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Engenharia do catabolismo do ácido hexurónico

Engineering hexuronic acid catabolism

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Dr. rer. nat. Peter Richard, Cientista Principal da VTT Technical Research Centre of Finland, Espoo, Finlândia e co-orientação científica da Professora Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora Auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Aspergillus niger, Ácido L-Galactónico, Fermentação em estado sólido, Fermentação submersa, Ácido D-Glucurónico, Engenharia metabólica

resumo

A engenharia metabólica é uma área emergente que visa o aperfeiçoamento de vias metabólicas para produção de compostos valiosos.

A produção mundial de casca de frutos cítricos é estimada em 15,000,000 de toneladas por ano, e o seu descarte causa problemas ambientais. O principal constituinte da casca de frutos cítricos é o ácido D-galacturónico. O objetivo deste projeto é converter o ácido D-galacturónico noutros químicos proveitosos, utilizando para tal bolores geneticamente modificados. *Aspergillus niger* foi escolhido por ser naturalmente um bom consumidor do ácido D-galacturónico e produtor das enzimas necessárias à hidrólise de casca de frutos cítricos.

No presente trabalho, estirpes de *Aspergillus niger* foram geneticamente modificados onde (i) o gene gaaB que codifica para a L-galactonato desidratase foi deletado (*ΔgaaB*) e (ii) o gene gaaB foi deletado e o gene gaaA que codifica para a D-galacturonato reductase se encontrava sobreexpresso (*ΔgaaB-gaaA)*. Estas estirpes foram utilizadas para fermentação submersa e em estado sólido para converter casca de laranja em L-galactonato num processo consolidado. As estirpes foram capazes de converter, até 87 %, de ácido D-galacturónico em L-galactonato por fermentação em estado sólido.

Outra via metabólica estudada foi a via eucariota do ácido glucurónico. Nesta via metabólica é uma descarboxilase que converte o 3-ceto-L-gulonato em L-xilulose. A reação ainda não está claramente caracterizada e o gene não é conhecido. Um teste enzimático acoplado foi realizado de forma a testar a sua atividade. Neste ensaio o ácido L-gulónico é o substrato inicial, uma L-gulonato-3-desidrogenase NAD-dependente (GDH) que produz o substrato para a descarboxilase. A L-xilulose reductase é então detetada por uma L-xilulose reductase NADPH-dependente de *Aspergillus niger* (*lxr*A). Para seguir a reação, o NADPH foi monitorizado a 340 nm. Para evitar a interferência do NADH que também absorve a 340 nm, Tio-NAD^+ foi usado para a desidrogenase.

GDH e *lxr*A ativas foram preparadas e o ensaio testado com precipitados sulfato de amónio de extrato de fígado bovino. A atividade da 3-ceto-L-gulonato descarboxilase não foi detetada.

keywords

Aspergillus niger, L-galactonate, Solid-state Fermentation, Submerged-state Fermentation. D-Glucuronic acid, Metabolic engineering

abstract

Metabolic engineering is an emerging field targeted to the improvement of pathways for the production of high-value compounds.

Citrus peel is produced at an estimated 15,000,000 t per year worldwide and its disposal causes environmental problems. The main constituent of citrus peel is D-galacturonic acid. The aim of this project is to convert D-galacturonic acid to useful chemicals using genetically engineered moulds. *Aspergillus niger* was chosen since it is naturally a good consumer of D-galacturonic acid and produces the enzymes required for citrus peel hydrolysis.

In the present study, engineered *Aspergillus niger* strains were used where (i) the gaaB coding for L-galactonate dehydrogenase was deleted (*ΔgaaB*) and (ii) the gaaB was deleted and the gaaA coding for D-galacturonate reductase was overexpressed (*ΔgaaB-gaaA).* These strains were used for solid-state and submerged state fermentation to convert orange peel to L-galactonate in a consolidate process. The strains were able to convert up to 87 % of D-galacturonic acid to L-galactonate by solid-state fermentation.

Another pathway that was studied was the eukaryotic D-Glucuronic acid pathway. In this pathway is a ddecarboxylase that converts 3-keto-L-gulonate to L-xylulose. The reaction is poorly characterized and the gene not known. It was tried to assay this activity in a coupled enzyme assay. In this assay L-gulonic acid is the initial substrate, an NAD-dependent L-gulonate-3-dehydrogenase (GDH) that produces the substrate for the decarboxylase. L-xylulose reductase is then detected by an NADPH-dependent L-xylulose reductase from *Aspergillus niger* (*lxr*A). To follow the reaction NADPH was monitored at 340 nm. To avoid the interference of NADH that also absorbs at 340 nm Thio-NAD^+ was used for the dehydrogenase.

Active GDH and *lxr*A were prepared and the assay tested with ammonium sulfate precipitates from bovine liver extract. A 3-keto-L-gulonate decarboxylase activity could not be detected.

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ΔgaaA	Delected D-galacturonate reductase of *Aspergillus niger*
ΔgaaB	Delected L-galactonate dehydratase of *Aspergillus niger*
ΔgaaB-gaaA	Delected L-galactonate dehydratase with overexpression of D-galacturonate reductase of *Aspergillus niger*
ATCC	American type culture collection
D-GalUA	D-galacturonic acid
gaaA	D-galacturonate reductase of *Aspergillus niger*
gaaB	L-galactonate dehydratase of *Aspergillus niger*
gaaC	2-keto-3-deoxy-L-galactonate aldolase of *Aspergillus niger*
gaaD	Glyceraldehyde reductase of *Aspergillus niger*
gar1	D-galacturonate reductase of *Trichoderma reesei*
GDH	L-gulonate-3-dehydrogenase
gld1	Glyceraldehyde reductase of *Trichoderma reesei*
HG	Homogalacturonan
HPLC	High performance liquid chromatography
LB	Luria broth

lgd1	L-galactonate dehydratase of *Trichoderma reesei*
*lxr*A	L-xylulose reductase of *Aspergillus niger*
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
NS	Nutritionally supplemented fermentation
PGNU	Polygalacturonase unit
qPCR	Real-time quantitative polymerase chain reaction
RGI	Rhamnogalacturonan I
RGII	Rhamnogalacturonan II
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SmF	Submerged-state fermentation
SSF	Solid-state fermentation
XGA	Xylogalacturonan

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I. BIOTECHNOLOGICAL CONVERSION OF D-GALACTURONIC ACID TO L-GALACTONATE

1. Introduction

1.1. Orange peel

Brazil is listed as the world's largest producer of oranges according to the Statistical Database of the Food and Agriculture Organization of the United Nations (FAOSTAT), representing 59 % of world orange output with values greater than 88,000,000 tons (Marín, et al., 2007).

The production of orange juice results in 50-60 % of peel, seeds and membrane residues which are discarded and ending up as significant agricultural wastes. In order to drop the waste rate, orange peel was traditionally treated as many other plant substrates: used as cattle feed. Indeed, that kind of utilization of orange wastes showed to be the most direct alternative, but several factories just burned or dumped such solid residues in landfills (Richard & Hilditch, 2009). The reason included the fact that only a percentage would be utilized, because it was reported that orange peel would contain toxins (also causing rumen parakeratosis) and processing to remove the toxin would increase energy costs (Lohrasbi, et al., 2010).

The economic and environmental problems motivated researchers to analyse the orange peel composition and the possibility to include it as a substrate for Biorefinery. The composition of orange peel is different in different orange species but it is now known that orange peel can be converted to high-value compounds, as it contains insoluble sugars in similar percentage of the middle lamella of primary cell walls of plants - alongside from organic polymers such as cellulose, hemicellulose and lignin, one-third is composed by

pectin (Hilditch, 2010). The variety of spices of orange peel is seen in their composition. Rivas et. al 2008, reported the composition of Valencia orange, containing approximately 17 % soluble sugar, 9 % cellulose, 10 % hemicellulose and 42 % pectin as the main components (Rivas, et al., 2008).

Homogalacturonan (HG) is the simplest of the four domains of pectin which consists of a linear backbone of α-(1,4)-linked D-galacturonic acid residues which can be methylated on O-6 and/or acetylated on O-2 and O-3 (de Vries & Jaap, 2011).

A ramified HG region with β-(1-3)-link of β-D-xylose residues, constructs xylogalacturonan (XGA). However, a more complex structure composed with alternate α-(1,2)-linked L-rhamnose might also be present in pectin main structure - the rhamnogalacturonan I (RGI) domain. RGI is also composed with galacturonic acid residues in those ramifications, linked to L-rhamnose residues being somehow substituted by L-arabinose, both attached at O-4 (de Vries & Jaap, 2011)

Rhamnogalacturonan II (RGII) backbone carries four side chains with rare sugars such as 2-*O*-methyl-L-fucose and 3-deoxy-D-*manno*-2-octulosonic acid, linked to nine α-(1,4)-linked D-galacturonic acid. The linkage of these sugars is not well known and a complete hydrolysis of complex structures as RGII evolves a refined cocktail of enzymes (Figure 1) (de Vries & Jaap, 2011).

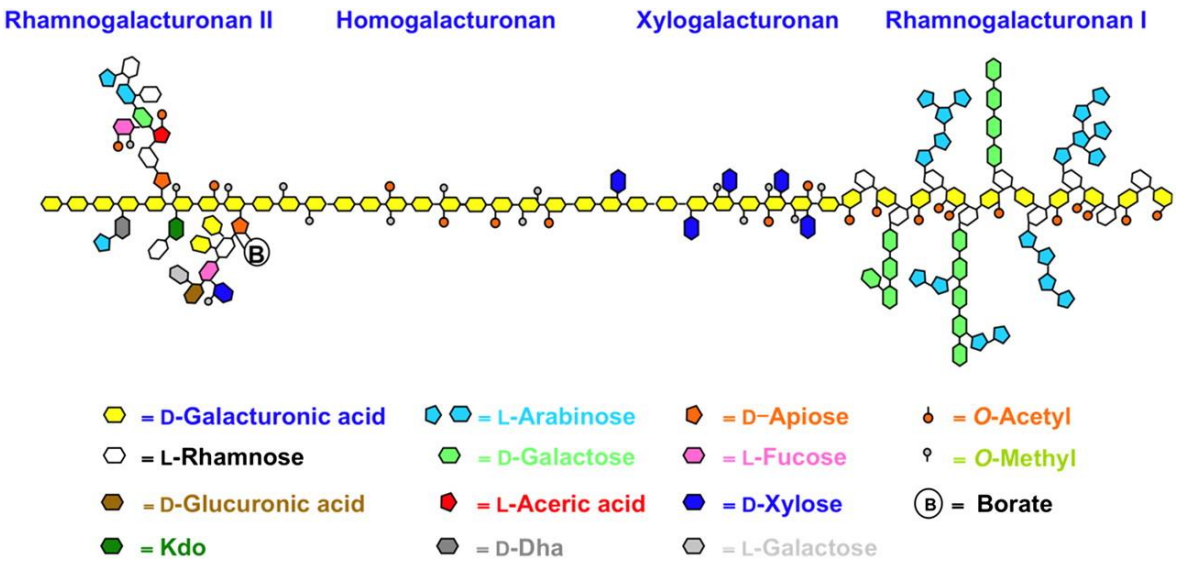


Figure 1 – Pectin: the structures of rhamnogalacturonan II, homogalacturonan, xylogalacturonan and rhamnogalacturonan I.

Pectin can be used for gelling, thickening and is widely used in the pharmaceutical, cosmetic and food industries. The main monosaccharide, D-galacturonic acid, finds limited use as chelating agent, nonetheless is a potential monomer as it represents 70 % of total pectin and can be converted to high-valuable compounds: D-Galacturonic acid may be electrolytically oxidised to galactaric (mucic) acid; the reduction of this monomer in the eukaryotic pathway results in the production of L-galactonic acid or L-galactonate; L-galactono-1,4-lactone that is formed upon acidification of L-galactonate can be chemically or through a fermentative process converted to L-ascorbic acid (vitamin C) (Burana-osota, et al., 2010) (Wiebe, et al., 2010).

