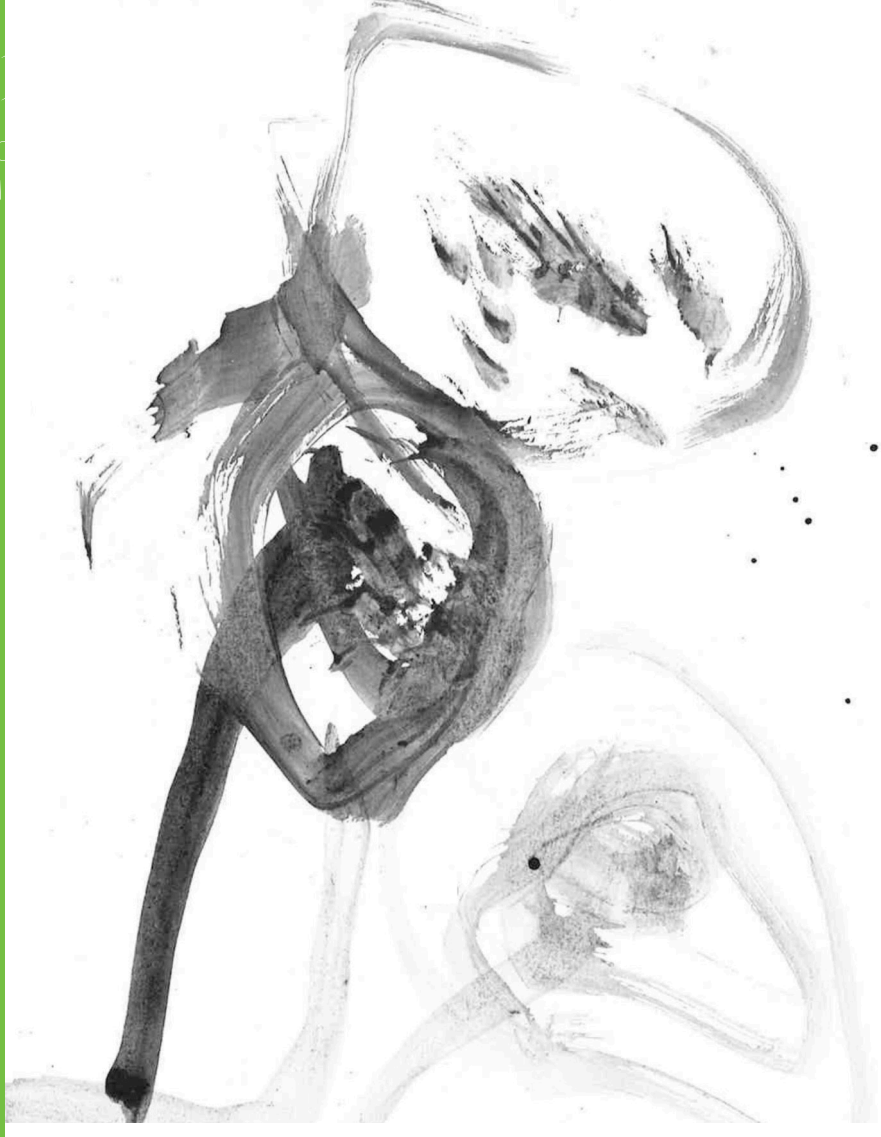
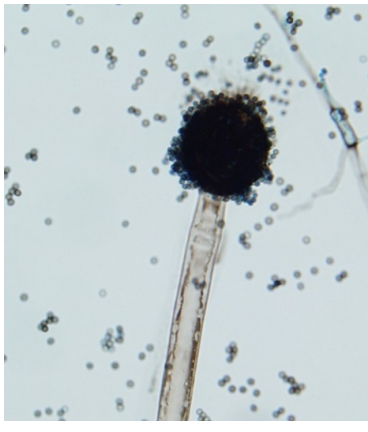


VISIONS • SCIENCE • TECHNOLOGY • RESEARCH HIGHLIGHTS

Dissertation
106

Metabolic engineering of the fungal D-galacturonate pathway

Joosu Kuivanen



VTT

VTT SCIENCE 106

Metabolic engineering of the fungal D-galacturonate pathway

Joosu Kuivanen

Doctoral dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the School of Chemical Technology for public examination and debate in Auditorium Puu2, at the Aalto University School of Chemical Technology (Espoo, Finland) on the 23rd of October, 2015, at 14.

ISBN 978-951-38-8340-9 (Soft back ed.)
ISBN 978-951-38-8341-6 (URL: http://www.vttresearch.com/impact/publications)

VTT Science 106

ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)

JULKAISIJA – UTGIVARE – PUBLISHER

Teknologian tutkimuskeskus VTT Oy
PL 1000 (Tekniikantie 4 A, Espoo)
02044 VTT
Puh. 020 722 111, faksi 020 722 7001

Teknologiska forskningscentralen VTT Ab
PB 1000 (Teknikvägen 4 A, Esbo)
FI-02044 VTT
Tfn +358 20 722 111, telefax +358 20 722 7001

VTT Technical Research Centre of Finland Ltd
P.O. Box 1000 (Tekniikantie 4 A, Espoo)
FI-02044 VTT, Finland
Tel. +358 20 722 111, fax +358 20 722 7001

Cover image: Saimi Kuivanen

Juvenes Print, Tampere 2015

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.

I would like to express my deepest gratitude to my supervisor Docent Peter Richard. Besides being an endless source of new ideas, Peter's general enthusiasm for science has inspired me a lot. I warmly thank Peter for our numerous fruitful discussions which are excited my own passion for science. Thank you also for being an excellent and supportive mentor to me during these years. I warmly thank Dr. Dominik Mojzita for guiding me to the world of filamentous fungi and for all the help relating to my work. Research Professor Merja Penttilä is warmly acknowledged for scientific guidance and for her inspirational visions of future biotechnology.

I am very grateful to my supervising professor Associate Professor Alexander Frey. I thank Alex for his very supportive attitude towards my work and for his invaluable effort to help me to get this thesis finished. I also wish to thank Professor Vera Meyer and Professor Michael Sauer for pre-examination of the thesis and for their useful comments and suggestions about how to improve it. Michael Bailey is acknowledged for revising the language of the thesis.

It has been a real privilege to collaborate with many skilful and passionate people and thus I would like to thank all other co-authors of my publications: Dr. Marilyn G. Wiebe, Professor Nadia Krieger, Professor David Mitchell, Dr. Satu Hilditch, Alessandra Biz, Francine Motter, Edgar Mallmann, Hugo Dantas, Yanming Wang and Hanna Keränen. I wish to express my special thanks to Marilyn who is the corresponding author in my first scientific publication from which I learned a lot about scientific data analysis and writing.

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

Supervising professor, Custos	Associate Professor Alexander Frey Department of Biotechnology and Chemical Technology Aalto University, Finland
Thesis advisor	Docent Peter Richard VTT Technical Research Centre of Finland Ltd
Preliminary examiners	Professor Vera Meyer Applied and Molecular Microbiology, Institute of Biotechnology Berlin University of Technology, Germany
	Professor Michael Sauer Department of Biotechnology University of Natural Resources and Life Sciences, Vienna, Austria
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].

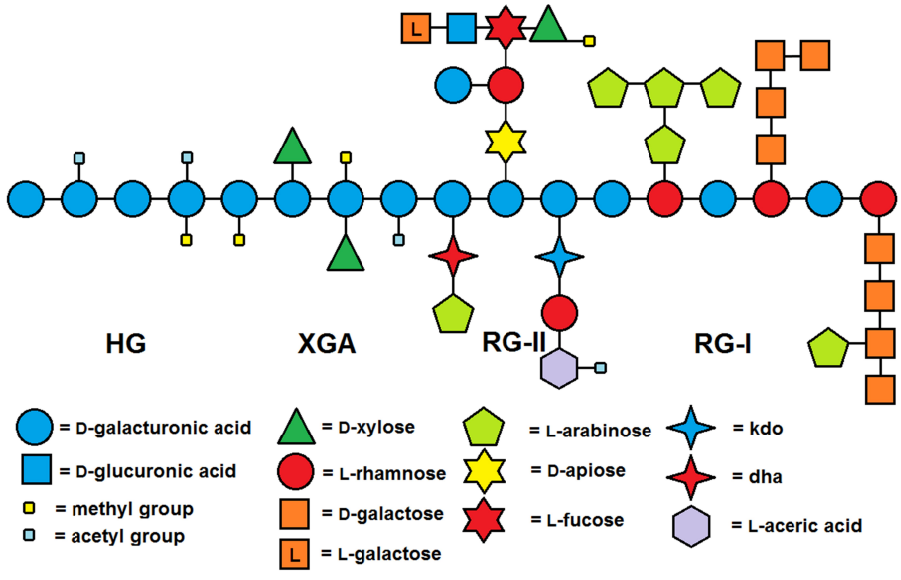


Figure 1. Pectin structure: homogalacturonan (HG), xylogalacturonan (XGA) rhamnogalacturonan II (RG-II) and rhamnogalacturonan I (RG-I).

