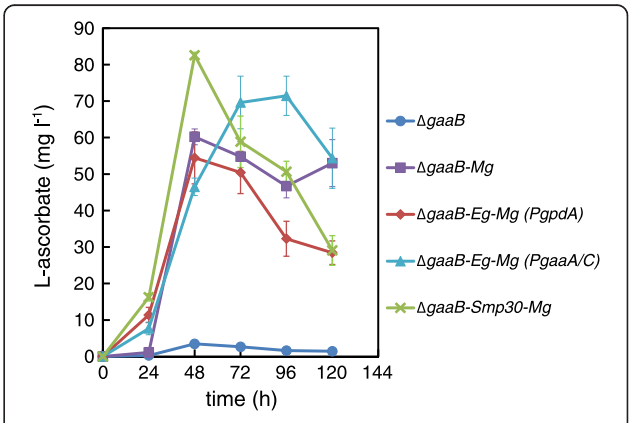

Figure 4 L-Ascorbate production by engineered *A. niger* strains in minimal medium supplemented with D-galUA and D-xylose. Error bars represent ± SEM (n = 3).

limitations and to test L-AA production from a pectin-rich residue, we used CPW as substrate containing a complex mix of different sugars and sugar acids in the form of polysaccharides. We decided to use Δ*gaaB-Eg-Mg* (*PgaaA/C*) strain in order to avoid unnecessary and energy wasting expression of the heterologous genes at the beginning of fermentation when free D-galUA is not yet available. In addition, we used higher fungal biomass in the inoculation in order to ensure CPW hydrolysis and speed up the process. L-AA production from CPW turned out to be more efficient than in defined media conditions with respect to the final concentrations (Figure 5). A lag phase of about 48 h was observed before the production started at a higher rate, which is probably due to rather slow pectin hydrolysis to D-galUA and utilization of the more preferred carbon sources, such as D-glucose present in CPW. The D-galUA content in CPW that was prepared as the CPW used in this study was reported to be 27% on a dry mass basis [20]. Other detected main components after pectinase hydrolysis were L-arabinose, D-galactose, D-glucose and L-rhamnose. Even though, L-AA production started later than when compared to the defined conditions with the Δ*gaaB-Eg-Mg* (*PgaaA/C*) strain the highest titer (around 170 mg l^{-1}) was achieved at 96 h as was the case of the cultures in the defined conditions. As in the case of defined conditions, a decrease in L-AA concentration was observed after 96 h probably due to the instability of the product. The CPW used as a substrate did not contain detectable amounts of L-AA. In the control fermentations with the parental strain Δ*gaaB*, the observed L-AA concentrations were below 15 mg l^{-1}.

Discussion

Synthetic L-AA is a widely used compound in several industries. Currently it is produced in a multistep process

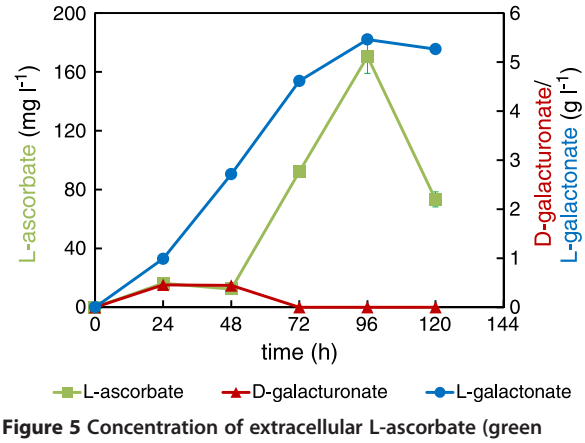

Figure 5 Concentration of extracellular L-ascorbate (green squares), D-galUA (red triangles) and L-galA (blue circles) from a culture of the strain Δ*gaaB-Eg-Mg* (*PgaaA/C*) in minimal medium supplemented with CPW. Error bars represent ± SEM (n = 3) and where not visible, are smaller than the symbol.

including chemical and biochemical steps. This study is the first report on an engineered filamentous fungus for direct L-AA production. To the best of our knowledge, the L-AA titer of 170 mg l^{-1} that was achieved in this work is the highest among engineered fungal strains. In addition, the pathway for L-AA biosynthesis originating from D-galUA was for the first time introduced to a heterologous host. In the previous literature, L-AA production was reported to be achievable from D-glucuronic acid and D-galUA using wild type strains of *A. niger* [22] or yeast [23]. However, the detection method for L-AA that was used in these studies (2,6-dichlorophenolindophenol method) could not distinguish between L-AA and other similar compounds, such as D-EA [24].