Kuivanen et. al 2012, first reported an extracellular accumulation of this precursor of L-ascorbic acid by an engineered strain of *Aspergillus niger* from polygalacturonate. Therefore, this compound has the potential to be produced from pectin rich residues and to be more widely used, as L-galactonate is currently an expensive chemical. Furthermore, L-galactonate has similar physicochemical properties to D-gluconic acid that is used as a food acidifier and as a chelator in dyes, detergents, solvents and paints (Kuivanen, et al., 2012). The removal of water from L-galactonate leads to the formation of keto-deoxy-L-galactonic acid (3-deoxy-L-threo-hex-2-ulosonic acid), which has potential as precursors in the synthesis of medicinal compounds (Wiebe, et al., 2010).

1.2. *Aspergillus niger*

The genus *Aspergillus* was firstly cataloged in Micheli's *Nova Plantarum Genera* in 1729, when Antonio Micheli reported a structure that resemble the holy water sprinkler – aspergillum. However, merely one century later Mayer observed the genus in birds, followed by John Hughs Bennett in 1847, describing aspergilloma (Bennett, 2009).

In the beginning of twentieth century, the fungi started to be classified. A review of the first classification proposed to include 14 groups within the genus, with some pathogenic fungi such as *A. fumigatus*, *A. flavus* and *A. parasiticus* and the black aspergilli *A. niger* and *A. tubingensis* (de Vries & Jaap, 2011)**.**

Nowadays, the number of species is uncertain, however is estimated to range from 260 to 837 (Krijgsheld, et al., 2013). Aspergillus is included in Ascomycota division, a

group of filamentous fungi famous for its ability to grow on rather simple substrates and in extreme cultivation conditions (Ryan & Ray, 2004). It is classified as a saprophytic aerobic mold that requires exogenous carbon for growth, producing hyphae and propagating asexually forming mycelium and spores (Levinson, 2008) (Ciganovich, 1999).

Aspergillus is known to be one of the most common molds on earth and as an interesting tool for metabolic engineering (Bennett, 2009) (Kuivanen, et al., 2012). Meyer, et al., 2010 shown that *Aspergillus* can grow in an extensive sort of temperature of 10-50ºC, pH of 2.0-10, salinity of 0-34 %, water activity of 0.6-1.0. Hence, as this filamentous fungus has been given GRAS state (generally regarded as safe) by the U.S. Food and Drug Administration, it makes them suitable organisms for industrial applications such as for solid-state and submerged-state fermentations (Meyer, et al., 2010).

Traditionally, *A. niger* biotechnological application is citric acid production, becoming increasingly important in microbial cell factory. The production of pectinases with solid-state and submerged-state fermentation is used for food industry, mainly for juice extraction and clarification (de Vries & Jaap, 2011). Moreover, the usage of *Aspergillus niger* in Biorefinery symbolizes an exciting challenge: the lack of efficient genetic engineering tools for mold and the production of new and commercially interesting metabolites by simple fermentative processes.

1.3. D-galacturonic acid pathway in *Aspergillus niger*

A bacterial pathway for the catabolism of D-galacturonic acid was described for *E. coli* involving five enzymes and pyruvate and D-glyceraldehyde-3-phospate as final products, in 1950's by Gilbert Ashwell. In the same period, an oxidative pathway for *Pseudomonas* and *Agrobacterium tumefaciens* was shown to exist, having *meso*-galactaric acid (mucic acid) as an intermediate.

A third pathway was described in the eukaryots *Trichoderma reesei*, *Botrytis cinerea* and *Aspergillus niger*. *A. niger* has also the ability to produce efficiently extracellular enzymes with the ability to hydrolyze pectin and to grow in D-galacturonic acid (Figure 2) (Richard & Hilditch, 2009). The pathway for D-galacturonic acid catabolism in *A. niger* is similar to the *Trichoderma reesei*, both reductive pathways. D-

galacturonic acid is firstly reduced to L-galactonate by the D-galacturonate reductase (gar1 or gaaA in *T. reesei* and *A. niger*, respectively).

In a second step, L-galactonate is converted to 2-keto-3-deoxy L-galactonate (3-deoxy-L-threo-hex-2-ulosonate) by the action of L-galactonate dehydratase (Tr lgd1 or An gaaB), which is then split by a 3-deoxy-L-threo-hex-2-ulosonate aldolase (Tr lgd1 or An gaaC) to pyruvate and L-glyceraldehyde. In the final step L-glyceraldehyde is converted by glyceraldehyde reductase to glycerol (Tr gld1 or An gaaD) (Kuivanen, et al., 2012) (Richard & Hilditch, 2009) (Kuorelahti, et al., 2006).

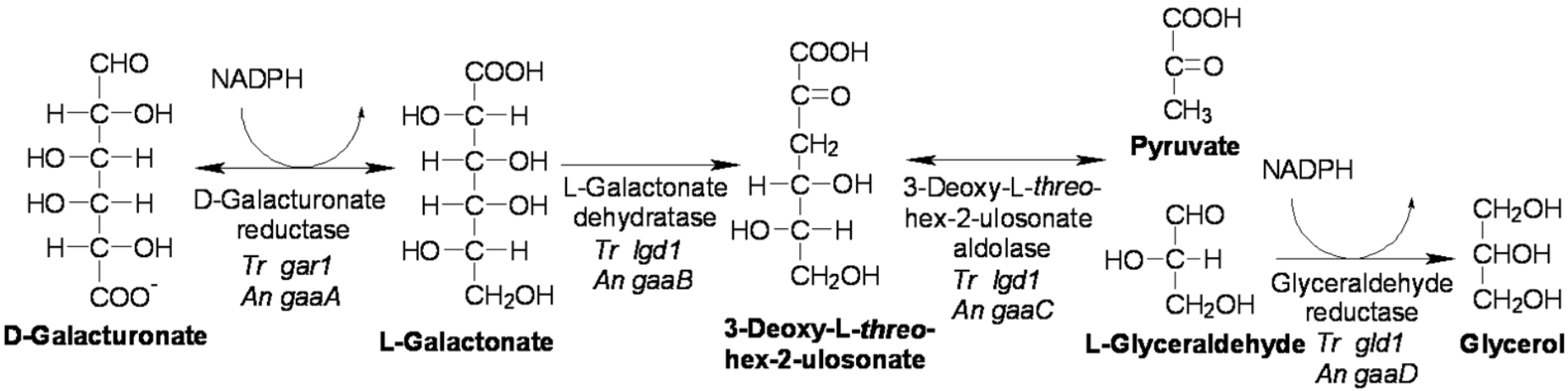


Figure 2 – The fungal pathway for D-galacturonic acid catabolism by Kuivanen, et al., 2012.

1.4. Objectives of the work

In 2012, Kuivanen et. al demonstrated the conversion of pure D-galacturonic acid and polygalacturonate to L-galactonate by submerged-state fermentation with the use of metabolically engineered fungal strains. The engineered *Aspergillus niger* strains were able to produce L-galactonate from pure D-galacturonic acid in a submerged-state fermentation (Kuivanen, et al., 2012).

Whilst *Trichoderma reesei* is generally used for the production of hydrolytic enzymes (Miettinen-Oinonen, 2004), *Aspergillus niger* shows the ability to degrade pectin, thus can be used for hydrolysis of pectin-rich residue such as orange peel. The resulting monomeric D-galacturonic acid would be converted to a higher value product: L-galactonate.

In this study, solid and submerged-state fermentation of orange peel using genetically engineered *Aspergillus niger* will be conducted, in order to produce L-galactonate. The accumulation of the higher value compound will be studied by disrupting the pathway. The deletion and overexpression of the gene encoding D-galacturonic acid pathway enzymes will be compared to *Aspergillus niger* wild type strain (Figure 3).

Furthermore, it will be tested whether the conversion of the resulting D-galacturonic acid to L-galactonic acid could be combined to a single fermentation process.

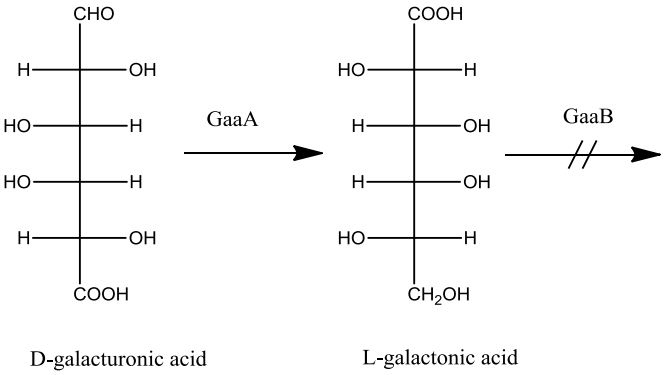


Figure 3 – Deletion of gaaB for the accumulation of L-galactonic acid in D-galacturonic acid pathway of *A. niger*.

2. Materials and Methods

2.1. *Aspergillus niger*

The filamentous fungus *Aspergillus niger* ATCC 1015 strain was used as wild type. In order to study D-galacturonic acid and L-galactonate production, strains ATCC 1015 Δ*gaaA*, Δ*gaaB* and Δ*gaaB-gaaA* were engineered. Respectively, it was deleted the gene encoding D-galacturonate reductase and the gene encoding L-galactonic acid dehydratase as described by Mojzita; and overexpressed the galacturonate reductase coding gene, *gaaA*, in the Δ*gaaB* as described by Kuivanen (Mojzita, et al., 2010) (Kuivanen, et al., 2012).

2.2. Treatment of orange peel

The orange peel was obtained from a local restaurant in Curitiba-Paraná, in Brazil and it was treated to increase the relative percentage of pectin and surface availability, to be further fermented by the filamentous fungi.

The orange peel was dried at 60°C and shredded with a grinder to a calibre ranging 0.85-2.36 mm. The ground orange peel was washed with water and dried three times, again at 60°C. Lastly, it was autoclaved for 40 min at 120°C.

2.3. Orange peel composition

In order to know orange peel composition and to define the maximal theoretical yield, the orange peel was enzymatically hydrolysed: 1 g of dried orange peel was hydrolysed with 127 PGNU ml^{-1} of a commercial pectinase (Pectinex Ultra SP-L, Novozymes) in the final volume of 30 ml. The reaction mixture was incubated overnight, mild-shaking (100 rpm) at 30 °C. The hydrolysed suspension was centrifuged at 13,500 rpm for 20 min and the supernatant was quantified by HPLC.

2.4. Solid-state fermentation

Aspergillus niger spores were collected with a solution of 0.8 % NaCl, 0.025 % Tween 20 and 20 % Glycerine and filtered with a sterile cotton-membrane. Spore suspension was counted with Cellometer® Auto M10, following manufacturer's instructions.

Solid-state fermentation (SSF) was carried out in 100 ml Erlenmeyer flasks containing 2 g of the dried orange peel. The substrate was moisturized and inoculated with 3 ml (g orange peel)$^{-1}$ of sterile water with 10^7 spores (g orange peel)$^{-1}$ (Figure 4). The Erlenmeyer flasks were incubated at 28 °C and a relative humidity of 96 %. Also, the effect of a nutritional supplementation in flask were studied by moisturizing the substrate with sterile water or a solution containing 3 g l^{-1} K_2HPO_4, 13 g l^{-1} $(NH_4)_2SO_4$, 5 g l^{-1} $MgSO_4*7H_2O$, 10 g l^{-1} KCl and 0.1 g l^{-1} $FeSO_4*7H_2O$. These fermentations were incubated at 28 °C without humidity control.

Figure 4 – Non-supplemented SSF in 100 ml Erlenmeyer flask after 4 days of fermentation.

Fixed bed column bioreactors (28 cm by 3.5 cm) with aeration were filled with 10 g of dried orange peel in order to enhance fermentation. The substrate was moisturized and inoculated as above. Columns were incubated at 28 °C in a water-bath and a sub-

compartment with water saturated air was blown through the substrate at 150 ml min^{-1} (Figure 5).

All solid-state fermentations performed had no pH adjustment. Moreover, each fermentation product was extracted with 30 ml (g orange peel)$^{-1}$ of sterile water and incubated at 28 °C, 250 rpm for an hour. The resulting liquid collected by vacuum filtration was analysed with HPLC to quantify D-galacturonic acid and L-galactonate.

Figure 5 – Fixed bed column bioreactors for SSF containing inoculated orange peel.

2.5. Submerged-state fermentation

Pre-grown mycelia of *A. niger* were used for submerged-state fermentation (SmF). 250 ml Erlenmeyer flasks containing 10 g yeast extract l^{-1}, 20 g peptone l^{-1} and 30 g gelatine l^{-1} were inoculated with spore suspension and incubated overnight at 28 °C, 250 rpm. The mycelia were extracted with alternating warm and cold sterile water rinses by vacuum filtration.