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.

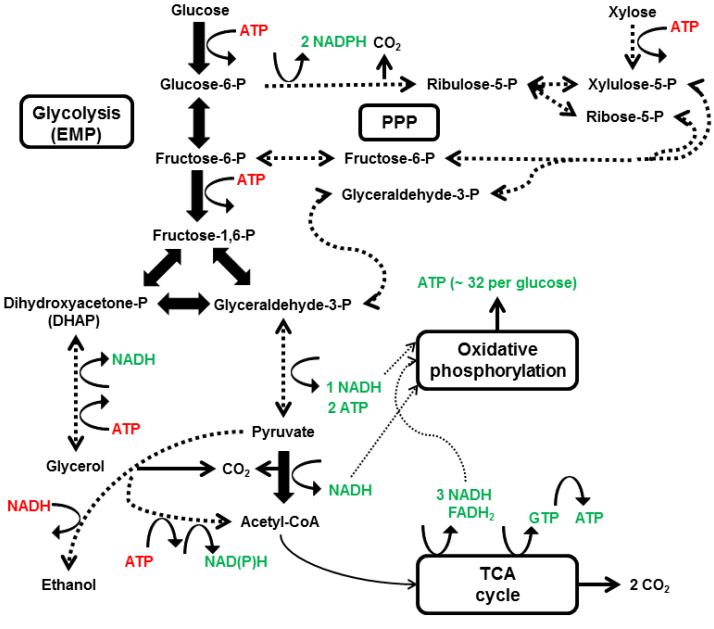


Figure 2. The central pathways of carbohydrate catabolism in the cell: Embden-Meyerhof-Parnas pathway (EMP), pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle and oxidative phosphorylation. Solid arrows represent single enzymatic reactions, dotted line arrows represent simplified steps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

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

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

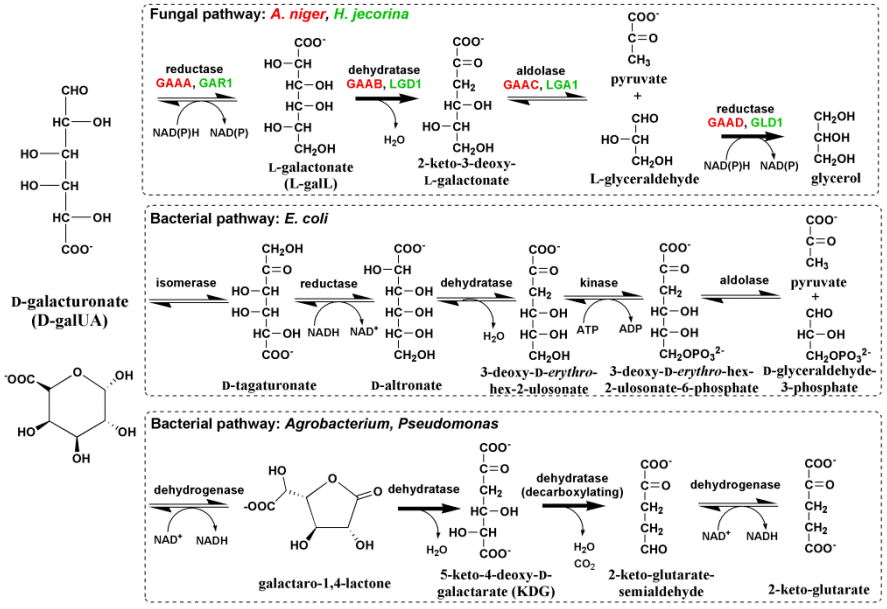


Figure 3. Fungal and bacterial pathways for D-galUA catabolism.

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.

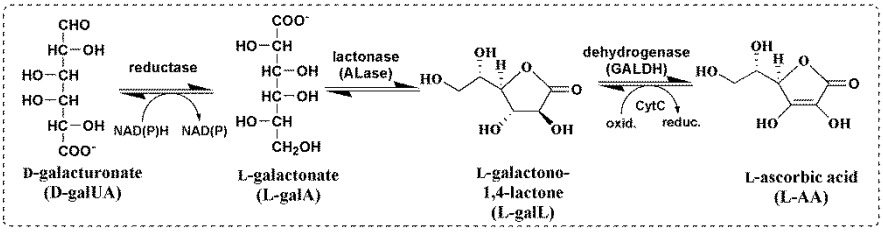


Figure 4. Pathway for conversion of D-galUA to L-AA in plants.

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).

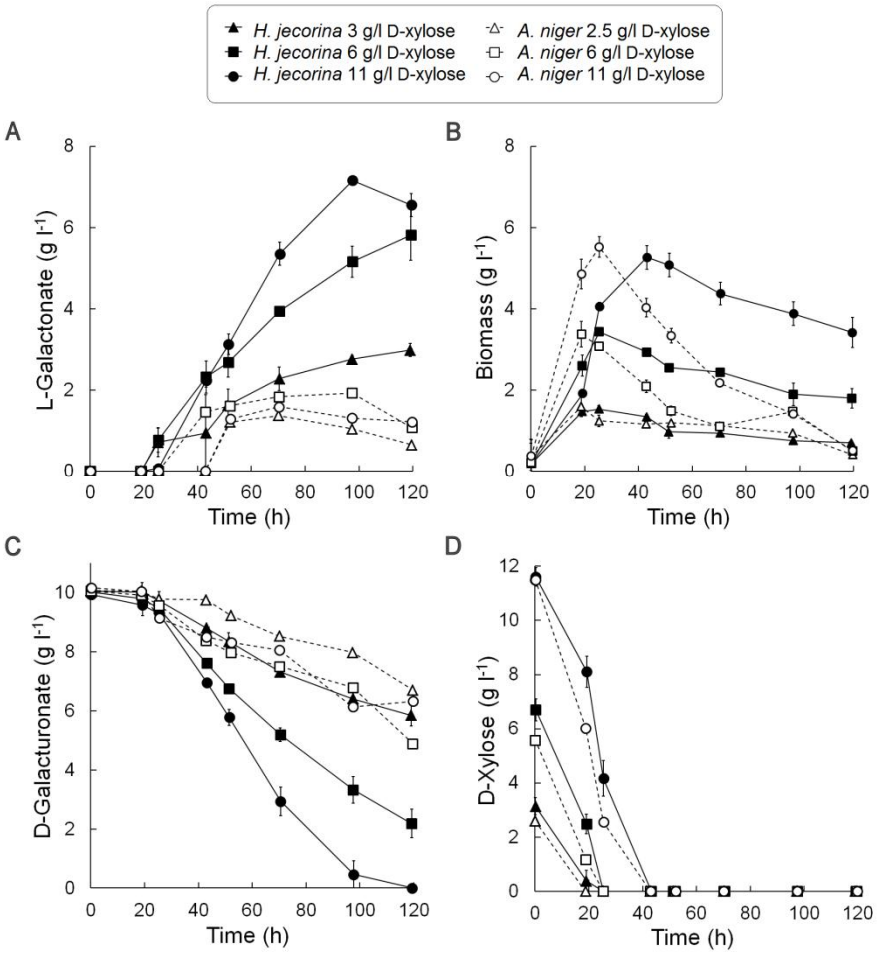


Figure 5. Bioreactor cultivations with *H. jecorina* Δ*lgd1* (solid symbols) and *A. niger* Δ*gaaB* (open symbols) strains. L-GalA (A), biomass (B), D-galUA (C) and D-xylose (D) concentrations were measured. The cultivations were started with an initial D-galUA concentration of 10 g l^{-1}. Different 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.