We decided to use the GALDH from *M. glabra* (acerola), which is known for its vitamin C rich fruits, including the native N-terminal mitochondrial signal peptide. The kinetic parameters of MgGALDH are not known but the expression level of *MgGALDH* is known to be higher in *M. glabra* tissue when compared to the corresponding GALDH gene (*AtGALDH*) expression in *Arabidopsis thaliana* [25]. *AtGALDH* gene codes for a protein having 75.25% homology with MgGALDH and kinetic parameters of K_m 0.17 mM and k_{cat} 134 s^{-1} for L-galL [26]. We have also tested the expression of *AtGALDH* gene in the *A. niger* Δ*gaaB* strain, however, L-AA was not observed in the resulting strains (data not shown). In plants, the GALDH locates on the inner membrane of mitochondria binding non-covalently FAD as a cofactor [26]. During the oxidation of L-galL to L-AA, electrons are shuttled to cytochrome c and further to the electron transport chain. The recombinant AtGALDH without mitochondrial targeting signal has been produced in *E. coli* as a cytoplasmic protein [26]. The resulting enzyme was active with L-galL when cytochrome c was added to the reaction mixture. However, in a living cell the functionality of a GALDH is probably dependent on its correct localization enabling interaction with mitochondrial cytochrome c. Thus it is crucial that the mitochondrial targeting signal in a plant GALDH is also functional in *A. niger*. Mitochondrial inner membrane proteins are most often targeted to their location by an N-terminal cleavable peptide [27]. The targeting peptide is typically positively charged among different organisms. However, in yeast, mitochondrial signal peptides are generally shorter, less alpha-helix forming and less hydrophobic when compared with the plant signal peptides [27]. The cleavage site for the signal peptides seem to be relatively conserved between yeast and plants consisting of tyrosine, leucine, phenylalanine and arginine residues [27]. In the case of *AtGALDH* and *MgGALDH*, the estimated signal peptides differ being 102 and 84 residues long, respectively [28]. The shorter signal peptide in *MgGALDH* may be more suitable for mitochondrial targeting in *A.*

niger, however, the localization of MgGALDH in the engineered *A. niger* strains was not investigated in this study.

Despite several attempts, the ALase activity for L-galL could not be detected from any of *EgALase* or *smp30* expressing strains. In addition to *A. niger* strains, we have also tried to express both of the ALases in yeast or *E. coli* strains, however, without detectable activity. The lactonase assay that was used in this work is based on a pH change and a pH indicator in the reaction mixture. The assay should provide high enough sensitivity for the activity and it has been routinely and successfully used in many studies. Consequently, the most likely explanations for the non-detectable ALase activity are inactivity of the protein in heterologous hosts or inactivation during cell disruption. In the literature, EgALase was produced at low temperature (15°C) in an *E. coli* strain co-expressing the Trigger Factor chaperone protein [16]. In the case of Smp30 from animal L-AA pathway, the recombinant protein was produced in *E. coli* co-expressing two chaperones GroEL and GroES [17]. In addition, it has been reported that production of human Smp30 protein in *E. coli* without chaperones led up to the insoluble and inactive proteins and formation of inclusion bodies [29,30]. Thus poor folding of the ALase proteins in a heterologous host is a possible explanation for the unsuccessful expression.