The submerged cultures containing 2 g of orange peel were moisturized with sterile water from 20 to 25 ml (g orange peel)$^{-1}$ in 250 ml Erlenmeyer flasks, without pH

adjustment. Then, 3.4 to 5.5 g of pre-grown mycelia (wet) l^{-1} was inoculated and incubated at 28 °C and 250 rpm (Figure 6).

So as to scale up the process, 20 g of orange peel were fermented in a 2 L Erlenmeyer flask containing 500 ml of sterile water with Δ*gaaB* (Table 1).

Figure 6 – Non-supplemented SmF in 250 ml Erlenmeyer flask after 4 days of fermentation.

Fermentation scale	Conditions	Strains	Concentration	Mycelia
Small scale	Water	Wild type	40 g l^{-1}	0.177 g
		Δ*gaaB*		
		Δ*gaaA*	50 g l^{-1}	0.100 g
		Δ*gaaB-gaaA*		
	Nutritional supplementation	Wild type	40 g l^{-1}	0.100 g
		Δ*gaaB*		
Big scale	Water	Δ*gaaB*	40 g l^{-1}	3.02 ± 0.268 g

Table 1 – Conditions used for different submerged-state fermentations.

In the submerged orange peel cultures 2 ml were collected to an Eppendorf with a sterile cut tip and centrifuged for 30 min at 13,500 rpm, in order to separate mycelia and insoluble substrate for further HPLC analysis.

2.6. L-galactonate purification

L-galactonate was purified from the resulting sample of big scale SmF by Δ*gaaB*, which every replicates were combined (approximately 1,5 L). Firstly, the sample was run to bound in DOWEX resin and washed to be eluted by fractioning with 0-3 M Formic acid at 2 ml min^{-1}. Prior to lyophilise the monomer, the fractions were analysed by HPLC to track the samples with highest concentration of L-galactonate, which those were merged.

Relative amount of L-galactono-1,4-lactone in the sample was quantified by the lactone assay as described by Beeson IV, 2011: 200 µl of freshly dissolved lactone mixed with 400 µl of 4 M hydroxylamine and 4 M NaOH, with 200 µl of 4 M HCl and Ferric Chloride 100 g l^{-1} and 200 µl 0.1 M HCl; quantified by spectrophotometry at 540 nm; for all experiments a standard curve was constructed (Beeson IV, et al., 2011).

2.7. Transcriptional analysis

The induction of engineered and wild type *Aspergillus niger* genes was studied: collecting 1 ml from SmF samples; and collecting similar portion from SSF samples. Consequently, on both samples, the mycelia were harvested by vacuum filtration, freeze with liquid nitrogen to store at -80 °C. RNeasy Plant Mini Kit (Qiagen) was used to extract RNA, following manufacturer's instructions. When extracted RNA, cDNA was translated using the First Strand cDNA Synthesis Kit (Roche) following manufacturer's instructions. For quantitative PCR (qPCR) 1:10 dilutions of cDNA with the LightCycler SYBR Green I Master mix were used for LightCycler 96 System.

The genes gaaA, gaaB, gaaC and three undescribed transporters of *A. niger* (JGI181673, JGI41809 and JGI194438) were quantified and the transcription levels were normalized to actin using *Advanced Relative Quantification Tool* software.

2.8. HPLC analysis

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

3. Results

A consolidated bioconversion of orange peel to L-galactonate was achieved using engineered strains of *Aspergillus niger* in solid-state (SSF) and submerged fermentation (SmF). In the engineered strains the gene coding L-galactonate dehydratase (*gaaB*) was deleted, consequently, blocking the native D-galacturonate pathway. The wild type parental strain (wt) was not capable of L-galactonate production.

3.1. Orange peel composition

3.1.1. Enzymatic hydrolysis

Orange peel's pectin was hydrolysed by commercial pectinases and an overnight incubation was done to be assured a complete release of monomers. The resulting D-galacturonic acid content was then used as a maximal theoretical yield for L-galactonate production. D-galacturonic acid and other constituents in the peel were quantified by HPLC. In addition to D-galacturonic acid, D-glucose, D-galactose, L-arabinose and L-rhamnose were found (Table 2).

Table 2 – Main components of hydrolysed orange peel detected by HPLC.

Soluble sugars	Concentration g (g orange peel)$^{-1}$
D-galacturonic acid	**0.237 ± 0.006**
L-arabinose	0.059 ± 0.002
D-galactose	0.054 ± 0.001
D-glucose	0.048 ± 0.001
L-rhamnose	0.013 ± 0.001

3.2. Conversion of orange peel to L-galactonate

A. niger ATCC1015 wild type and engineered strains *ΔgaaB* and *ΔgaaB-gaaA* were grown on orange peel in SSF and SmF. L-galactonate production was compared between both fermentation methods and nutritional conditions.

3.2.1. Solid-state fermentation (SSF)

3.2.1.1. SSFs without nutritional supplementation

In the flask scale solid-state fermentations without nutritional supplementation, the wild type released higher amount of D-galacturonic acid compared to the engineered strains, with 0.114 ± 0.005 g (g orange peel)$^{-1}$ after 10 days of fermentation (Figure 7). Unexpectedly, wt produced higher D-galacturonic acid concentrations than Δ*gaaA*, with 0.114 ± 0.005 to 0.056 ± 0.001 g (g orange peel)$^{-1}$, respectively. Both of the L-galactonate producing strains, Δ*gaaB* and Δ*gaaB-gaaA*, had similar D-galacturonic acid concentrations in the fermentation after 4 days (0.019 ± 0.001 and 0.023 ± 0.010 g (g orange peel)$^{-1}$, respectively).

Wt and Δ*gaaA* did not produce L-galactonate in the flask scale fermentation. However, after 10 days Δ*gaaB* and Δ*gaaB-gaaA* produced 0.069 ± 0.002 and 0.098 ± 0.001 g of L-galactonate (g orange peel)$^{-1}$, respectively. The fermentation with Δ*gaaB-gaaA* lasted 21 days, producing 0.147 ± 0.002 g (g orange peel)$^{-1}$.

Δ*gaaB-gaaA* was able to produce more D-galacturonic acid and L-galactonate in big than in small scale fermentations: the highest concentration of D-galacturonic acid was seen after 10 days, where the strain produced 0.041 ± 0.003 g (g orange peel)$^{-1}$ and after 21 days, the strain produced 0.166 ± 0.008 g L-galactonate (g orange peel)$^{-1}$ (Figure 8).

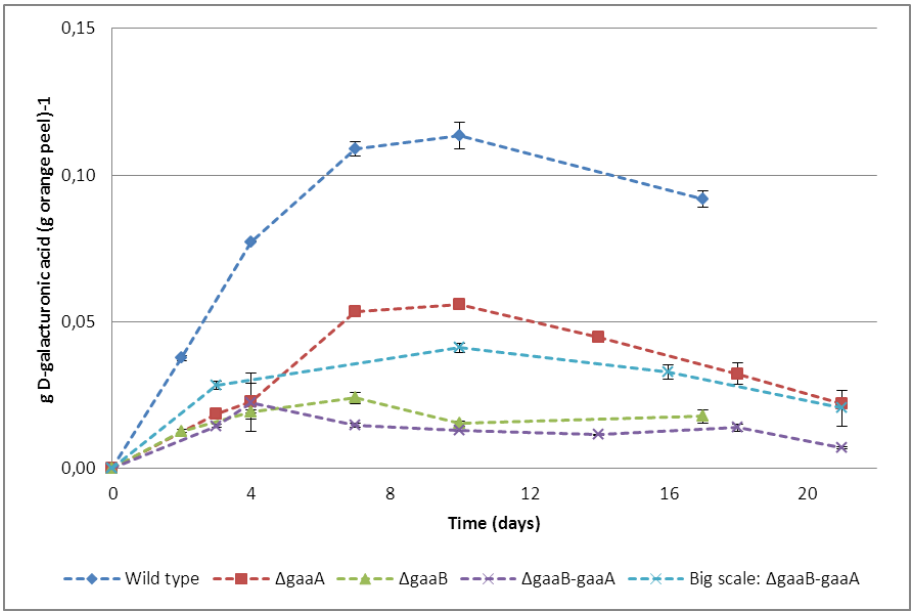


Figure 7 – D-galacturonic acid production in SSF without nutritional supplementation.

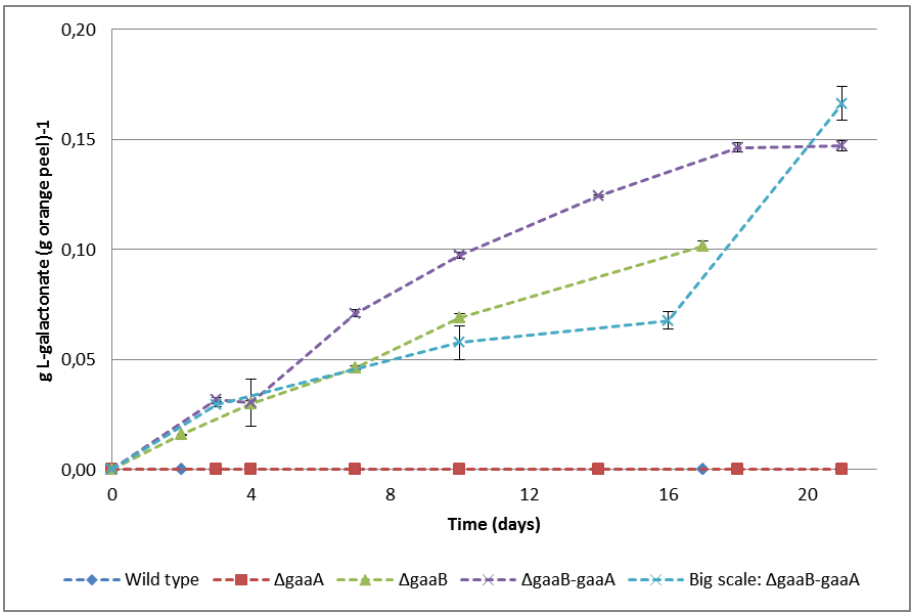


Figure 8 – L-galactonate production in SSF without nutritional supplementation.

3.2.1.2. SSFs with nutritional supplementation

In order to improve the fermentation efficiency, SSFs were supplemented with minerals. After 24 hours, product yields were practically similar with Δ*gaaB* and Δ*gaaB-gaaA* in small and big scale fermentations (Figure 9 and 10). However, after 72 hours, the strains in the big scale fermentation produced more 0.010 g D-galacturonic acid (g orange peel)$^{-1}$ than in small scale fermentation (Figure 9).

Δ*gaaB-gaaA* was able to convert slightly more L-galactonate than Δ*gaaB* after 96 hours with 0.002 and 0.0018 g (g orange peel)$^{-1}$ h^{-1}, respectively (Figure 10). In the small scale supplemented fermentation, Δ*gaaB* kept producing L-galactonate yielding 0.205 g ± 0.002 (g orange peel)$^{-1}$ after 10 days, producing more than Δ*gaaB-gaaA* which maximum yield was 0.194 ± 0.005 g (g orange peel)$^{-1}$ after 8 days.

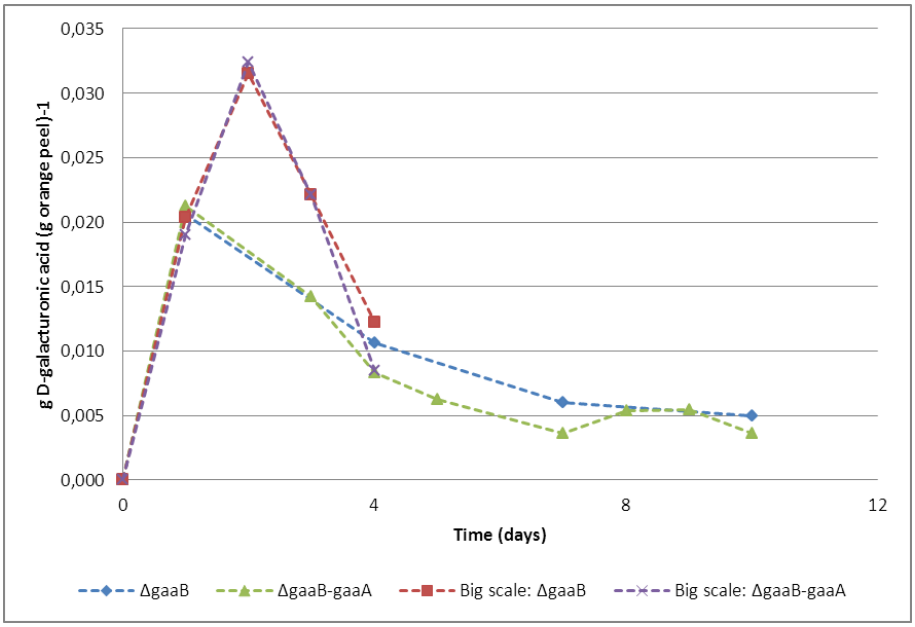


Figure 9 – D-galacturonic acid production in SSF with nutritional supplementation.