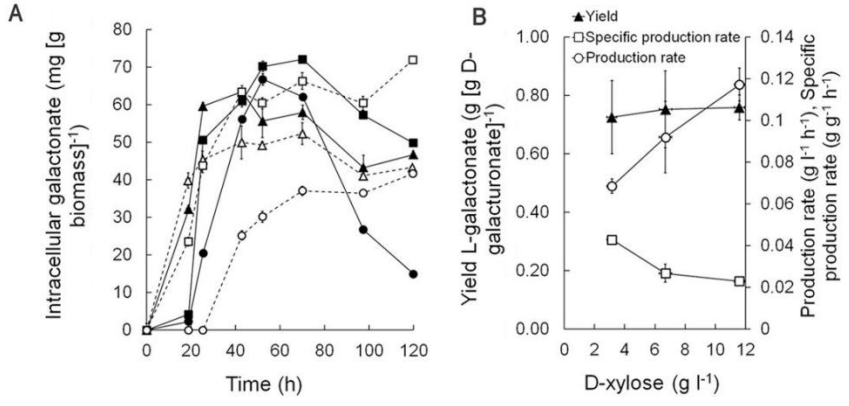


Figure 6. Intracellular L-GalA concentrations in bioreactor cultivations of *H. jecorina* Δ*lgd1* and *A. niger* Δ*gaaB* (A) and yields (triangle; L-galA / consumed D-galUA), initial specific production rates (square; g L-galA / g biomass / h) and initial production rates (circle; g L-galA / l / h) in 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*.

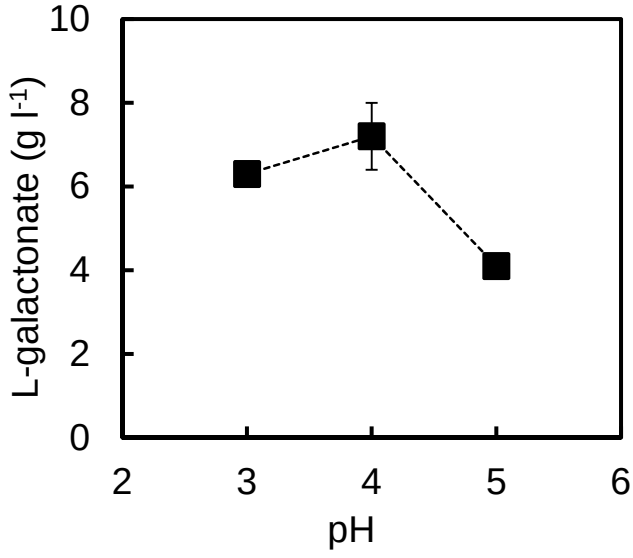


Figure 7. Effect of pH on L-galA production in flask cultivations with *A. niger* Δ*gaaB* after 144 h. A buffered minimal medium containing 20 g l^{-1} of D-galUA and 5 g l^{-1} of D-xylose was used. Error bars represent ± the standard error of the mean (SEM, n=3).

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.

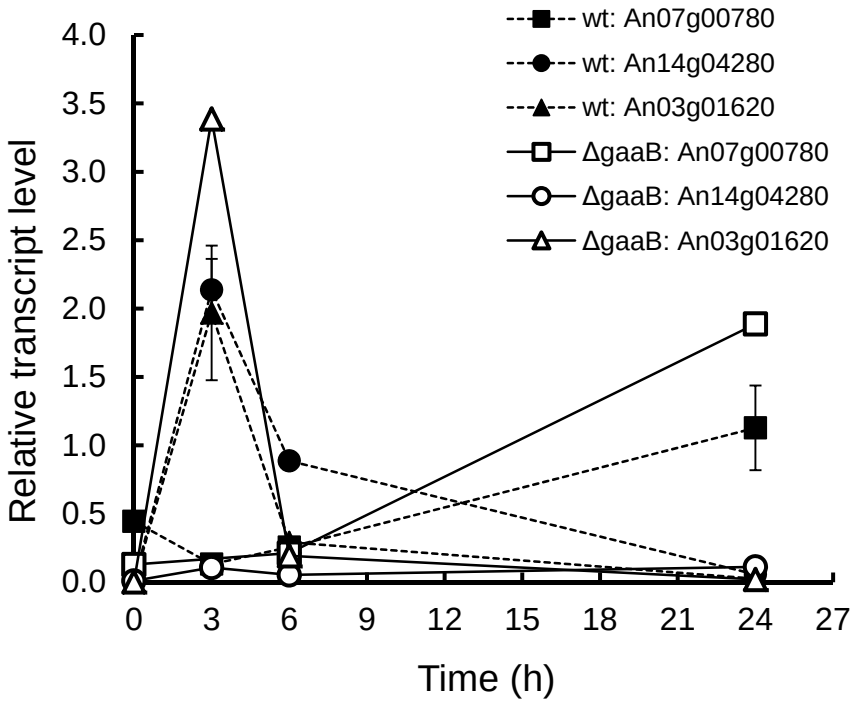


Figure 8. Relative transcript levels of the 3 putative D-galUA transporters in *A. niger* strains in flask cultivations at pH 3 in the medium containing 20 g l^{-1} D-galUA and 5 g l^{-1} D-xylose. Error bars represent ± the standard error of the mean (SEM, n=3).

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.

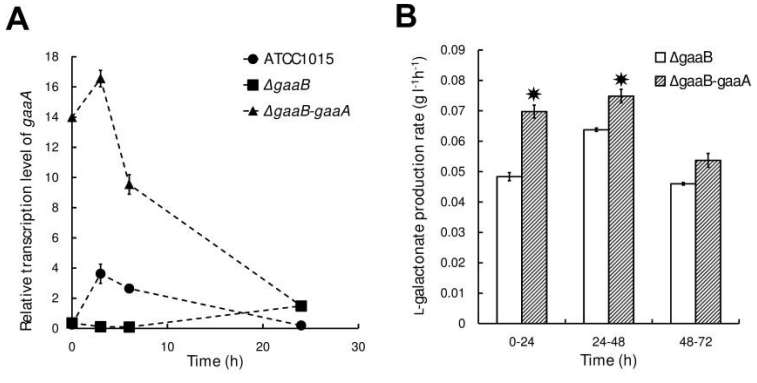


Figure 9. The relative transcript levels of *gaaA* in wild type (ATCC 1015), Δ*gaaB* and Δ*gaaB-gaaA* strains (A) and initial L-galA productivities in Δ*gaaB* and Δ*gaaB-gaaA* strains (B) in flask cultures at pH 3 in the medium containing 20 g l^{-1} D-galUA and 5 g l^{-1} D-xylose. The initial productivities 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.

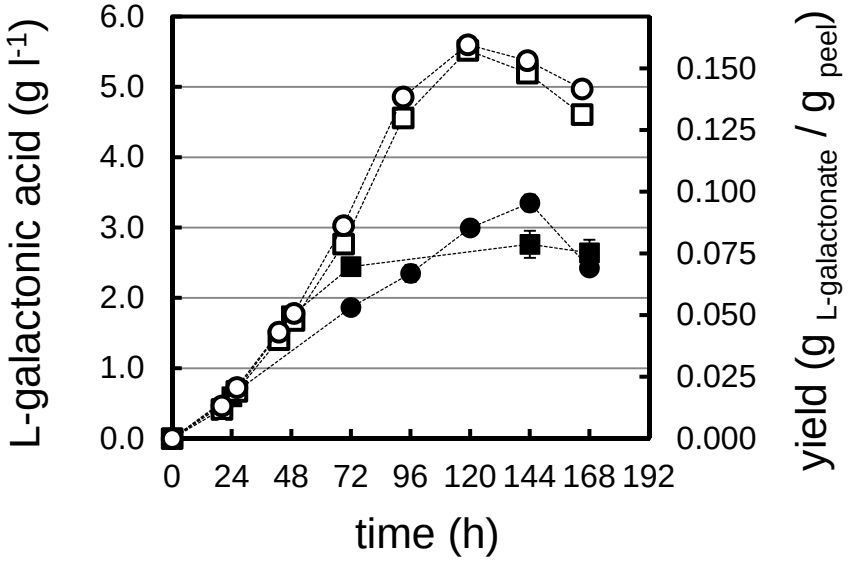


Figure 10. L-GalA production in SmFs on CPW with the engineered strains Δ*gaaB* (squares) and Δ*gaaB-gaaA* (circles) with (open symbols) or without (solid symbols) nutritional supplementation at pH 3. Error bars represent the standard error of the mean (SEM, n=3); if not visible they are smaller than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