Regardless of the non-detectable ALase activity, several *A. niger* strains with a functional GALDH were capable of L-AA production. The lactonization reaction might be spontaneous and may take place extracellularly. In the lactonization reaction, protons are assimilated, which mean that a low pH shifts the equilibrium towards the lactone form. This would explain the fact that only low pH enabled L-AA production. Thus it is possible that L-galA is first secreted out from the cells and spontaneously formed L-galL is then transported back to the cells and oxidized to L-AA. On the other hand, the parental strain *ΔgaaB* is known for its higher capacity to produce L-galA at low pH [19,20] which may provide more substrate for the L-AA pathway also in the engineered strains when cultured at low pH. The production improved when CPW was used as substrate. In addition to the higher fungal biomass in the inoculation, the improvement was possibly due to the additional carbon sources that were constantly released from the substrate during the fermentation providing energy such as reducing power for the strain. The product titer was still relatively low and possible limited by the poor lactonization reaction from L-galA to L-galL. In addition, L-AA concentrations started to decrease during the fermentation. This is likely due to the oxidation that occurs by the exposure to air. Thus it would be beneficial for the process to optimize factors, such as, aeration and timing of the harvesting. Nevertheless, this is the first demonstration of a consolidated bioprocess for L-AA production.

Conclusions

We have engineered *A. niger* strains to redirect the D-galUA pathway to L-AA synthesis. The native catabolic D-galUA pathway was disrupted and the biosynthetic L-AA pathway from plants originating from D-galUA was introduced. In addition, we built a strain having the L-AA pathway under D-galUA inducible expression. The resulting strains were capable of L-AA production from pure D-galUA but also from CPW that is a pectin-rich biomass residue. Final L-AA titers up to around 170 mg l^{-1} were achieved with the engineered strains.

Methods

Strains

The *Aspergillus niger* strain ATCC 1015 (CBS 113.46) was used as a wild type. The engineered strain of ATCC 1015 with the deleted L-galA dehydratase *gaaB* [19,20] was used as a host strain for the L-AA production strains that are listed in Table 1. All the plasmids were produced in *E. coli* TOP10 cells and the homologous recombination for the plasmid construction was carried out in the *S. cerevisiae* strain ATCC 90845.

Plasmid construction

For introducing the biosynthetic L-AA pathway in *A. niger*, the heterologous genes coding for a GALDH or an ALase, listed in Table 1, were custom synthetized as codon optimized ORFs (Genscript) and inserted to the JKp1-hph plasmid [19] under the *gpdA* promoter. Alternatively, the GALDH from *Malpighia glabra* (*MgGALDH*) and the ALase from *Euglena gracilis* (*EgALase*) were both expressed under the native bidirectional promoter (657 bp) of the *A. niger* D-galUA reductase (*gaaA*) and the 2-keto-3-deoxy-L-galactonate aldolase (*gaaC*) genes that are clustered in the genome. In the expression cassette, *MgGALDH* was orientated as *gaaA* and *EgALase* as *gaaC* in *A. niger* genome. Terminators that were used in the bidirectional cassette for *MgGALDH* and *EgALase* were the native *gaaA* and *gaaC* terminators, respectively, both 505 bp downstream from the stop codon. All of the expression cassettes contained hygromycin B phosphotransferase (*hph*) gene under the *gpdA* promoter for the selection of transformants.

The codon optimized ORFs were released with *SacI* and *XmaI* (both NEB) and ligated to the JKp1 plasmid. The resulting plasmids JKp1-EgALase/Smp30/MgGALDH were linearized with *SpeI* (NEB). For constructing the cassette with D-galUA inducible expression, the bidirectional *gaaA/C* promoter and terminators of *gaaA* and *gaaC* genes were amplified from *A. niger* ATCC1015 genomic DNA with the primers P1-P6 as described in Table 2. The *EgALase*, *MgGALDH* and the *hph* gene (from JKp1) were amplified with the primers P7-P12 (Table 2). All the six amplified fragments, containing 40 bp flanks for homologous recombination, and an *EcoRI* and *BamHI* digested platform