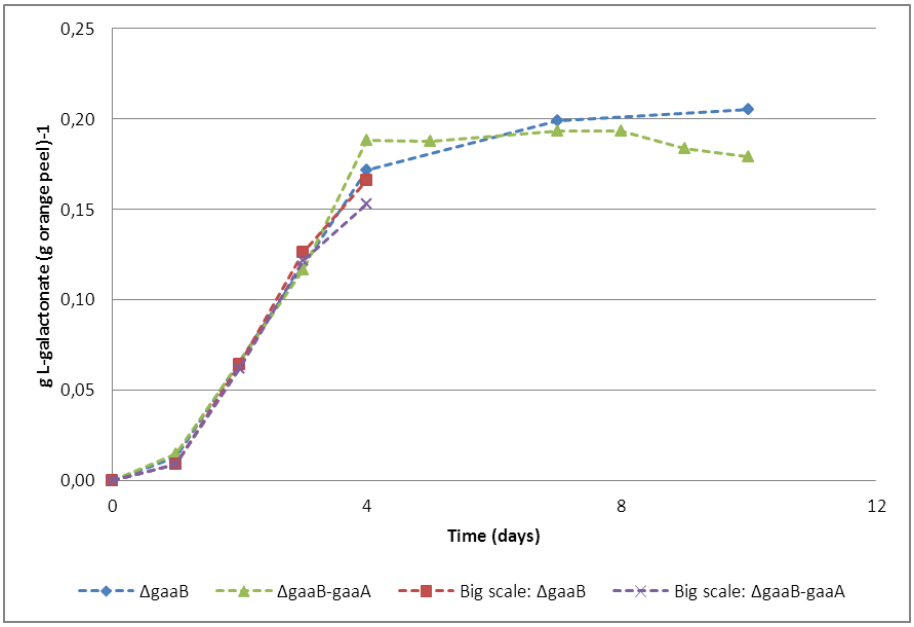


Figure 10 – L-galactonate production in SSF with nutritional supplementation.

3.2.2. Submerged fermentation (SmF)

3.2.2.1. SmFs without nutritional supplementation

In SmF on orange peel, released D-galacturonic acid was consumed during the first 48 hours by wt (Figure 11). Δ*gaaB* showed similar performance regardless the fermentation scale, but higher concentrations of D-galacturonic acid were detected in small than in big scale fermentation: 0.010 ± 0.001 g (g orange peel)$^{-1}$ to 0.007 ± 0.000 g (g orange peel)$^{-1}$, respectively (Figure 11). Other engineered strains such as Δ*gaaA* and Δ*gaaB-gaaA* had higher yields of D-galacturonic acid comparing to wt: 0.024 ± 0.003, 0.033 ± 0.005 and 0.013 ± 0.007 g (g orange peel)$^{-1}$, respectively.

Aside from wild type and Δ*gaaA* that did not produce L-galactonate, ΔgaaB yielded 0.055 ± 0.004 while Δ*gaaB-gaaA* yielded 0.084 ± 0.003 g (g orange peel)$^{-1}$ (Figure 12). The average production rates during the first 72 hours were 0.71 and 0.65 mg (g orange peel)$^{-1}$ h^{-1}, respectively.

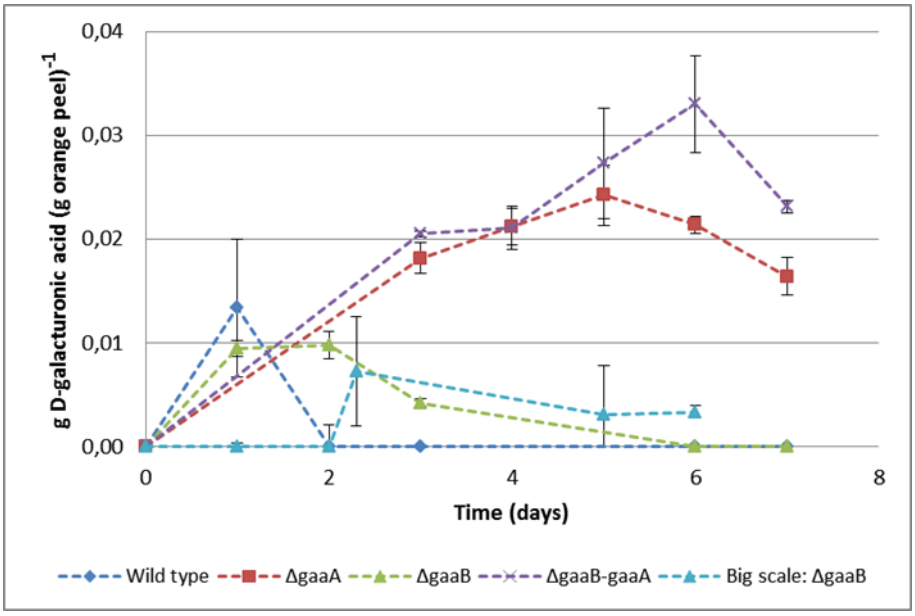


Figure 11 – D-galacturonic acid production in SmF without nutritional supplementation.

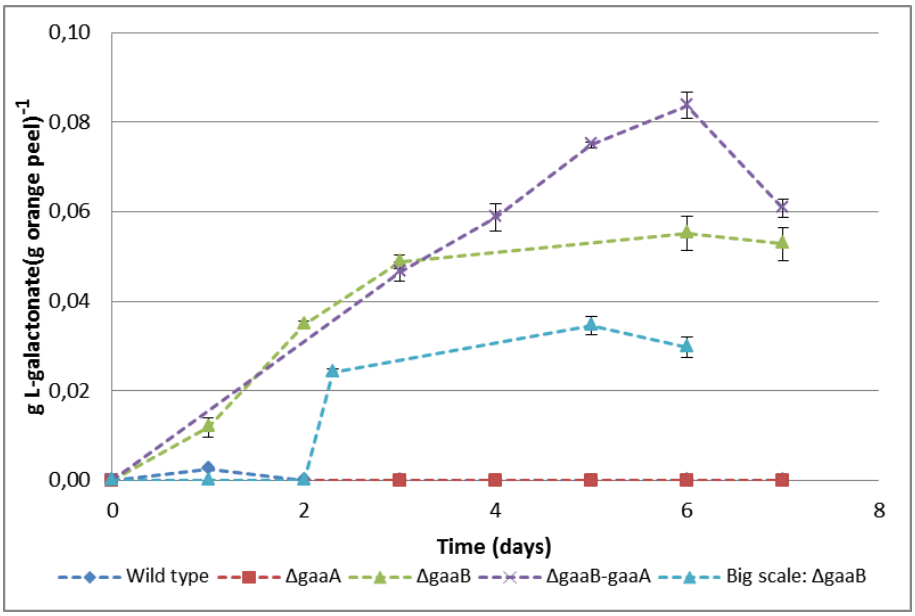


Figure 12 – L-galactonate production in SmF without nutritional supplementation.

3.2.2.2. SmFs with nutritional supplementation

SmFs with nutritional supplementation were investigated with wt and Δ*gaaB*. The highest production of D-galacturonic acid was observed to be on the second day of fermentation when wild type produced 0.025 ± 0.002 g (g orange peel)$^{-1}$, whereas Δ*gaaB* produced 0.010 ± 0.001 g (g orange peel)$^{-1}$ (Figure 13).

Significantly higher amounts of L-galactonate were present on the cultures using Δ*gaaB* (0.106 ± 0.003 g (g orange peel)$^{-1}$ after 8 days) (Figure 14).

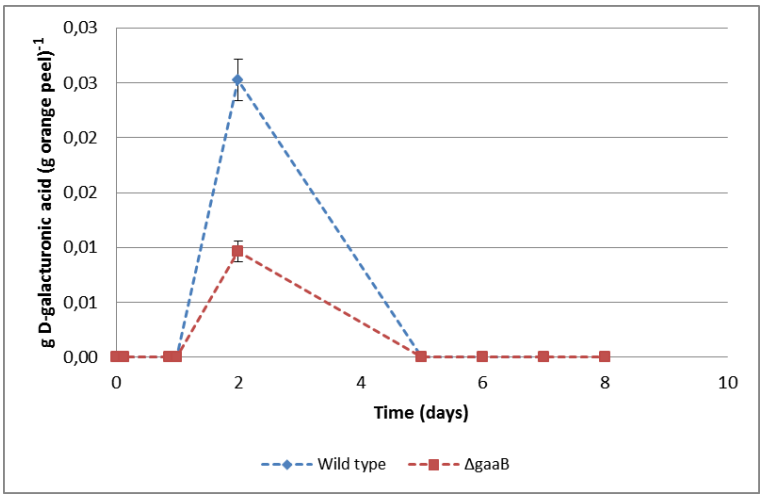


Figure 13 – D-galacturonic acid production in SmF with nutritional supplementation.

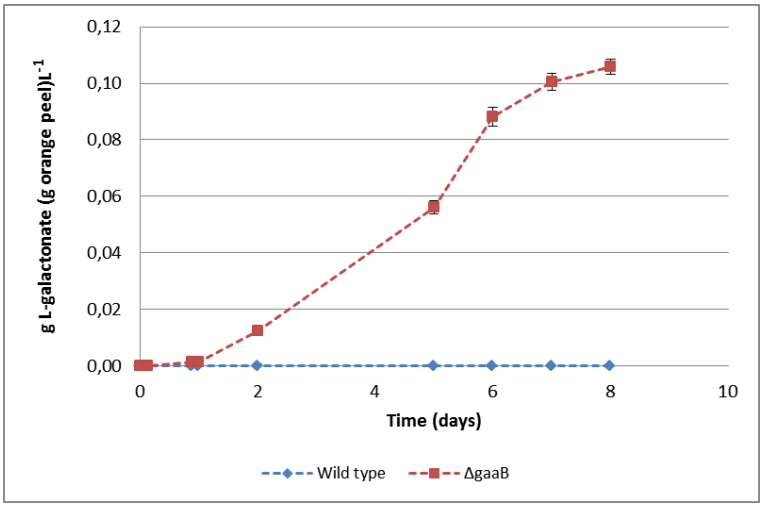
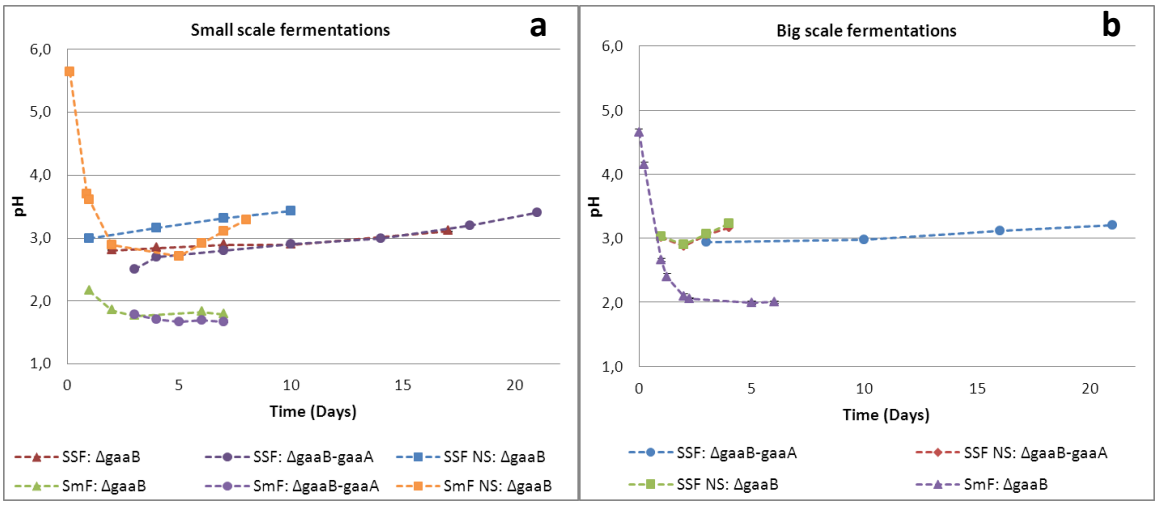


Figure 14 – L-galactonate production in SmF with nutritional supplementation.