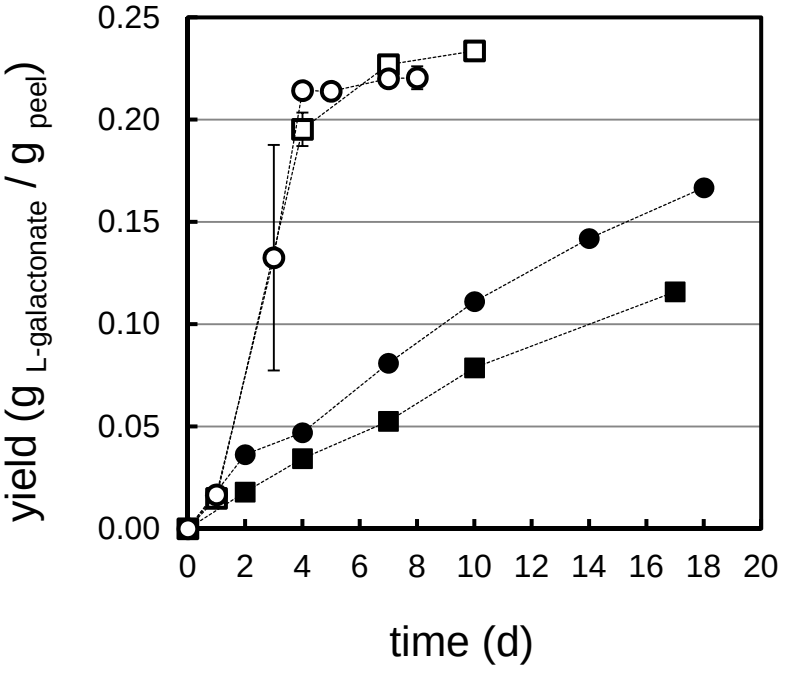


Figure 11. L-GalA production in SSFs on CPW with the engineered strains Δ*gaaB* (squares) and Δ*gaaB-gaaA* (circles) with (open symbols) or without (solid symbols) nutritional supplementation. Error bars represent the standard error of the mean (SEM, n=3), if not visible they are smaller than the symbol.

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.

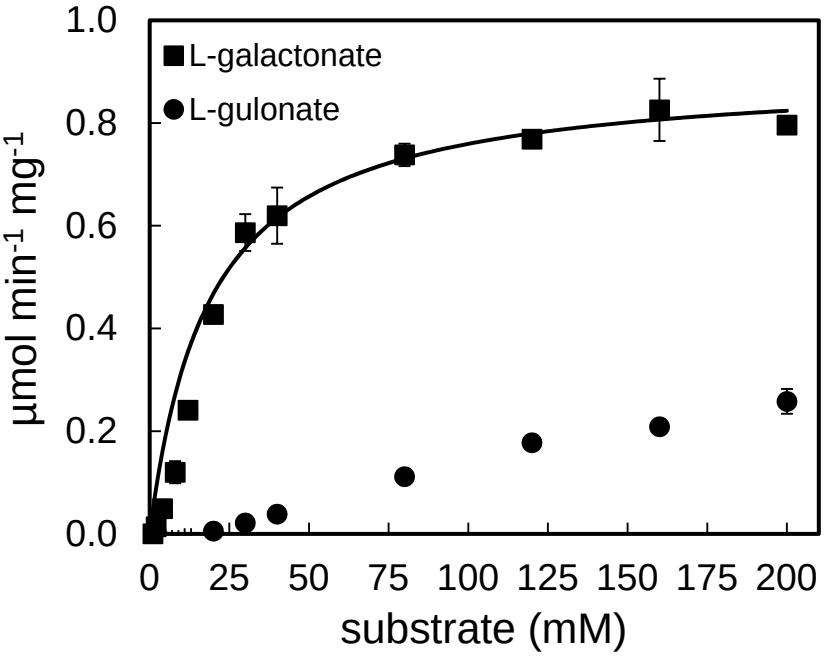


Figure 12. YjjN dehydrogenase activity from purified protein against L-galA (squares) and L-gulonic acid (circles) in the presence of NAD^+. Errors represent ±SD, n=3.

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*.

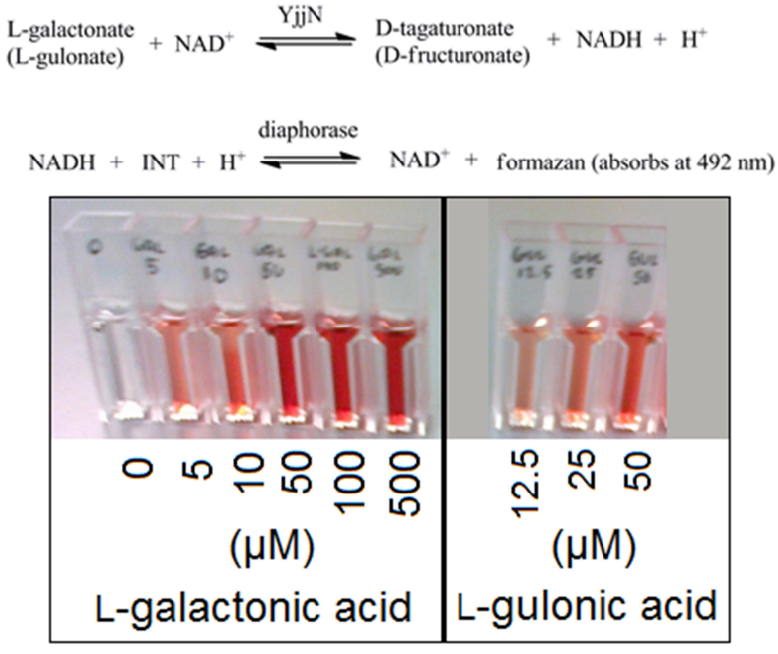


Figure 13. The coupled YjjN/diaphorase assay for L-galA and L-gulonic acid quantification.

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].

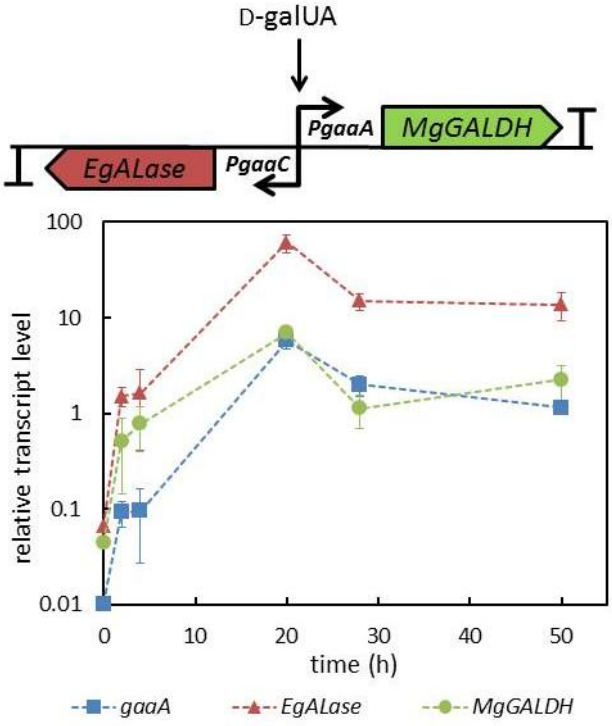


Figure 14. The D-galUA-inducible expression cassette for *EgALase* and *MgGALDH* and relative transcript levels of *gaaA* (blue squares), *EgALase* (red

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.