Table 2 Primers used in this work

Primer	Sequence	Description
P1	AGGCATCTGTCTGAGAGGCAACCGTGGCGA	Amplification of *gaaA/C* promoter, forward
	GAGTCCGCATTCTTTGATCTGCTGTTAGTT	
P2	GGTGACGAAGTGTGCGATTGAGCGTGATAA	Amplification of *gaaA/C* promoter, reverse
	AACGAAACATTGTGATTGCTGTGGTGTAAA	
P3	TAAGTTGGAGAAGTTGTTTCCGTCGCTCGAT	Amplification of *gaaA* terminator, forward
	GCCATTTGAATACCTTAGAGAAGCTTGTATG	
P4	CGTCTCTCCGCATGCCAGAAAGAGTCACCGG	Amplification of *gaaA* terminator, reverse
	TCACTGTACCATCTCCATCTCCTTCCCG	
P5	GCCCCCCCTCGAGGTCGACGGTATCGATAAGC	Amplification of *gaaC* terminator, forward
	TTGATATCGAATTCCTGTTGGAGAGAGGGTGTGT	
P6	CCCAGCCCCAGGTCCGCCACCCGCAGAGTTCCGT	Amplification of *gaaC* terminator, reverse
	TTGTGATCCATTGTATCATATAGATTATGAC	
P7	ATGCGGACTCTCGCCACG	Amplification of *Eg* ALase, forward
P8	TCACAAACGGAACTCTGCGG	Amplification of *Eg* ALase, reverse
P9	ATGTTTCGTTTTATCACGCT	Amplification of *Mg* GALDH, forward
P10	TCAAATGGCATCGAGCGAC	Amplification of *Mg* GALDH, reverse
P11	GTACAGTGACCGGTGACTCT	Amplification of *hph*, forward
P12	GCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGA	Amplification of *hph*, reverse
	ACTAGTGGATCCTTGGAGATTTCAGTAACGTT	
P13	GAGGTCGACGGTATCGATAAGC	Sequencing of the bidirectional *Eg/Mg* cassette
P14	TGATACAATGGATCACAAACGG	"
P15	CAACAGAGAACAGACCGCCA	"
P16	GTGTTGCGAAGCTGTAGTTGG	"
P17	ATTTACACCACAGCAATCAC	"
P18	AAAGAAGCGTGTTCGAGTCC	"
P19	ATACGGAGGATGAAGCCCTC	"
P20	GCCAGCGGAAGGAGATTACG	"
P21	GGCAGTGATTGAGGCTGTGG	"
P22	AGTAAGCGAAGGAGAATGTG	"
P23	AGTACTTTGCTACATCCATACTCC	"
P24	ATTCGGACCGCAAGGAATCG	"
P25	TGTCGGGCGTACACAAATCG	"
P26	AGCCGTGTTTCAATGTCGTG	"
P27	CGCTCTAGAACTAGTGGATC	"
P28	CAACATTGTCATGTCTGGTGG	qPCR of *actin*, forward
P29	GGAGGAGCAATGATCTTGAC	qPCR of *actin*, reverse
P30	AGGACACGATTACTCTACTTG	qPCR of *gaaA*, forward
P31	GAGCCCATATAATGGAAGTAC	qPCR of *gaaA*, reverse
P32	TCCGGGTGGACCCCGCTAAG	qPCR of *EgALase*, forward
P33	TGAAACACGGCTCCGGCGTC	qPCR of *EgALase*, reverse
P34	TAGCAAGTGGCGCGGTGTCC	qPCR of *MgGALDH*, forward
P35	TCGTGATCTCACCGCCCCGA	qPCR of *MgGALDH*, reverse

plasmid pRS426 were transformed to yeast using the Gietz method [31] and the transformants were selected on SCD-URA plates. Several yeast colonies were collected and the resulting plasmid Bidir-EgALase-MgGALDH was rescued and sequenced with the primers P13-P25 (Table 2). The resulting expression cassette was linearized with *EcoRI* and *BamHI* (both NEB) before *A. niger* was transformed.

All the resulting expression cassettes were introduced into the *A. niger* Δ*gaaB* strain by protoplast transformation method [32]. Transformants were screened for integration of the cassette by growth in the presence of 400 μg ml^{-1} hygromycin B (Calbiochem). Strains having both genes (*smp30* or *EgALase* + *MgGALDH*) expressed in JKp1 under *gpdA* promoter were generated by co-transformation. Integration of the transformed cassettes into the genome was confirmed with colony PCR using Phire direct PCR kit (Thermo Scientific) and the primers P26 and P27 (Table 2).