3.2.3. pH in the fermentations

The pH in the fermentations were similar for Δ*gaaB* and Δ*gaaB-gaaA* when the same conditions were used, ranging from 2.5 to 3.5 in SSFs and from 1.70 to 2.15 in SmFs (Figure 15a and 15 b). The nutritional supplementation in the cultures led up to the higher initial pH (up to 5.65) due to the buffering capacity of the solution (Figure 15a). Afterwards, fermentations were acidified close to the pH of the fermentations without nutritional supplementation. Moreover, all the pH values in the up-scale fermentations were reported to be similar to the pH values in small scale fermentations (Figure 15b).



NS: Nutritionally supplemented fermentation

Figure 15 – SSF and SmF pH analysis.

3.3. L-galactonate purification

As described, L-galactonate was purified from 500 ml of the resulting fermentation broth by Δ*gaaB*. After loading the broth with 1.192 g l^{-1} L-galactonate to a DOWEX column and eluting with a linear formic acid gradient the fractions were analysed by HPLC to identify the fractions containing L-galactonate (Figure 16). The samples 13 to 16, with higher concentration of the L-galactonate were combined and a solution with a final volume of 40 ml was lyophilized.

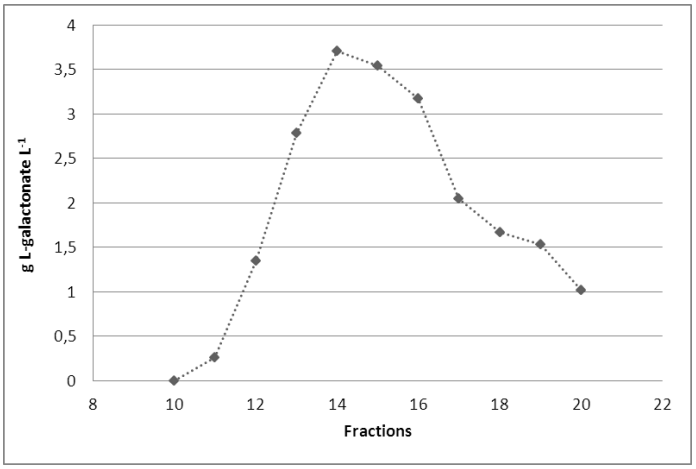
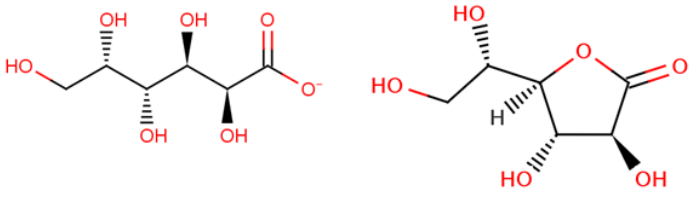


Figure 16 – L-galactonate samples from fractioning quantified by HPLC.

The combined fractions were lyophilized in two separate falcon tubes. The samples were dissolved with 40 ml of water each for HPLC analysis. The concentration was 1.284 g l^{-1} and the total purified L-galactonate was estimated to be 0.103 g. Finally, the purification yield was calculated from the initial broth concentration as being 17.2 %.

Furthermore, a lactone assay was made in order to relate the amount of L-galactonate in linear and lactone form: 69 % was found in its linear form, whereas 31 % was as L-galactono-1,4-lactone (Figure 17).



L-galactonate L-galactono-1,4- lactone

Figure 17 – L-galactonate chemical structure.

3.4. Transcriptional analysis

The gene expression in wt and *ΔgaaB* was analyzed from a supplemented solid-state fermentation of orange peel by taking samples at 3, 4 and 7 days. This study was expected to cover L-galactonate production process, as the maximum L-galactonate concentrations would be reached after 4 days in supplemented solid-state fermentation. The genes gaaA, gaaB, gaaC and the transporters JGI181673, JGI41809 and JGI94438 of *Aspergillus niger* were normalized to actin expression, expecting that the expression level of these genes would be high.

The cDNA from the samples taken at 7 days of fermentation provided no valid data with qPCR, but the study allowed an analysis of gene expression trend. The expression of gaaA in wt was higher compared to *ΔgaaB*, after 3 days. However, both strains increased to similar D-galactoronate reductase expressions levels, after 4 days (Figure 18). Despite the variations between the replicates for gaaB induction, there were still high expression of the gene in wt after 3 and 4 days. In contrast, L-galactonate dehydratase (gaaB) was not induced in the *ΔgaaB*, as expected. The relative transcription of gaaC was significantly higher in wt than in *ΔgaaB*, nevertheless its expression in *ΔgaaB* had increased from 3 to 4 days. JGI181673 expression levels were also high in wt, but a decrease of its expression was seen after 4 days, despite replicate variations; the induction of the transporter in gaaB deletion strain was present, but in low levels. For the time-points studied, *ΔgaaB* expressed the JGI41809 after 3 days of fermentation, but in lower levels than wt, that expressed the gene after 3 days and it significantly increased after 4 days. JGI94438 expression analysis was only possible for the 3rd day of fermentation, showing that both wt and *ΔgaaB* express the transporter during L-galactonate production.

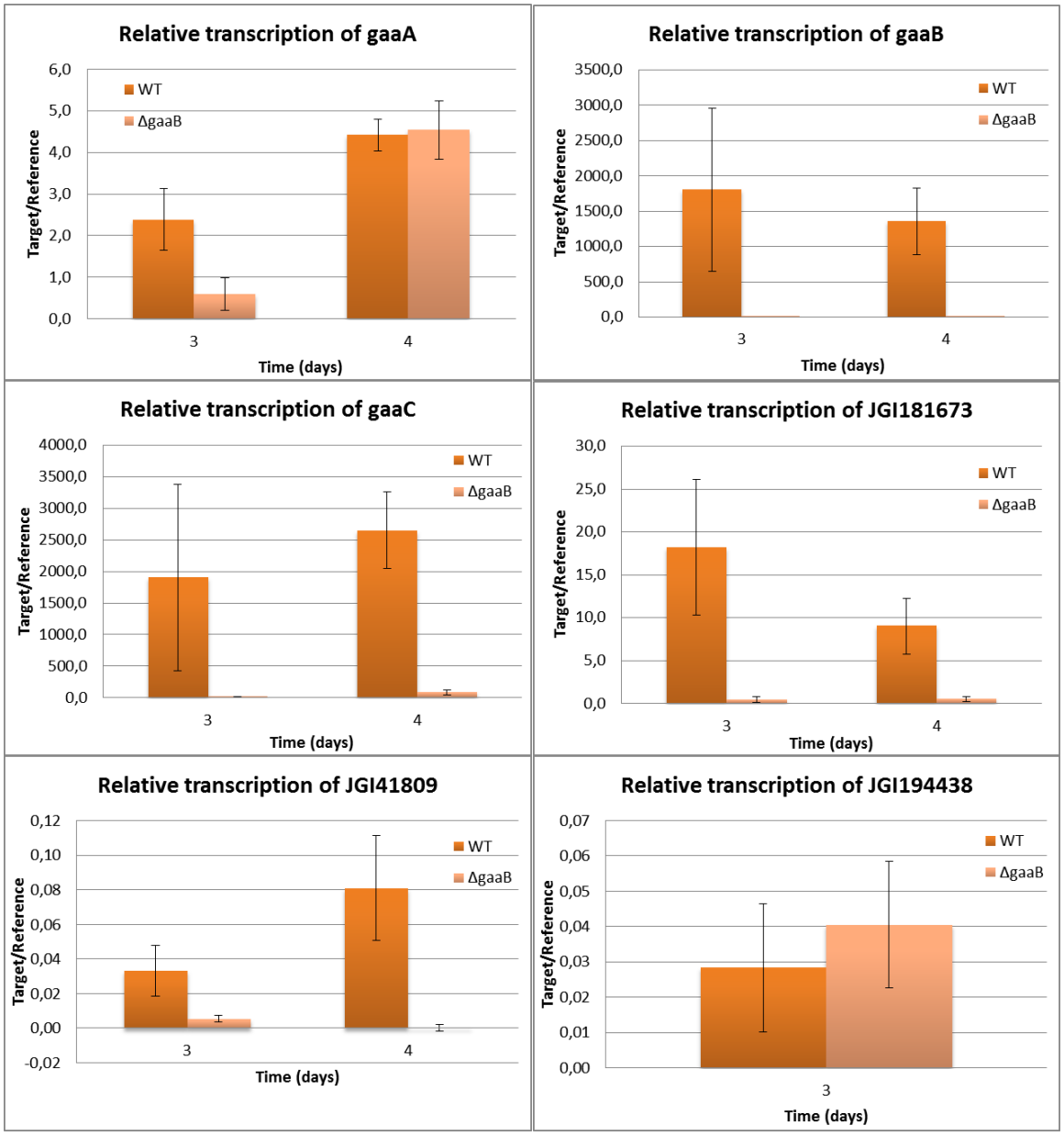


Figure 18 – Transcriptional analysis of gaaA, gaaB, gaaC, JGI181673, JGI41809 and JGI94438 in wild type and *ΔgaaB* from SSF of orange peel.

4. Discussion

The use of pectinases showed to be insufficient for complete hydrolysis of orange peel, since only 41,1 % of total orange peel amount was hydrolysed and detected by HPLC. The presence of polysaccharides such as cellulose and the use of pectinases, which are unable to hydrolyse it, were responsible for such low yield. However, a more efficient hydrolysis was expected by the strains, since *Aspergillus niger* secretes several types of enzymes.

Unexpectedly, Δ*gaaA* did not produce higher concentrations of D-galacturonic acid than wt in solid-state fermentations. Simultaneously, a relative output was reported: the more L-galactonate produced, the less D-galacturonic acid was detected, for each strain in each time-point. The up-scale fermentation contributed to prove that in the absence of supplements D-galacturonic acid production stops after 10 days. During that process *Aspergillus niger* grows, pectin is degraded and a simultaneous conversion process of D-galacturonic acid to L-galactonate occurs.

In the supplemented column fermentation D-galacturonic acid was higher after 2 and 3 days than in the flask fermentation. The lack of oxygen availability in the inner part due to seen compaction might be the reason for prolonged hydrolysis process. This fact did not decelerate the production of L-galactonate, because the similar yields of both sugar acids were detected after 4 days. Nevertheless, the period of the fermentation was shorter and most importantly, the yields were higher for both studied monomers when nutritional supplement was added.