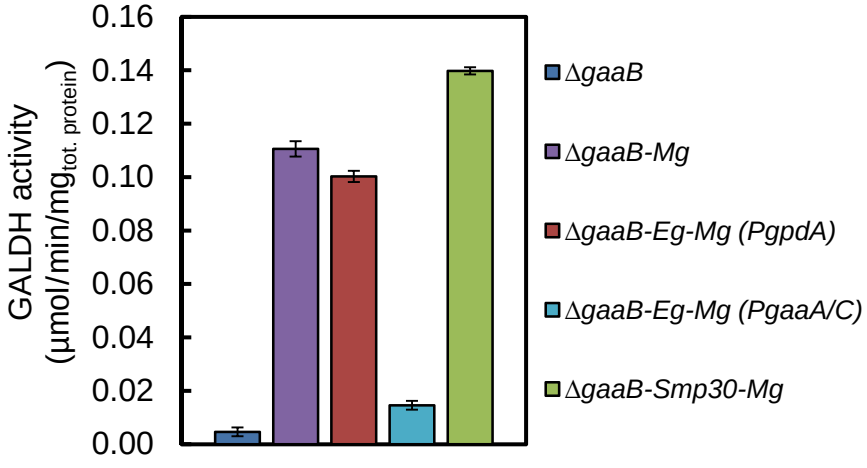


Figure 15. GALDH activity for L-galL in the crude extracts of Δ*gaaB*, Δ*gaaB-Mg* Δ*gaaB-Eg-Mg* (*PgpdA*), Δ*gaaB-Eg-Mg* (*PgaaA/C*) and Δ*gaaB-Smp30-Mg* strains cultured in minimal medium supplemented with D-galUA and D-xylose for 20 h. GALDH activity of the strains expressing *MgGALDH* differed significantly 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.

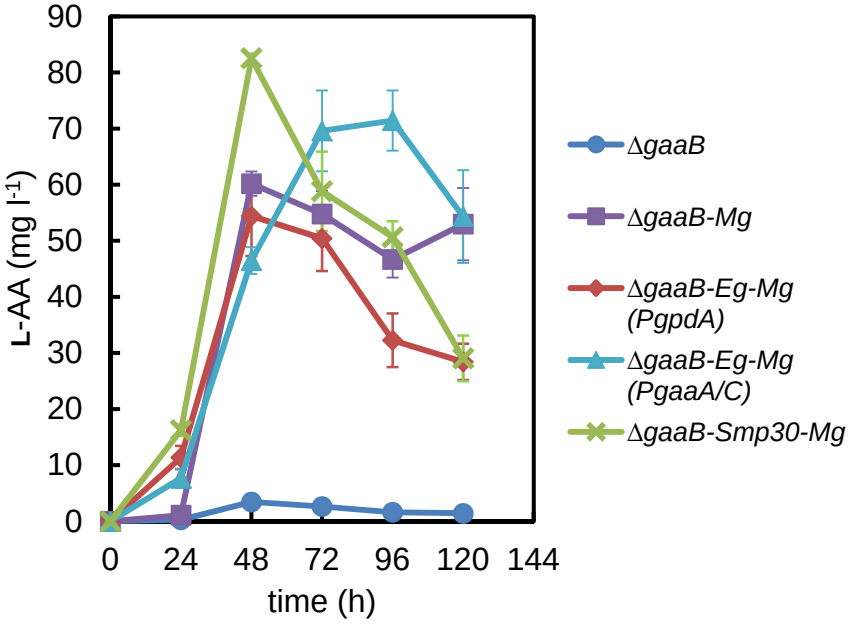


Figure 16. L-AA production by engineered *A. niger* strains in minimal medium supplemented with D-galUA and D-xylose. Error bars represent ± SEM (n=3).

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.

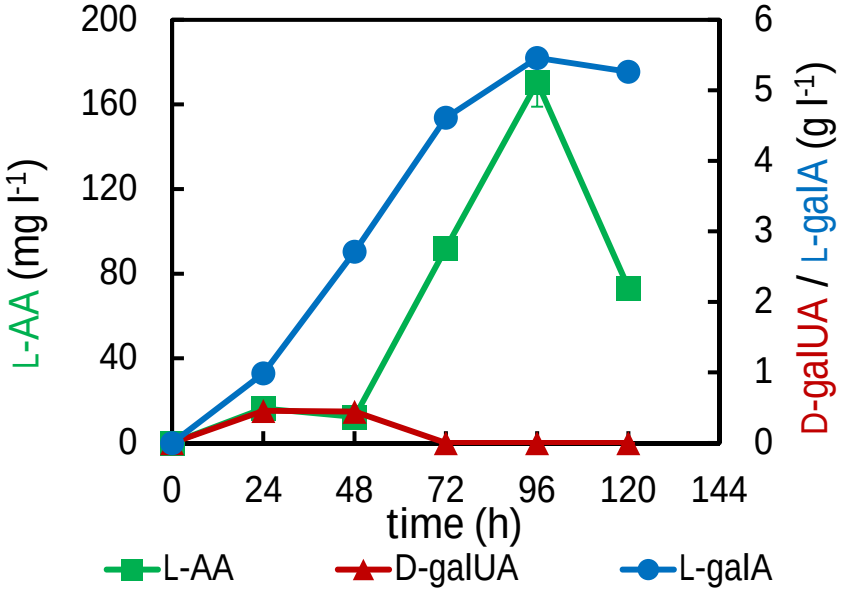


Figure 17. Concentrations of extracellular L-AA (green squares), D-galUA (red triangles) and L-galA (blue circles) in a culture of the strain Δ*gaaB-Eg-Mg* (*PgaaA/C*) in minimal medium supplemented with CPW. Error bars represent ± SEM (n=3) and where not visible, are smaller than the symbol.

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.

Acknowledgements

This study was financially supported by the Academy of Finland through the research program Sustainable Energy (grants 131869 and 271025).

References

1. Jong E, Higson A, Walsh P, Wellisch M (2012) Product developments in the bio-based chemicals arena. Biofuels, Bioproducts and Biorefining 6: 606-624

2. Werpy T, Petersen G (2004) Top value-added chemicals from biomass: Volume I - Results of screening for potential candidates from sugars and synthesis gas. Available at: http://www1.eere.energy.gov/biomass/pdfs/35523.pdf [April 1, 2015]

3. Holladay JE, White JF, Bozell JJ, Johnson D (2007) Top value-added chemicals from biomass: Volume II - Results of screening for potential candidates from biorefinery lignin. Available at: www1.eere.energy.gov/biomass/pdfs/pnnl-16983.pdf [April 1, 2015]

4. Bozell JJ, Petersen GR (2010) Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "top 10" revisited. Green Chem 12: 539-554

5. Geiser DM, Klich MA, Frisvad JC, Peterson SW, Varga J, Samson RA (2007) The current status of species recognition and identification in Aspergillus. Stud Mycol 59: 1-10

6. Lubertozzi D, Keasling JD (2009) Developing Aspergillus as a host for heterologous expression. Biotechnol Adv 27: 53-75

7. Ward OP, Qin WM, Dhanjoon J, Ye J, Singh A (2006) Physiology and biotechnology of Aspergillus. Adv Appl Microbiol 58: 1-75

8. Sheir-Neiss G, Lai MH, Morris NR (1978) Identification of a gene for β-tubulin in Aspergillus nidulans. Cell 15: 639-647

9. Morris NR, Lai MH, Elizabeth Oakley CE (1979) Identification of a gene for α-tubulin in Aspergillus nidulans. Cell 16: 437-442

10. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, Berriman M, Abe K, Archer DB, Bermejo C (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature 438: 1151-1156

11. Galagan JE, Calvo SE, Cuomo C, Ma L, Wortman JR, Batzoglou S, Lee S, Baştürkmen M, Spevak CC, Clutterbuck J (2005) Sequencing of Aspergillus nidulans and comparative analysis with A. fumigatus and A. oryzae. Nature 438: 1105-1115

12. Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto K, Arima T, Akita O, Kashiwagi Y (2005) Genome sequencing and analysis of Aspergillus oryzae. Nature 438: 1157-1161