Media and culture conditions

Luria Broth (LB) culture medium supplemented with 100 μg ml^{-1} of ampicillin and cultural conditions of 37°C and 250 rpm were used for *E. coli* cultures. For yeast pre-cultures, YPD medium (10 g yeast extract l^{-1}, 20 g peptone l^{-1} and 20 g D-glucose l^{-1}) was used and after transformation SCD-URA plates (uracil deficient synthetic complete media supplemented with 20 g D-glucose l^{-1}) were used for uracil auxotrophy selection. Yeast cultures were carried out at 30°C and all the liquid cultures at 250 rpm. For the *A. niger* submerged fermentations, spores were generated on potato-dextrose plates and $0.9*10^8$ spores were inoculated into 50 ml of YP medium (10 g yeast extract l^{-1}, 20 g peptone l^{-1}) containing 30 g gelatin l^{-1}. Mycelia were pre-grown in 250-ml Erlenmeyer flasks by incubating overnight at 28°C, 200 rpm and harvested by vacuum filtration, rinsed with sterile water and weighted. In the *A. niger* transformations and fermentations, *A. nidulans* defined minimal medium [33], containing (in g l^{-1}) 6 $NaNO_3$, 0.52 KCl, 0.52 $MgCl_2$ and 1.52 KH_2PO_4 was used. In the transformations the minimal medium was supplemented with 1.2 M D-sorbitol, 10 g l^{-1} of D-glucose and 20 g l^{-1} of agar and the pH was adjusted to 6.5. The minimal medium used in the fermentations for L-AA production, enzymatic assays and transcriptional analysis was supplemented with 20 g l^{-1} of D-galUA and 5 g l^{-1} of D-xylose or with 40 g l^{-1} of dried citrus processing waste (CPW). The pH was adjusted to 3. The CPW contained peel and pulp from the processed oranges and was received from the Federal University of Paraná in Curitiba, Brazil. The submerged cultures supplemented with pure D-galUA and D-xylose were inoculated with 1 g l^{-1} (cdw) and the submerged cultures supplemented with CPW were inoculated with 3 g l^{-1} (cdw) of pre-grown mycelia.

Enzymatic assays

For enzymatic assays, *A. niger* mycelia were harvested by vacuum filtration. Crude extracts were prepared in 100 mM sodium phosphate buffer pH 7 with protease inhibitor (Complete, Roche). The cells were disrupted with 0.4 mm diameter glass beads using a bead beater (Precellys 24, Bertin Technologies) and solid residues were removed by centrifugation. The GALDH assay was based on the cytochrome c reduction at 550 nm as described by Leferink et al. [26]. Crude extracts from the each strain were supplemented with 500 μM of FAD (flavin adenine dinucleotide), which is an essential cofactor for MgGALDH, and were tested for the activity by following the cytochrome c reduction after addition of 5 mM L-galL. The enzymatic assay for the lactonase activity (from EgALase and Smp30) was based on a pH change in the reaction from L-galA to L-galL that absorbs protons and was assayed as described by Ishikawa et al. [16]. The pH was followed at 405 nm using p-nitrophenol as a pH indicator and the correspondence between Δ405 nm and the absorption of protons were quantified using the titration with HCl.

Transcriptional analysis

Samples of 2 ml were collected from the cultures and the mycelium was harvested by vacuum filtration. The filtered mycelium was 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 with RNase free water (Roche) and were used for RT-qPCR using a LightCycler II with the LightCycler SYBR green I Master mix (both Roche). The expression of *gaaA*, *EgALase*, *MgGALDH* and actin (ATCC 1015 200483-mRNA) were quantified using the primers P28-P35 (Table 2). The signal from each of the target genes was normalized to actin using the accompanying software (Advance Relative Quantification tool).

Chemical analyses

Samples of 2 ml were removed at intervals and mycelium was separated from the supernatant by centrifugation. The concentration of D-galUA and L-galA was determined by HPLC using a Fast Acid Analysis Column (100 mm × 7.8 mm, BioRad Laboratories, Hercules, CA) linked to an Aminex HPX-87H organic acid analysis column (300 mm × 7.8 mm, BioRad Laboratories) with 5.0 mM H_2SO_4 as eluent and a flow rate of 0.5 ml min^{-1}. The column was maintained at 55°C. Peaks were detected using a Waters 410 differential refractometer and a Waters 2487 dual wavelength UV (210 nm) detector. The L-AA content was measured using the commercial Ascorbic Acid Assay Kit II (Sigma-Aldrich) following the manufacturer's instructions.