Regardless the addition of nitrogen sources, approximately the same amount of D-galacturonic acid was produced by *ΔgaaB* in SSF, but a faster conversion of D-galacturonic acid to L-galactonate might occur in supplemented fermentations. On the other hand, those supplements in submerged fermentation propelled 2 times more L-galactonate production than in the non-supplemented fermentation by *ΔgaaB*. This experiment was also important to demonstrate that nutritional supplementation does not

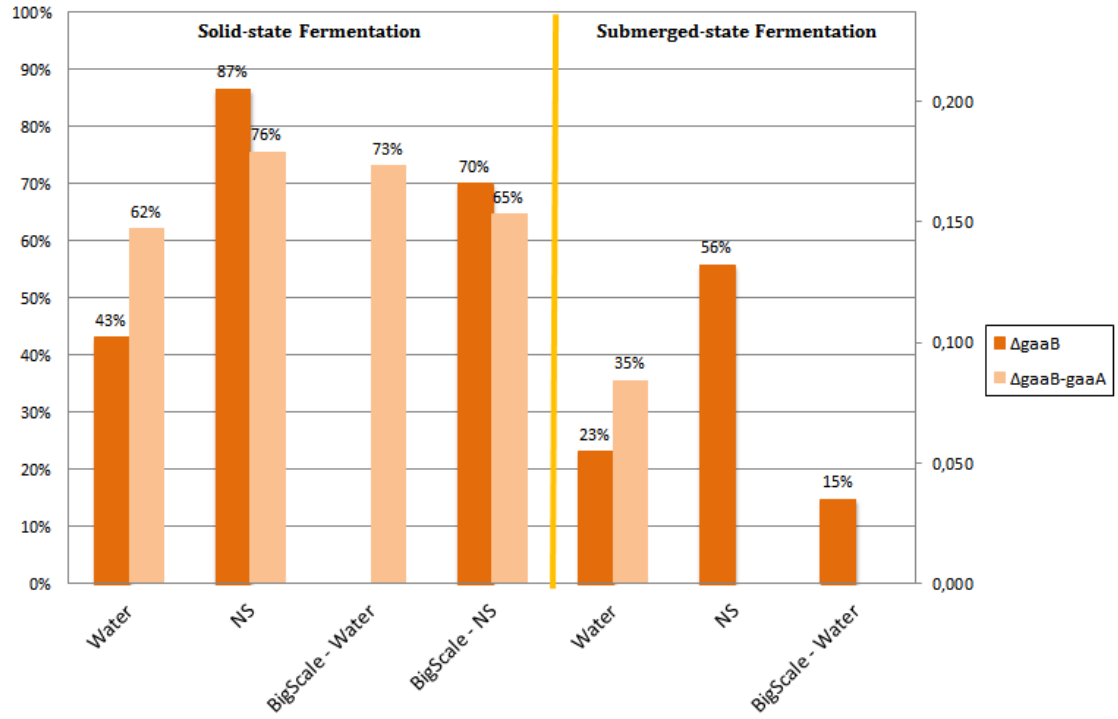
contribute as good as seen in solid-state fermentation to shorten the fermentation period. An explanation might be that L-galactonate production is pH dependent and higher initial pH delayed supplemented fermentation.

The pH in the fermentations showed that *Aspergillus niger* is a pH dependent organism, producing higher L-galactonate concentrations at low pH, minimizing considerably the risk of contamination. The measurement of pH for solid-state cultures was only possible through the extracted samples. Hence, there is a change of pH when water is added, increasing the actual pH of the fermentation process. That fact, might explain the lower pH range seen in submerged-state fermentation.

4.1. Comparison of SSF and SmF

Solid-state fermentation allowed the production of higher yields of L-galactonate. The source of nutrients endorsed L-galactonate production by Δ*gaaB* to 87 % of L-galactonate in solid-state fermentation, in contrast to the 56 % produced with submerged-state fermentation, from the maximum theoretical yield – 0.237 g D-galacturonic acid (g orange peel)$^{-1}$ (Figure 19). However, when no supplementation was added, Δ*gaaB-gaaA* produced higher L-galactonate concentrations than Δ*gaaB* in both solid and submerged-state fermentations.

An advantage of submerged-state fermentation is that initial production rates are higher than solid-state fermentation. However, highest production rates are seen in solid-state cultures when supplementation is added. Secondly, on submerged-state fermentation D-galacturonic acid is in some cases totally consumed, which can provide more pure L-galactonate in the medium. However, a complete conversion of D-galacturonic acid in submerged-state fermentation did not implied higher yields of L-galactonate. A possible explanation for the insufficient conversion yield is an incomplete pectin hydrolysis by the strain. The fact that in solid-state fermentation D-galacturonic acid was not totally converted might be based on less homogenous secretion of enzymes that can be present closer to hyphae and unavailable D-galacturonic acid can no longer be further converted. Moreover, the presence of other carbon sources beyond D-galacturonic acid might lead to unclear L-galactonate collateral consumption.



NS: Nutritionally supplemented fermentation

Figure 19 – Production yield of L-galactonate in SSF and SmF for *ΔgaaB* and *ΔgaaB-gaaA*.

The reproducibility was satisfactory for both methods and the performance can be easily predicted when changing the growth factors. Highest concentrations of L-galactonate were reached in solid-state fermentation without supplementation approximately after 19 days. In contrast, submerged-state fermentation takes 5 days, using the same conditions. The use of nitrogen supplementation shortens solid-state fermentation to 4 days, whereas with submerged-state fermentation a reverse effect was seen, only increasing the production yields.

The use of nutritional supplementation to shorten fermentation period might be considered if the cost evolved in the use of the additive is lower than the purified L-galactonate. Even though the downstream process resulted in a purification yield of 17.2 %, it can be optimized. The explanation for a still low yield would be based on the usage of submerged-state culture that had lower L-galactonate yields than a solid-state culture, an unspecific DOWEX ion-exchange resin and the selection of only the most concentrated fractions.

Solid-state fermentation also uses fewer resources: in submerged-state fermentation it was used 50 ml of water and higher consumption of energy to shake flasks; in solid-state fermentation it was only used 36 ml (including extraction process) and flask shaking was not needed. The more this process is up-scaled, the larger is the difference between the methods. However, up-scaling the process does not significantly increase L-galactonate production yields.

Concluding, the production of L-galactonate in solid-state fermentation using nutritional supplementation would be the most profitable.

II. IDENTIFICATION OF THE MISSING LINK IN THE EUKARYOTIC D-GLUCURONIC ACID PATHWAY

1. Introduction

Glucose is used for the formation of glycogen, pentoses and mucopoly saccharides in the liver (Rao, 2006). In some mammals, when glucose is not degraded by glycolysis it can be oxidized by an alternative pathway: the uronic acid pathway. No energy is produced by the pathway, but it provides formation of UDP-glucuronate, which is involved in the conjugation of bilirubin, steroid hormones, heparin, heparan and xenobiotics. It is also responsible for the biosynthesis of L-ascorbic acid (Vitamin C) and the unutilized D-glucuronic acid is converted to D-xylose-5-phosphate, which is metabolized via the Pentose Phosphate pathway (Rao, 2006).

Glucose is converted to D-glucuronic acid by a series of reactions (Chatterjea, 2004). D-glucuronic acid is converted to L-gulonate by the NADPH-dependent glucuronate reductase, identical to galacturonate reductase (gaaA) (Puri, 2011). As L-galactonate, L-gulonate is a precursor of Vitamin C. In humans and other primates as well as guinea pigs, Vitamin C is not synthesized, because of genetic deficiency of the enzyme L-gulonolactone oxidase, which converts L-gulono-1,4-lactone to L-ascorbic acid (Figure 20) (Chatterjea, 2004) (Linster & Schaftingen, 2006).

L-gulonate is a hexonic acid that can be converted to xylitol, by a series of reactions. The reactions are: (i) a NAD-dependent dehydrogenase, (ii) a decarboxylase where the enzyme activity was described but the corresponding gene is not known and an NADPH-depentent L-xylulose reductase (Linster & Schaftingen, 2006). Ishikura, et al., 2005, has cloned the gene encoding rabbit liver L-gulonate-3-dehydrogenase, describing

the enzyme responsible for the catabolism of L-gulonate to 3-keto-L-gulonate. The enzyme showed to be identical to rabbit lens λ-crystallin, being the most active GDH among rabbit tissues (Ishikura, et al., 2005).

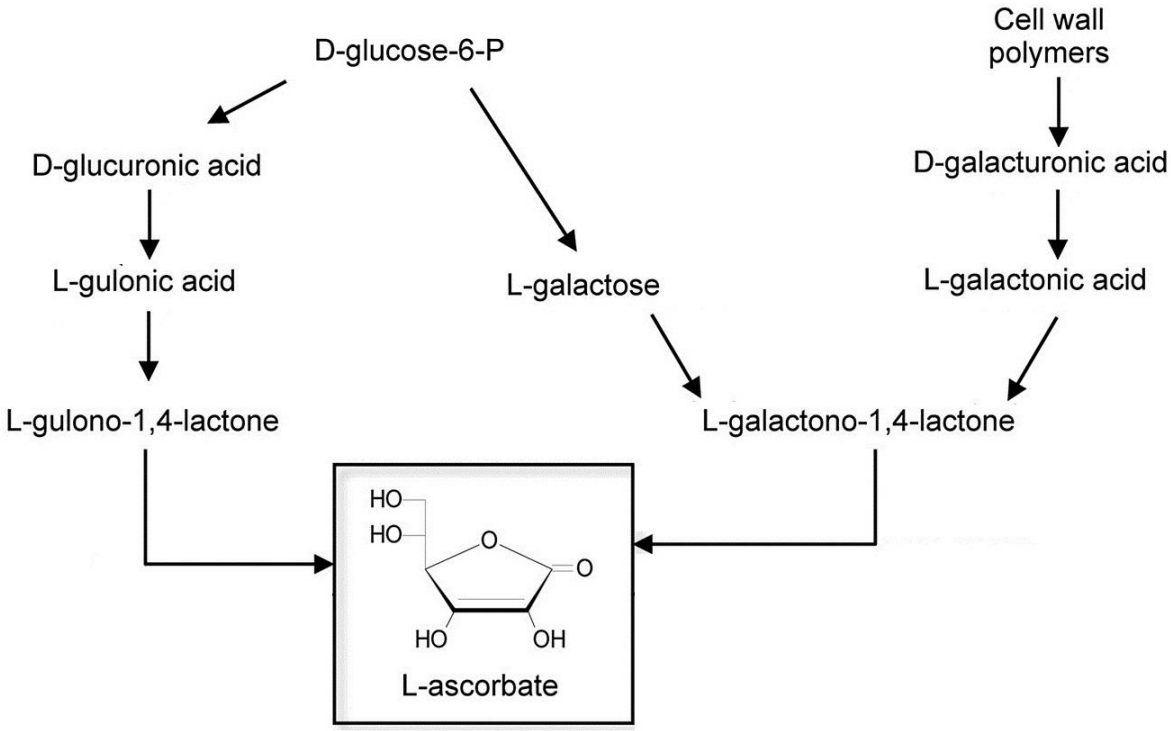


Figure 20 – Synthesis of Vitamin C from D-glucose-6-phosphate and D-galacturonic acid **(Aragüez, et al., 2013)**.

3-keto-L-gulonate is converted to L-xylulose by a poorly described decarboxylase (Figure 22). In the 1960's, Ashwell et al., Dwarsky et al., and Kagawa et al. purified decarboxylases, but molecular identity of the decarboxylase in the D-glucuronic acid pathway is still unknown (Winkelman & Ashwell, 1961) (Dworsky & Hoffmann-Ostenhof, 1967) (Kagawa & Shimazono, 1970). Ashwell et al., described a reaction in which L-xylonic acid was converted by rat liver enzymes to L-erythroascorbic acid (Winkelman & Ashwell, 1961). Kagawa et al. presented evidence for an enzyme in rat liver which decarboxylated 2,3-diketo-L-gulonic acid to L-xylonic acid and L-lyxonic acid (Figure 21) (Kagawa & Shimazono, 1970). Some characteristics of the purified enzyme from were reported: the optimal pH was 7.0 in phosphate buffer; 90 % of activity was lost at 65ºC after 5 min; the Km was 1.5 x 10^{-2} mM; and activity was found in liver and kidney of dog,

hog, ox, guinea pig, rabbit, rat, monkey and man. However, the relation of those purified decarboxylases to the one in the pentose pathway remains to be determined.

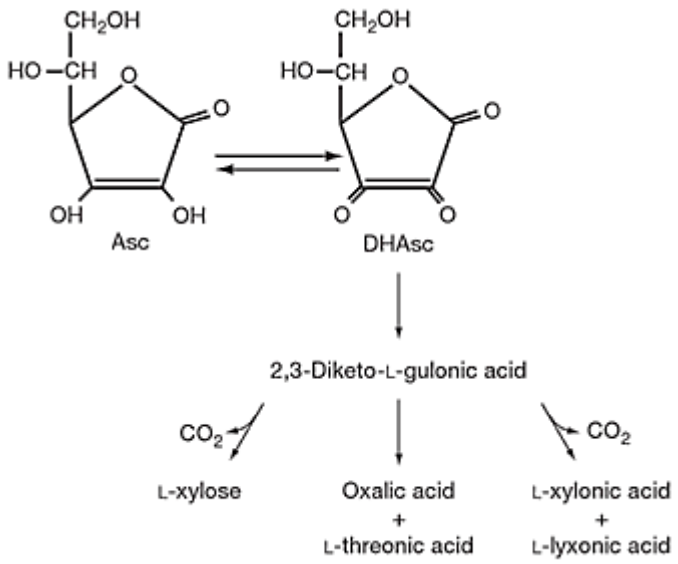


Figure 21 – Metabolism of D-glucuronic acid via L-ascorbic acid. Asc: L-ascorbic acid; DHAsc: L-dehydroascorbic acid **(Lennarz & Lane, 2013)**.