13. Amaike S, Keller NP (2011) Aspergillus flavus. Annu Rev Phytopathol 49: 107-133

14. Machida M, Yamada O, Gomi K (2008) Genomics of Aspergillus oryzae: learning from the history of Koji mold and exploration of its future. DNA Res 15: 173-183

15. Fleißner A, Dersch P (2010) Expression and export: recombinant protein production systems for Aspergillus. Appl Microbiol Biotechnol 87: 1255-1270

16. Knuf C, Nookaew I, Brown SH, McCulloch M, Berry A, Nielsen J (2013) Investigation of malic acid production in Aspergillus oryzae under nitrogen starvation conditions. Appl Environ Microbiol 79: 6050-6058

17. Manzoni M, Rollini M, Bergomi S, Cavazzoni V (1998) Production and purification of statins from Aspergillus terreus strains. Biotechnol Tech 12: 529-532

18. Okabe M, Lies D, Kanamasa S, Park EY (2009) Biotechnological production of itaconic acid and its biosynthesis in Aspergillus terreus. Appl Microbiol Biotechnol 84: 597-606

19. Abarca ML, Accensi F, Cano J, Cabañes FJ (2004) Taxonomy and significance of black aspergilli. Antonie Van Leeuwenhoek 86: 33-49

20. Pel HJ, De Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K (2007) Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. Nat Biotechnol 25: 221-231

21. Andersen MR, Salazar MP, Schaap PJ, van de Vondervoort PJ, Culley D, Thykaer J, Frisvad JC, Nielsen KF, Albang R, Albermann K, Berka RM, Braus GH, Braus-Stromeyer SA, Corrochano LM, Dai Z, van Dijck PW, Hofmann G, Lasure LL, Magnuson JK, Menke H, Meijer M, Meijer SL, Nielsen JB, Nielsen ML, van Ooyen AJ, Pel HJ, Poulsen L, Samson RA, Stam H, Tsang A, van den Brink JM, Atkins A, Aerts A, Shapiro H, Pangilinan J, Salamov A, Lou Y, Lindquist E, Lucas S, Grimwood J, Grigoriev IV, Kubicek CP, Martinez D, van Peij NN, Roubos JA, Nielsen J, Baker SE (2011) Comparative genomics of citric-acid-producing Aspergillus niger ATCC 1015 versus enzyme-producing CBS 513.88. Genome Res 21: 885-897

22. 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: 1-8

23. Withers JM, Swift RJ, Wiebe MG, Robson GD, Punt PJ, van den Hondel, Cees AMJJ, Trinci AP (1998) Optimization and stability of glucoamylase production by recombinant strains of Aspergillus niger in chemostat culture. Biotechnol Bioeng 59: 407-418

24. van Dijck PW, Selten G, Hempenius RA (2003) On the safety of a new generation of DSM Aspergillus niger enzyme production strains. Regulatory Toxicology and Pharmacology 38: 27-35

25. Currie JN (1917) The citric acid fermentation of Aspergillus niger. J Biol Chem 31: 15-37

26. Dhillon GS, Brar SK, Verma M, Tyagi RD (2011) Recent advances in citric acid bio-production and recovery. Food and Bioprocess Technology 4: 505-529

27. Berovic M, Legisa M (2007) Citric acid production. Biotechnol Annu Rev 13: 303-343

28. Karaffa L, Kubicek CP (2003) Aspergillus niger citric acid accumulation: do we understand this well working black box? Appl Microbiol Biotechnol 61: 189-196

29. Ramachandran S, Fontanille P, Pandey A, Larroche C (2006) Gluconic acid: properties, applications and microbial production. Food Technology and Biotechnology 44: 185-195

30. Singh OV, Kumar R (2007) Biotechnological production of gluconic acid: future implications. Appl Microbiol Biotechnol 75: 713-722

31. Houbraken J, Frisvad JC, Samson RA (2011) Fleming's penicillin producing strain is not Penicillium chrysogenum but P. rubens. IMA Fungus: The Global Mycological Journal 2: 87

32. Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC (2009) Review of secondary metabolites and mycotoxins from the Aspergillus niger group. Analytical and bioanalytical chemistry 395: 1225-1242

33. Abarca M, Accensi F, Bragulat M, Cabañes FJ (2001) Current importance of ochratoxin A-producing Aspergillus spp. Journal of Food Protection® 64: 903-906

34. Agrawal R, Joseph R (2000) Bioconversion of alpha pinene to verbenone by resting cells of Aspergillus niger. Appl Microbiol Biotechnol 53: 335-337

35. Mishra D, Rhee YH (2014) Microbial leaching of metals from solid industrial wastes. Journal of Microbiology 52: 1-7

36. Prabha VL, Punekar N (2004) Genetic transformation in Aspergilli: tools of the trade. Indian Journal of Biochemistry and Biophysics 41: 205-215

37. Meyer V, Arentshorst M, El-Ghezal A, Drews A, Kooistra R, van den Hondel, Cees AMJJ, Ram AF (2007) Highly efficient gene targeting in the Aspergillus niger kusA mutant. J Biotechnol 128: 770-775

38. Van Hartingsveldt W, Mattern IE, van Zeijl CM, Pouwels PH, van den Hondel, Cees AMJJ (1987) Development of a homologous transformation system for Aspergillus niger based on the pyrG gene. Molecular and General Genetics MGG 206: 71-75

39. Dave K, Ahuja M, Jayashri T, Sirola RB, Punekar NS (2012) A novel selectable marker based on Aspergillus niger arginase expression. Enzyme Microb Technol 51: 53-58

40. Meyer V, Wanka F, van Gent J, Arentshorst M, van den Hondel CA, Ram AF (2011) Fungal gene expression on demand: an inducible, tunable, and metabolism-independent expression system for Aspergillus niger. Appl Environ Microbiol 77: 2975-2983

41. Liu L, Liu J, Qiu R, Zhu X, Dong Z, Tang G (2003) Improving heterologous gene expression in Aspergillus niger by introducing multiple copies of protein-binding sequence containing CCAAT to the promoter. Lett Appl Microbiol 36: 358-361

42. Sagt CM, ten Haaft PJ, Minneboo IM, Hartog MP, Damveld RA, van der Laan JM, Akeroyd M, Wenzel TJ, Luesken FA, Veenhuis M, van der Klei I, de Winde JH (2009) Peroxicretion: a novel secretion pathway in the eukaryotic cell. BMC Biotechnol 9: 48-6750-9-48

43. De Jongh W, Nielsen J (2008) Enhanced citrate production through gene insertion in Aspergillus niger. Metab Eng 10: 87-96

44. Meijer S, Otero J, Olivares R, Andersen MR, Olsson L, Nielsen J (2009) Overexpression of isocitrate lyase—glyoxylate bypass influence on metabolism in Aspergillus niger. Metab Eng 11: 107-116

45. Meijer S, Nielsen ML, Olsson L, Nielsen J (2009) Gene deletion of cytosolic ATP: citrate lyase leads to altered organic acid production in Aspergillus niger. J Ind Microbiol Biotechnol 36: 1275-1280

46. Li A, van Luijk N, ter Beek M, Caspers M, Punt P, van der Werf M (2011) A clone-based transcriptomics approach for the identification of genes relevant for itaconic acid production in Aspergillus. Fungal Genetics and Biology 48: 602-611

47. Li A, Pfelzer N, Zuijderwijk R, Punt P (2012) Enhanced itaconic acid production in Aspergillus niger using genetic modification and medium optimization. BMC biotechnology 12: 57

48. Blumhoff ML, Steiger MG, Mattanovich D, Sauer M (2013) Targeting enzymes to the right compartment: Metabolic engineering for itaconic acid production by Aspergillus niger. Metab Eng 19: 26-32

49. Kobayashi K, Hattori T, Honda Y, Kirimura K (2014) Oxalic acid production by citric acid-producing Aspergillus niger overexpressing the oxaloacetate hydrolase gene oahA. J Ind Microbiol Biotechnol 41: 749-756

50. Mohnen D (2008) Pectin structure and biosynthesis. Curr Opin Plant Biol 11: 266-277

51. O'Neill M, Albersheim P, Darvill A (1990) 12 The Pectic Polysaccharides of Primary Cell Walls. Carbohydrates 2: 415

52. O'Neill MA, Ishii T, Albersheim P, Darvill AG (2004) Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. Annu.Rev.Plant Biol. 55: 109-139