Abbreviations
L-AA: L-ascorbic acid; D-EA: D-erythroascorbic acid; D-galUA: D-galacturonic acid; L-galA: L-galactonic acid; L-galL: L-galactono-1,4-lactone; GALDH: L-galactono-1,4-lactone dehydrogenase; ALase: Aldonolactonase; CPW: Citrus processing waste.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
JK and PR designed and JK carried out all the experimental work and analyzed the data. JK and PR drafted the manuscript. PR and MP designed the fundamental concept and participated in the coordination of the study. All the authors read and approved the final manuscript.

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VTT Science 106

Title	**Metabolic engineering of the fungal D-galacturonate pathway**
Author(s)	Joosu Kuivanen
Abstract	Industrial biotechnology is one of the enabling technologies for biorefineries. In addition to biofuels, several platform and fine chemicals can be produced from biomass taking advantage of metabolic pathways in the cell. However, genetic engineering is often needed to redirect the cellular metabolism towards a product of interest. In this thesis, one of these metabolic pathways catabolizing constituents of pectin – the catabolic D-galacturonate pathway in filamentous fungi– was engineered and redirected to desired end products. Biotechnological production of L-galactonic acid, a potential platform chemical, was demonstrated in this thesis for first time. The production was obtained in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*) by deleting the second gene, encoding a dehydratase, from the fungal D-galacturonate pathway. In addition to the production from pure D-galacturonic acid, a consolidated bioprocess from citrus processing waste, a pectin-rich biomass was investigated. L-galactonic acid can be lactonised and further oxidised to L-ascorbic acid (vitamin C) via chemical or biochemical routes. In this thesis, an *A. niger* strain was engineered for direct conversion of D-galacturonic acid to L-ascorbic acid by introducing two plant genes: L-galactono-1,4-lactone lactonase and dehydrogenase. The resulting strains were capable of L-ascorbic acid production from pure D-galacturonic acid or citrus processing waste. In addition to lactonization, two other biochemical reactions towards L-galactonic acid are known: dehydration, which is the second reaction in the fungal D-galacturonate pathway, and oxidation to D-tagaturonic acid, which occurs in the catabolic L-galactonic acid pathway in bacteria. Both of these biochemical reactions and responsible enzymes from *A. niger* and *Escherichia coli* were investigated more detailed. As a result, the substrate specifities for four dehydratases from *A. niger* were determined and the bacterial L-galactonate-5-dehydrogenase was characterised and applied in a colorimetric assay for L-galactonic and L-gulonic acids. Pectin-rich biomass has potential as a raw material for the production of renewable chemicals. This thesis presents new ways to utilise this residual biomass by using industrial biotechnology. In addition, the thesis broadens basic understanding of the fungal catabolic D-galacturonate pathway and how it can be engineered for production of useful chemicals.
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VTT Science 106