L-Xylulose reductase is responsible for the NADPH-dependent reduction of L-xylulose to xylitol, which its inherited deficiency causes e*ssential pentosuria* (Figure 22). The xylitol production is blocked, consequently accumulating L-xylulose that is excreted in the urine. In 1902, Archibald Garrod described Pentosuria, however during the century the clinically benign condition was treated as Diabetes Mellitus with insulin, (Puri, 2011). As long as a standard test did not differentiate between the reducing sugars D-glucose and L-xylulose, the insulin treatment led to hypoglycemia among the patients (Pierce, et al., 2011)

Xylitol is further catabolized by an NAD^+ requiring xylitol dehydrogenase to D-xylulose (Linster & Schaftingen, 2006). Finally, after phosphorylation of D-xylulose by an ATP requiring xylulokinase, it enters the hexose monophosphate shunt, also called pentose phosphate pathway as D-xylulose-5-phosphate (Bhagavan, 2002).

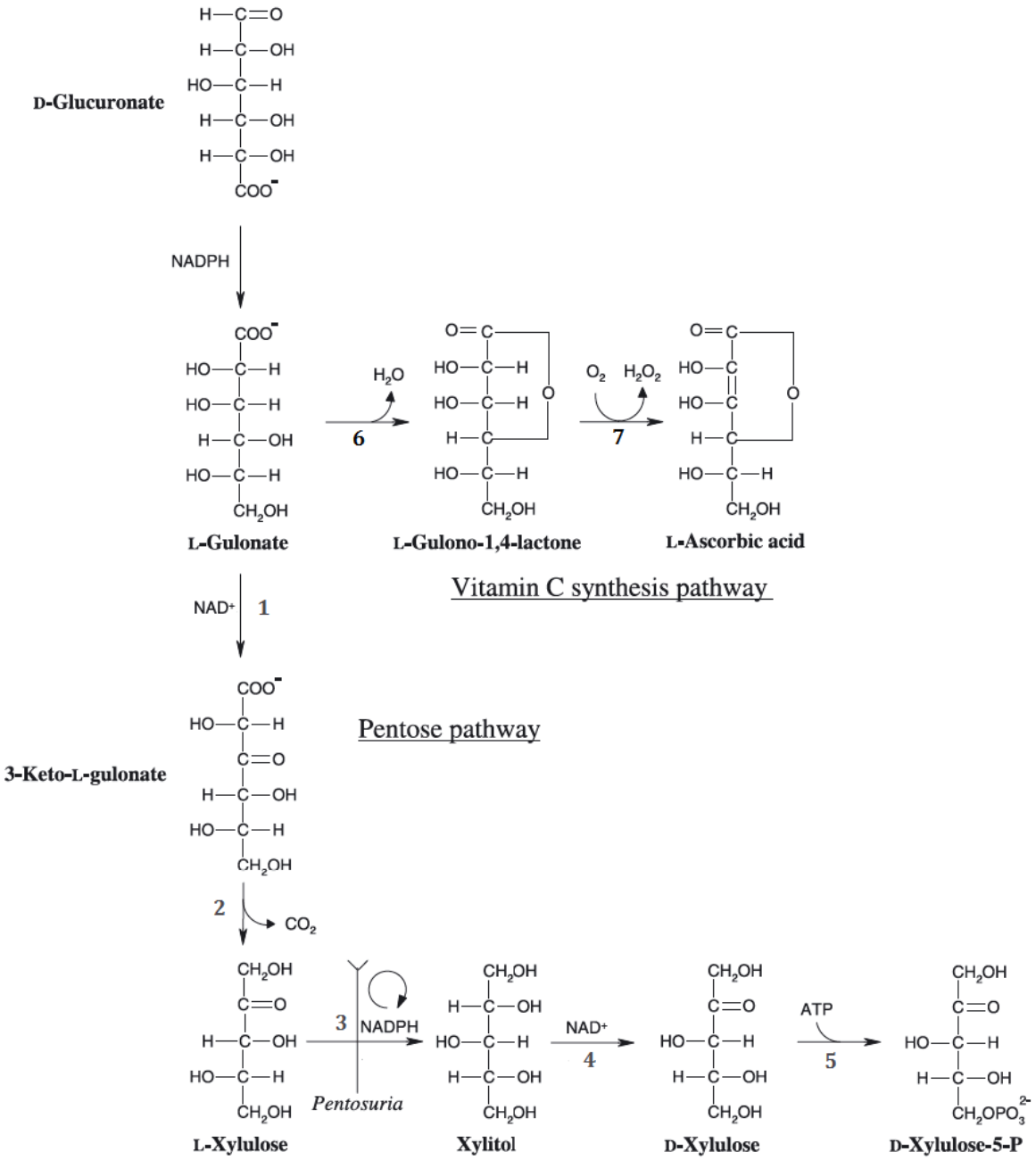


Figure 22 – Glucuronic acid pathway. 1. L-Gulonate-3-dehydrogenase; 2. Decarboxylase; 3. L-xylulose reductase; 4. Xylitol dehydrogenase; 5. D-Xylulokinase; 6. Gulonolactonase; 7. L-Gulonolactone oxidase **(Linster & Schaftingen, 2006)**.

1.1. Objectives of the work

The eukaryotic D-glucuronic acid pathway has the potential to be applied in metabolic engineering, since D-glucuronic acid can be catabolized to its high-value products: L-ascorbic acid (Vitamin C) and L-xylulose. In addition, characterizing the pathway would make it possible to link uronic acid metabolism to the pentose phosphate pathway. For this reason, it is important to characterize the missing link in the D-glucuronic acid pathway, the poorly known decarboxylase that converts 3-keto L-gulonate to L-xylulose.

In order to identify of the decarboxylase it should be tried to convert 3-keto L-gulonate to L-xylulose with liver decarboxylase, following NADPH consumption at 340 nm, when L-xylulose reductase is added. The consumption of NADPH would show that L-xylulose was formed. This assay could then be used for further purification. However 3-keto L-gulonate is not commercially available and could not be purified. For that reason 3-keto L-gulonate had to be formed in the assay mix by L-gulonate-3-dehydrogenase which is an NAD-dependent enzyme. Since the NADH absorbance is equivalent to NADPH at 340 nm, such a coupled assay could not be used to monitor the activities. Therefore, the conversion of L-gulonate to 3-keto L-gulonate was carried out by substituting NAD^+ by thio-nicotinamide-adenine dinucleotide (Thio-NAD^+), where the maximum absorbance of the reduced form (Thio-NADH) is at 398 nm and no absorbance changes occur at 340 nm.

In this study rabbit L-gulonate-3-dehydrogenase (GDH) and L-xylulose reductase of *Aspergillus niger* (*lxr*A) described by Mojzita, et al., 2010, will be purified in order to be able to purify a 3-keto-L-gulonate decarboxylase from bovine liver (Mojzita, et al., 2010). The aim is then to test if this coupled assay can be used to assay the activity in liver extract and to purify this activity.

2. Materials and Methods

2.1. Purification of L-Gulonate-3-Dehydrogenase (GDH)

The QIA*expressionist* protocol (Qiagen) was used to purify the L-gulonate-3-dehydrogenase from rabbit. The protein was His-tagged and expressed from the pBAT4 vector. The *E. coli* strain BL21 (DH3) was grown in 400 ml of Luria Broth (LB) with 100 µg ml^{-1} of ampicillin and the expression induced with 1mM IPTG for 24 hours at 28ºC. The cells were harvested by centrifugation and lysed using Zymolyase using the following lysis buffer: 0.1 mg/ml of Lysozyme; 0.1 % Triton X-100; 0.5 M NaCl; 0.5 mM EDTA; 20 mM Tris pH 8.0. Subsequently, the cells were lysed using sonication with a microtip sonicator. The lysed cells were centrifuged (in a Sorval centrifuge with the SS34 rotor at 20 000 rpm at 4ºC). From the supernatant the His-tagged protein was purified using the Ni-NTA resin (Qiagen) according to the protocol of the manufacturer.

2.2. Cloning and purification of *Aspergillus niger*' L-Xylulose Reductase (*lxr*A)

The pBAT4 vector and L-xylulose reductase of *Aspergillus niger* (*lxrA*) with N-terminal His-tag, in pYX212 vector were digested with EcoRI and BamHI and the vector was dephosphorylated with Shrimp alkaline phosphatase (SAP) by ArcticZymes, to prevent vector relegation (Figure 23 and 24). The digests were separated in an agarose gel and the pBAT4 and *lxr*A purified from the gel using the MinElute Gel Extraction Kit (Qiagen), following manufacturer's instructions. The digested open reading frame (ORF) and pBat4 vector were ligated using with the T4 DNA Ligase (New England BioLabs) at 4ºC, for overnight.

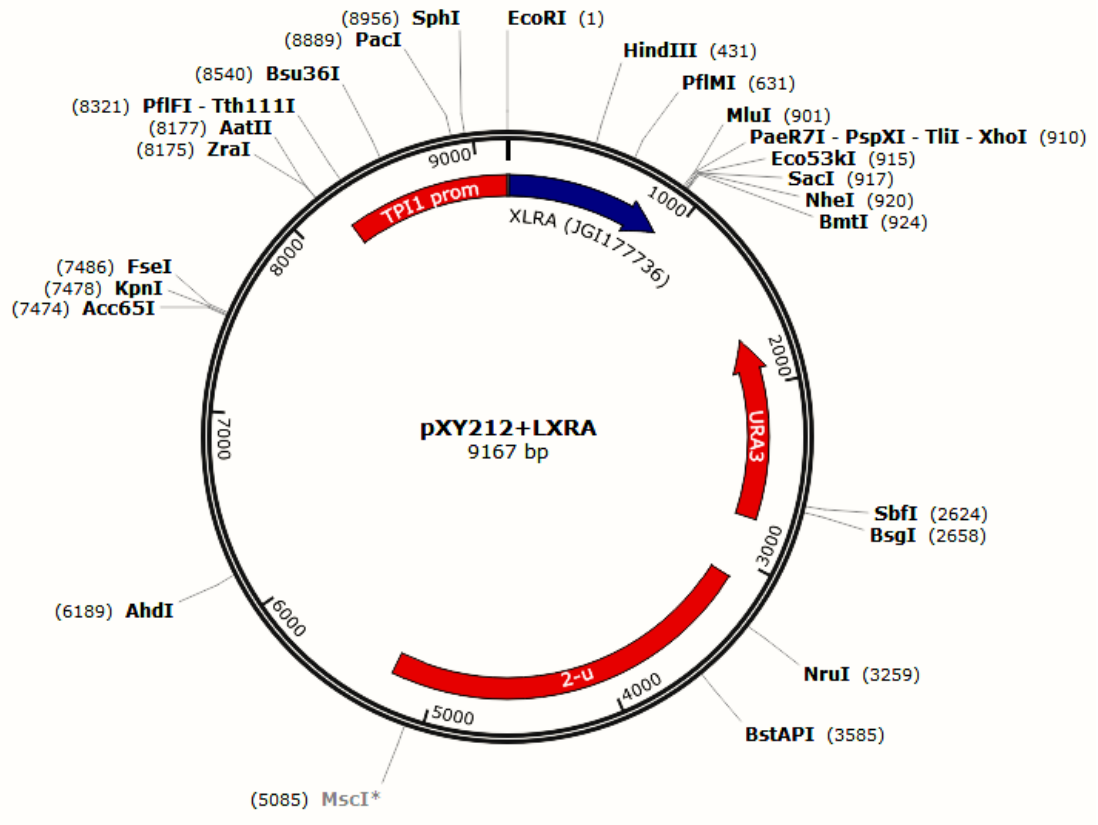


Figure 23 – *Lxr*A in the original vector.