53. Guillon F, Thibault J, Rombouts FM, Voragen AG, Pilnik W (1989) Enzymic hydrolysis of the “hairy” fragments of sugar-beet pectins. Carbohydr Res 190: 97-108

54. Colquhoun IJ, Ralet M, Thibault J, Faulds CB, Williamson G (1994) Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. Carbohydr Res 263: 243-256

55. Coenen G, Bakx E, Verhoef R, Schols H, Voragen A (2007) Identification of the connecting linkage between homo-or xylogalacturonan and rhamnogalacturonan type I. Carbohydr Polym 70: 224-235

56. Duan J, Zheng Y, Dong Q, Fang J (2004) Structural analysis of a pectic polysaccharide from the leaves of Diospyros kaki. Phytochemistry 65: 609-615

57. Culleton H, McKie V, de Vries RP (2013) Physiological and molecular aspects of degradation of plant polysaccharides by fungi: what have we learned from Aspergillus? Biotechnology journal 8: 884-894

58. de Vries RP, Visser J (2001) Aspergillus enzymes involved in degradation of plant cell wall polysaccharides. Microbiol Mol Biol Rev 65: 497-522

59. van der Vlugt-Bergmans CJ, Meeuwsen PJ, Voragen AG, van Ooyen AJ (2000) Endo-xylogalacturonan hydrolase, a novel pectinolytic enzyme. Appl Environ Microbiol 66: 36-41

60. Khanh NQ, Ruttkowski E, Leidinger K, Albrecht H, Gottschalk M (1991) Characterization and expression of a genomic pectin methyl esterase-encoding gene in Aspergillus niger. Gene 106: 71-77

61. Searle-van Leeuwen M, Vincken J, Schipper D, Voragen A, Beldman G (1996) Acetyl esterases of Aspergillus niger: purification and mode of action on pectins. Progress in biotechnology 14: 793-798

62. Martens-Uzunova ES, Schaap PJ (2009) Assessment of the pectin degrading enzyme network of Aspergillus niger by functional genomics. Fungal Genetics and Biology 46: 170-179

63. Zhou M, de Vries RP, Wiegenga A, Robert V (2014) Fungal growth database. Available at: http://www.fung-growth org [April 1, 2015]

64. Hondmann DH, Busink R, Witteveen CF, Visser J (1991) Glycerol catabolism in Aspergillus nidulans. J Gen Microbiol 137: 629-636

65. Kuorelahti S, Kalkkinen N, Penttilä M, Londesborough J, Richard P (2005) Identification in the mold Hypocrea jecorina of the first fungal D-galacturonic acid reductase. Biochemistry 44: 11234-11240

66. Martens-Uzunova ES, Schaap PJ (2008) An evolutionary conserved d-galacturonic acid metabolic pathway operates across filamentous fungi capable of pectin degradation. Fungal Genetics and Biology 45: 1449-1457

67. Kuorelahti S, Jouhten P, Maaheimo H, Penttilä M, Richard P (2006) l-galactonate dehydratase is part of the fungal path for d-galacturonic acid catabolism. Mol Microbiol 61: 1060-1068

68. Hilditch S, Berghall S, Kalkkinen N, Penttila M, Richard P (2007) The missing link in the fungal D-galacturonate pathway: identification of the L-threo-3-deoxy-hexulosonate aldolase. J Biol Chem 282: 26195-26201

69. Liepins J, Kuorelahti S, Penttilä M, Richard P (2006) Enzymes for the NADPH-dependent reduction of dihydroxyacetone and D-glyceraldehyde and L-glyceraldehyde in the mould Hypocrea jecorina. Febs Journal 273: 4229-4235

70. Mojzita D, Penttila M, Richard P (2010) Identification of an L-arabinose reductase gene in Aspergillus niger and its role in L-arabinose catabolism. J Biol Chem 285: 23622-23628

71. Kuivanen J, Mojzita D, Wang Y, Hilditch S, Penttila M, Richard P, Wiebe MG (2012) Engineering filamentous fungi for conversion of D-galacturonic acid to L-galactonic acid. Appl Environ Microbiol 78: 8676-8683

72. Benz JP, Protzko RJ, Andrich JM, Bauer S, Dueber JE, Somerville CR (2014) Identification and characterization of a galacturonic acid transporter from Neurospora crassa and its application for Saccharomyces cerevisiae fermentation processes. Biotechnology for biofuels 7: 1-14

73. Gruben BS, Zhou M, Wiebenga A, Ballering J, Overkamp KM, Punt PJ, de Vries RP (2014) Aspergillus niger RhaR, a regulator involved in l-rhamnose release and catabolism. Appl Microbiol Biotechnol 98: 5531-5540

74. Ashwell G, Wahba AJ, Hickman J (1960) Uronic acid metabolism in bacteria. I. Purification and properties of uronic acid isomerase in Escherichia coli. J Biol Chem 235: 1559-1565

75. 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. Appl Biochem Biotechnol 1-7

76. Hickman J, Ashwell G (1960) Uronic acid metabolism in bacteria. II. Purification and properties of D-altronic acid and D-mannonic acid dehydrogenases in Escherichia coli. J Biol Chem 235: 1566-1570

77. Smiley JD, Ashwell G (1960) Uronic acid metabolism in bacteria. III. Purification and properties of D-altronic acid and D-mannonic acid dehydrases in Escherichia coli. J Biol Chem 235: 1571-1575

78. Cynkin MA, Ashwell G (1960) Uronic acid metabolism in bacteria. IV. Purification and properties of 2-keto-3-deoxy-D-gluconokinase in Escherichia coli. J Biol Chem 235: 1576-1579

79. Meloche HP, Wood WA (1964) Crystallization and Characteristics of 2-Keto-3-Deoxy-6-Phosphogluconic Aldolase. J Biol Chem 239: 3515-3518

80. Kilgore WW, Starr MP (1959) Uronate oxidation by phytopathogenic pseudomonads. Nature 183: 1412-1413

81. Zajic JE (1959) Hexuronic dehydrogenase of Agrobacterium tumefaciens. J Bacteriol 78: 734-735

82. Chang YF, Feingold DS (1969) Hexuronic acid dehydrogenase of Agrobacterium tumefaciens. J Bacteriol 99: 667-673

83. Chang YF, Feingold DS (1970) D-glucaric acid and galactaric acid catabolism by Agrobacterium tumefaciens. J Bacteriol 102: 85-96

84. Molhoj M, Verma R, Reiter WD (2004) The biosynthesis of D-Galacturonate in plants. functional cloning and characterization of a membrane-anchored UDP-D-Glucuronate 4-epimerase from Arabidopsis. Plant Physiol 135: 1221-1230

85. Agius F, González-Lamothe R, Caballero JL, Muñoz-Blanco J, Botella MA, Valpuesta V (2003) Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. Nat Biotechnol 21: 177-181

86. Valpuesta V, Botella MA (2004) Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant. Trends Plant Sci 9: 573-577

87. Ishikawa T, Nishikawa H, Gao Y, Sawa Y, Shibata H, Yabuta Y, Maruta T, Shigeoka S (2008) The pathway via D-galacturonate/L-galactonate is significant for ascorbate biosynthesis in Euglena gracilis: identification and functional characterization of aldonolactonase. J Biol Chem 283: 31133-31141

88. Edwards MC, Doran-Peterson J (2012) Pectin-rich biomass as feedstock for fuel ethanol production. Appl Microbiol Biotechnol 95: 565-575

89. USDA (2013) (United States Department of Agriculture). Citrus: World markets and trade 8. Available at: http://www.fas.usda.gov/psdonline/circulars/citrus.pdf [April 1, 2015]

90. Mamma D, Christakopoulos P (2014) Biotransformation of Citrus By-Products into Value Added Products. Waste and Biomass Valorization, 2014, 5.4: 529-549

91. 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: 1-8

92. Pourbafrani M, Forgács G, Horváth IS, Niklasson C, Taherzadeh MJ (2010) Production of biofuels, limonene and pectin from citrus wastes. Bioresour Technol 101: 4246-4250

93. Grohman K, Cameron R, Kim Y, Widmer W, Luzio G (2013) Extraction and recovery of pectic fragments from citrus processing waste for co–production with ethanol. Journal of Chemical Technology and Biotechnology 88: 395-407

94. Staunstrup, J (2009) 49th Annual short course for the food industry: The international citrus and beverage conference.