Nimeke	**Mikrosienten D-galakturonaattireitin metabolian muokkaus**
Tekijä(t)	Joosu Kuivanen
Tiivistelmä	Teollinen biotekniikka on yksi tärkeistä teknologioista, jotka mahdollistavat biomassan jalostamisen erilaisiksi lopputuotteiksi. Bioteknologiaa käyttäen biomassasta voidaan biopolttoaineiden lisäksi tuottaa useita eri kemikaaleja hyödyntämällä solujen metaboliareittejä. Hyödyntämällä solujen geneettistä muokkausta, solun metabolia saadaan ohjattua myös sellaisten yhdisteiden tuottamiseen, joita solut eivät luonnostaan tuota. Tässä työssä yksi solun metaboliareitti – mikrosienten D-galakturonihapon kataboliareitti – oli geneettisen muokkauksen kohteena ja se ohjattiin haluttujen yhdisteiden tuottoon. D-galakturonihappo on pektiinin pääkomponentti, joka taas on yleinen kasvibiomassan heteropolysakkaridi. Tässä työssä L-galaktonihapon bioteknologinen tuotanto osoitettiin ensimmäisen kerran hyödyntäen *Aspergillus niger* ja *Hypocrea jecorina* -homeita, joista D-galakturonihapporeitin toinen, dehydrataasientsyymiä koodaava geeni oli poistettu. Puhtaasta D-galakturonihaposta tuottamisen lisäksi, L-galaktonihapon tuottamista suoraan pektiinipitoisesta biomassasta tutkittiin. L-galaktonihappo voidaan laktonisoida ja edelleen hapettaa L-askorbiinihapoksi (vitamiini C) käyttäen kemiallisia tai biokemiallisia reaktioita. Tässä työssä *A. niger* -home muokattiin tuottamaan L-askorbiinihappoa D-galakturonihaposta. Dehydrataasientsyymiä koodaavan geenin poiston lisäksi kaksi heterologista geeniä, laktonaasi ja dehydrogenaasi, tuotiin *A. niger* -homeseen. Muokatut kannat pystyivät tuottamaan L-askorbiinihappoa puhtaasta D-galakturonihaposta sekä pektiinipitoisesta biomassasta. Laktonisaation lisäksi L-galaktonihapolle tiedetään kaksi muuta biokemiallista reaktiota: dehydrataatio, joka esiintyy mikrosienten D-galakturonihapon kataboliareitillä, ja hapetus D-tagaturonihapoksi, joka esiintyy L-galaktonihapon kataboliareitillä bakteereissa. Molemmat näistä reaktioista olivat tarkemman tutkimuksen kohteena *A. niger* -homeessa ja *Escherichia coli* -bakteerissa. Tuloksena neljälle *A. niger* dehydrataasille määritettiin substraattispesifisyydet ja bakteriaalinen L-galaktonaatti-5-dehydrogenaasi karakterisoitiin ja sitä hyödyntäen kehitettiin menetelmä L-galaktoni- ja L-gulonihappojen kolorimetriseen määrittämiseen. Pektiinipitoinen biomassa on potentiaalinen uusiutuva raaka-aine kemikaalien tuottamiseksi. Tämä väitöskirja tuo esille uusia tapoja, joilla jäännösbiomassa voidaan hyödyntää entistä tehokkaammin käyttäen teollista bioteknologiaa. Lisäksi väitöskirja laajentaa perusymmärrystä sienien D-galakturonihapon metaboliareitistä ja siitä, kuinka sitä voidaan muokata tuottamaan hyödyllisiä kemikaaleja.
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Metabolic engineering of the fungal D-galacturonate pathway

Several chemicals can be produced from biomass taking advantage of metabolic pathways in the cell. Genetic engineering is often needed to redirect the cellular metabolism towards a product of interest. In this thesis the catabolism of D-galacturonic acid that is the main constituent in pectin was altered in filamentous fungi and redirected to desired end products. Biotechnological production of L-galactonic acid was demonstrated for first time. The production was obtained in *Aspergillus niger* and *Hypocrea jecorina* by deleting the second gene, encoding a dehydratase, from the fungal D-galacturonate pathway. In addition, two heterologous genes, lactonase and dehydrogenase, were introduced in *A. niger* resulting in strains capable of producing L-ascorbic acid (vitamin C) from D-galacturonic acid. In addition, the engineered *A. niger* strains were capable of producing these products in a consolidated bioprocess from pectin-rich biomass.

Two biochemical reactions towards L-galactonic acid are known in microorganisms: dehydration in fungi and oxidation in bacteria. Both of these biochemical reactions and responsible enzymes from *A. niger* and *Escherichia coli* were investigated more detailed. As a result, the substrate specifities for four dehydratases from *A. niger* were determined and the bacterial L-galactonate-5-dehydrogenase was characterised and applied in a colorimetric assay for L-galactonic and L-gulonic acids.

Pectin-rich biomass has potential as a raw material for the production of renewable chemicals. This thesis presents new ways to utilise this residual biomass by using industrial biotechnology. In addition, the thesis broadens basic understanding of the fungal catabolic D-galacturonate pathway and how it can be engineered for production of useful chemicals.

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