95. López J, Li Q, Thompson I (2010) Biorefinery of waste orange peel. Crit Rev Biotechnol 30: 63-69

96. Van Maris AJA, Abbott DA, Bellissimi E, Brink Jvd, Kuyper M, Luttik MAH, Wisselink HW, Scheffers WA, Dijken JPv, Pronk JT (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: current status. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 90: 391-418

97. Kamel S, Mentaverri R, Lion JM, Brazier M and Courtois-Sambourg J (2009) USE OF A COMPOSITION OF OLIGOMERS OF GALACTURONIC ACID AS AN AGENT FOR INHIBITING DEGRADATION OF COLLAGEN. Patent application WO2009004152

98. Castillo DH and Trevino CI (2011) USE OF GALACTURONIC ACID FOR REMOVING METALLIC IONS (AL, AS, BA, CD, CU, CR, FE, MN, HG, PB, NA, ZN) FROM WATER RESULTING FROM HUMAN CONSUMPTION WELLS. Patent application MX2010004385

99. Huisjes EH, Luttik MA, Almering MJ, Bisschops MM, Dang DH, Kleerebezem M, Siezen R, van Maris AJ, Pronk JT (2012) Toward pectin fermentation by Saccharomyces cerevisiae: Expression of the first two steps of a bacterial pathway for d-galacturonate metabolism. J Biotechnol 162: 303-310

100. Souffriau B, den Abt T, Thevelein JM (2012) Evidence for rapid uptake of d-galacturonic acid in the yeast Saccharomyces cerevisiae by a channel-type transport system. FEBS Lett 586: 2494-2499

101. Mojzita D, Wiebe M, Hilditch S, Boer H, Penttilä M, Richard P (2010) Metabolic engineering of fungal strains for conversion of D-galacturonate to meso-galactarate. Appl Environ Microbiol 76: 169-175

102. Guoxiang, W.Li Z and Chunyang Z. (2011) CN20101575384,

103. Bonhoure J, Abboud H, Aussenac TR, Coste CU, Hoang L, RalainIrina R, Rannou AC (2013) NEW METHOD FOR SYNTHESISING MUCIC ACID USING OZONE. Patent application WO2013FR50571

104. Li X, Wu D, Lu T, Yi G, Su H, Zhang Y (2014) Highly Efficient Chemical Process To Convert Mucic Acid into Adipic Acid and DFT Studies of the Mechanism of the Rhenium-Catalyzed Deoxydehydration. Angewandte Chemie 126: 4284-4288

105. Wiebe MG, Mojzita D, Hilditch S, Ruohonen L, Penttilä M (2010) Bioconversion of D-galacturonate to keto-deoxy-L-galactonate (3-deoxy-L-threo-hex-2-ulosonate) using filamentous fungi. BMC biotechnology 10: 63

106. Barratt RW, Johnson GB, Ogata WN (1965) Wild-type and mutant stocks of Aspergillus nidulans. Genetics 52: 233

107. Csiba M, Cleophax J, Petit S, Gero S (1993) An expedient and practical three-step synthesis of vitamin C from a byproduct of the sugar industry: the L-galactono-1, 4-lactone pathway. J Org Chem 58: 7281-7282

108. Roland JF, Cayle T, Dinwoodie RC, Mehnert DW (1986) Fermentation production of ascorbic acid from l-galactonic substrate. US Patent 4,595,659

109. Sloothaak J, Schilders M, Schaap PJ, de Graaff LH (2014) Overexpression of the Aspergillus niger GatA transporter leads to preferential use of D-galacturonic acid over D-xylose. AMB Express 4: 1-9

110. Burgstaller W (2006) Thermodynamic boundary conditions suggest that a passive transport step suffices for citrate excretion in Aspergillus and Penicillium. Microbiology 152: 887-893

111. Viniegra-González G, Favela-Torres E (2006) Why solid-state fermentation seems to be resistant to catabolite repression? Food Technology and Biotechnology 44: 397

112. Solis-Pereira S, Favela-Torres E, Viniegra-González G, Gutiérrez-Rojas M (1993) Effects of different carbon sources on the synthesis of pectinase by Aspergillus niger in submerged and solid state fermentations. Appl Microbiol Biotechnol 39: 36-41

113. Martens-Uzunova ES, Schaap PJ (2008) An evolutionary conserved D-galacturonic acid metabolic pathway operates across filamentous fungi capable of pectin degradation. Fungal Genetics and Biology 45: 1449-1457

114. Watanabe S, Saimura M, Makino K (2008) Eukaryotic and bacterial gene clusters related to an alternative pathway of nonphosphorylated L-rhamnose metabolism. J Biol Chem 283: 20372-20382

115. Rakus JF, Fedorov AA, Fedorov EV, Glasner ME, Hubbard BK, Delli JD, Babbitt PC, Almo SC, Gerlt JA (2008) Evolution of Enzymatic Activities in the Enolase Superfamily: l-Rhamnonate Dehydratase. Biochemistry 47: 9944-9954

116. Guimaraes M, Veiga L (1990) An Oxidative Pathway for L-Fucose Dissimilation in Fungi. Arq Biol Tecnol 33: 645-655

117. Gella FJ, Olivella MT, Pegueroles F, Gener J (1981) Colorimetry of diaphorase in commercial preparations and clinical chemical reagents by use of tetrazolium salts. Clin Chem 27: 1686-1689

118. Cooper RA (1979) The pathway for L-galactonate catabolism in Escherichia coli K-12. FEBS Lett 103: 216-220

119. Reed JL, Patel TR, Chen KH, Joyce AR, Applebee MK, Herring CD, Bui OT, Knight EM, Fong SS, Palsson BO (2006) Systems approach to refining genome annotation. Proceedings of the National Academy of Sciences 103: 17480-17484

120. Choi MS, Saxena A, Chilukuri N (2010) A strategy for the production of soluble human senescence marker protein-30 in Escherichia coli. Biochem Biophys Res Commun 393: 509-513

121. He XX, Luo G, Zhou S (2013) Soluble Expression of Recombinant Human Smp30 for Detecting Serum Smp30 Antibody Levels in Hepatocellular Carcinoma Patients. Asian Pacific Journal of Cancer Prevention 14: 2383-2386

Series title and number

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.
ISBN, ISSN	ISBN 978-951-38-8340-9 (Soft back ed.) ISBN 978-951-38-8341-6 (URL: http://www.vttresearch.com/impact/publications) ISSN-L 2242-119X ISSN 2242-119X (Print) ISSN 2242-1203 (Online)
Date	August 2015
Language	English, Finnish abstract
Pages	82 p. + app. 49 p.
Name of the project	
Commissioned by	
Keywords	filamentous fungi, Aspergillus niger, pectin, D-galacturonic acid, L-galactonic acid, L-ascorbic acid, metabolic engineering
Publisher	VTT Technical Research Centre of Finland Ltd P.O. Box 1000, FI-02044 VTT, Finland, Tel. 020 722 111

Julkaisun sarja ja numero

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.
ISBN, ISSN	ISBN 978-951-38-8340-9 (nid.) ISBN 978-951-38-8341-6 (URL: http://www.vtt.fi/julkaisut) ISSN-L 2242-119X ISSN 2242-119X (Painettu) ISSN 2242-1203 (Verkkojulkaisu)
Julkaisuaika	Elokuu 2015
Kieli	Englanti, suomenkielinen tiivistelmä
Sivumäärä	82 s. + liitt. 49 s.
Projektin nimi	
Rahoittajat	
Avainsanat	filamentous fungi, Aspergillus niger, pectin, D-galacturonic acid, L-galactonic acid, L-ascorbic acid, metabolic engineering
Julkaisija	Teknologian tutkimuskeskus VTT Oy PL 1000, 02044 VTT, puh. 020 722 111

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.

ISBN 978-951-38-8340-9 (Soft back ed.)
ISBN 978-951-38-8341-6 (URL: http://www.vttresearch.com/impact/publications)
ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)