E. coli TOP 10 electrocompetent cells were transformed with the ligation mix and, plated on LB-agar with ampicillin, at 37ºC, for overnight. The insert was screened by Colony PCR using DyNAzyme II DNA Polymerase, following manufacturer's instructions, using primers of the TPI promoter and terminator region. A Miniprep of the cells containing the plasmid was made using QIAprep Spin Miniprep Kit (Qiagen), following manufacturer's instructions. Sequencing of *lxr*A was done (in GATC Biotech) in order to know if the insert was correctly in pBAT4. The plasmid was then transformed to BL21 (DH3) cells for further purification of the enzyme.

The purification of the His-tagged protein followed also the QIA*expressionist* protocol (Qiagen), inducing 1 mM IPTG for overnight at 28ºC, with the same lysis buffer used for GDH purification.

Figure 24 – pBAT4 polylinker region.

2.3. Purification of liver Decarboxylase

The purification of the liver decarboxylase was made following a modified protocol from Winkelman and Ashwell's 3-keto-L-gulonate decarboxylase. A fresh bovine liver (100 g) was ground to homogeneity in a Warring blender at 4ºC with 300 ml of 10 mM sodium phosphate at pH=7 and added proteinase inhibitor (Complete, Roche). The mixture was centrifuged at 10000 x g for 10 min, at 4ºC. To the supernatant ammonium sulphate was added in 3 steps, in order to remove proteins that precipitate before the decarboxylase. The steps were 0-50 % ammonium sulphate saturation, 50-60 % and 60-85 %. After each step the mixture was centrifuged at 10 000 x g at 4ºC. After the first two centrifugations the pellet were discarded. The pellet from the 60 to 85 % saturation was dissolved in a small amount of water and desalted using PD-10 Desalting Columns (GE Healthcare, Life Sciences).

2.4. Protein concentration

The concentration of purified L-xylulose reductase of *Aspergillus niger* and L-gulonate-3-dehydrogenase was measured using Bio-Rad Protein Assay (Bio-Rad), following manufacturer's instructions and BSA as a protein standard.

2.5. Enzyme activity assay

The activity of L-gulonate-3-dehydrogenase was assayed by spectrophotometry using 1 mM L-gulonate as substrate, buffered with 50 mM Tris-HCl at pH 8. GDH activity was tested using the cofactors 1mM NAD^+ or 1mM Thio-NAD^+ at 340 or 398 nm, respectively.

L-xylulose reductase of *Aspergillus niger* activity was assayed using 10 mM L-xylulose as substrate, buffered with 50 mM Tris-HCl at pH 8 and 200 µM NADPH. The disapearance of NADPH was measured at 340 nm.

2.6. SDS-PAGE

The purified L-xylulose reductase of *Aspergillus niger* was concentrated with Vivaspin 500 µl (Sartorius Stedim Biotech) by centrifugation at 13,500 rpm for 10 min. The sample containing 50 µl of concentrated *lxr*A was run in Bio-Rad SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in order to know its molecular weight and purity, following manufacturer's instructions.

3. Results

L-xylulose reductase of *Aspergillus niger*, which is responsible for the conversion of L-xylulose to xylitol in the eukaryotic D-glucuronic acid pathway was successfully engineered for expression in *E. coli*. In addition, NADPH-dependent *lxr*A and NAD-dependent GDH were purified and had activity in the crude extract.

3.1. L-gulonate-3-dehydrogenase (GDH)

GDH had a concentration of 19.0 mg l^{-1}. The activity of the purified GDH was measured by the formation of NADH at 340 nm, for 120 seconds (Figure 25). The NAD-dependent enzyme showed a Δabs of 0.229 min^{-1} cm^{-1} with L-gulonate as substrate and NAD^+ as cofactor.

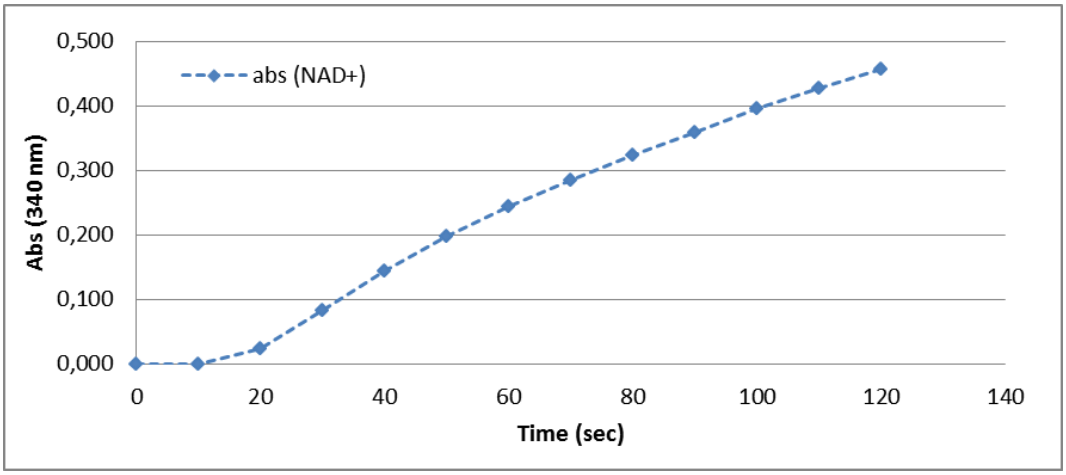


Figure 25 – Activity of L-gulonate-3-dehydrogenase with NAD^+ at 340 nm.

At the same time, the activity of GDH was measured using Thio-NAD^+ as cofactor (Figure 26). The Δabs was calculated to be lower than using NAD^+ (0.026 min^{-1} cm^{-1}). In addition, the activity was calculated from Thio-NAD^+ molar extinction coefficient of 11900 and GDH concentration.

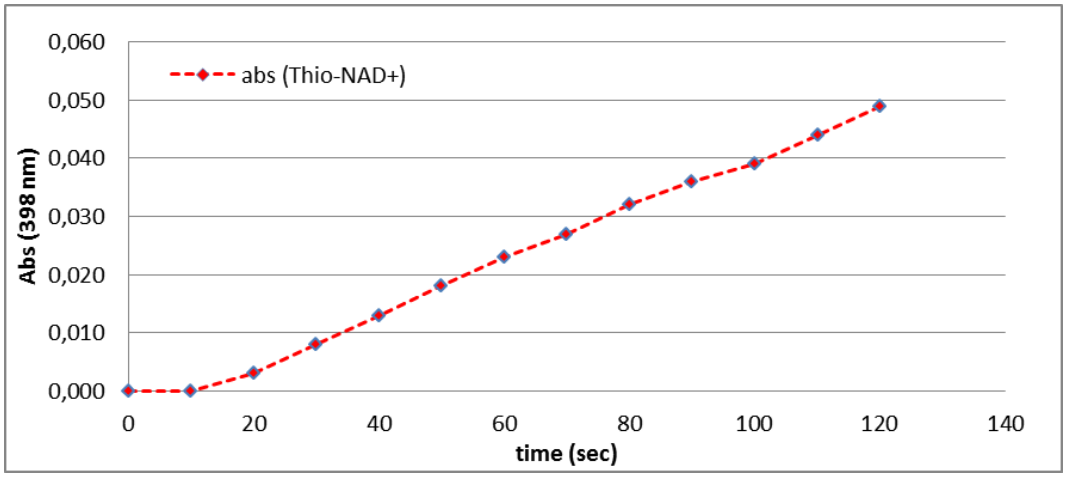


Figure 26 – Activity of GDH with Thio-NAD^+ at 398 nm.

A lower activity of the enzyme was reported, as 2.18 x 10^{-6} mol l^{-1} min^{-1}, 0.12 U mg^{-1} was tested using the Thio-NAD^+ (Table 3).

The activity was measured following the equation:

$$Activity = \frac{\Delta Abs}{\in \ x \ t_{min}}$$

The activity of GDH was calculated from molar extinction coefficient (∈) of NAD^+ (6220 M^{-1} cm^{-1}) showing to be 3.68 x 10^{-5} mol l^{-1} min^{-1}, 1.94 U mg^{-1} (Table 3).

Table 3 – Activity of GDH with NAD^+ and Thio-NAD^+.

GDH cofactors	Activity		
	(mol l^{-1} min^{-1})	**(U l^{-1})**	**(U mg^{-1})**
NAD^+	3.68 x 10^{-5}	36.817	1.94
Thio-NAD^+	2.18 x 10^{-6}	2.185	0.12

3.2. L-xylulose reductase of *Aspergillus niger* (*lxr*A)

The digested pBAT4, *lxr*A and pYX212 were run in agarose gel and, as expected, *lxr*A fragment had approximately 1 kb (Figure 27). The plasmid was transformed to BL21 (DH3) cells and colony PCR was made. The PCR products were screened in an agarose gel, identify 4 colonies containing *lxr*A (Figure 28). The sequence was confirmed and *lxr*A was purified.

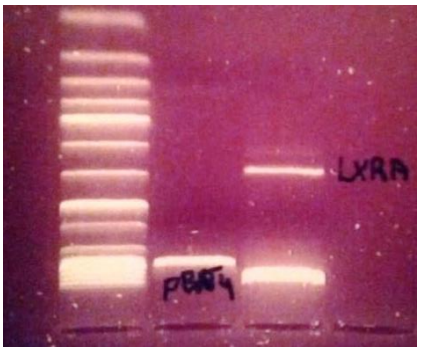


Figure 27 – Digested pBAT4 and *lxr*A.

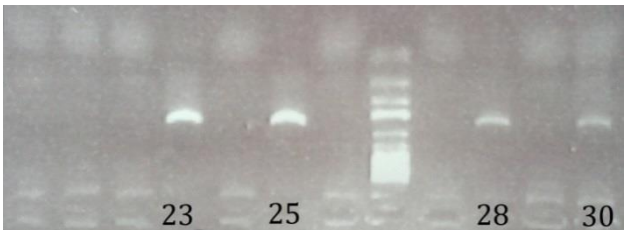


Figure 28 – Electrophoresis of colony PCR product of plasmid construct of *lxr*A.

L-xylulose reductase of *Aspergillus niger* showed to be active using 10 mM L-xylulose and 200 µM NADPH (Figure 29). Simultaneously, a control without L-xylulose was tested to ensure that NADPH degradation did not mislead to $NADP^+$ formation by the enzyme. In this colorimetric assay the absorbance was read at 340 nm and a molar extinction coefficient of 6220 M^{-1} cm^{-1} was used. By monitoring NADPH formation, the Δabs was estimated as 0.065 min^{-1} cm^{-1}. *Lxr*A concentration was 2.31 mg l^{-1} and had an activity of 1.05 x 10^{-5} mol l^{-1} min^{-1}, corresponding to 4.52 U mg^{-1} (Table 4).

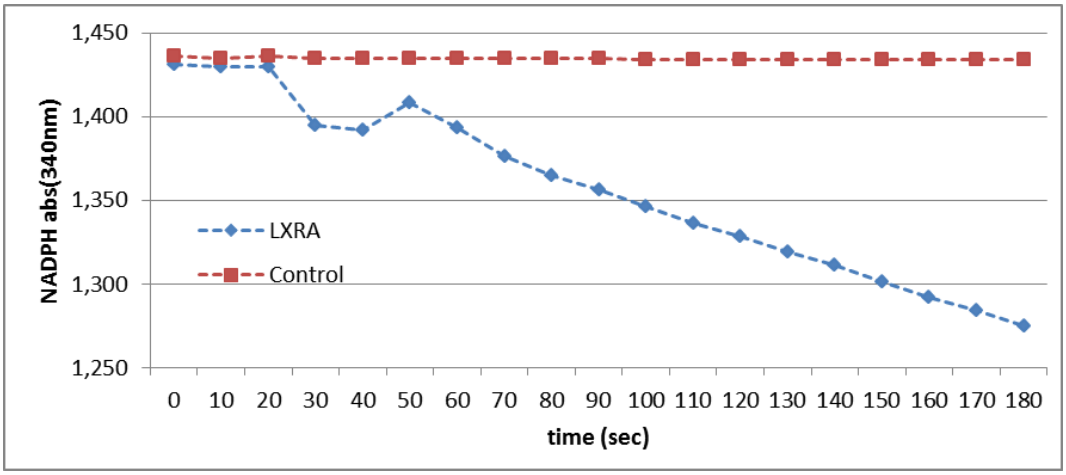


Figure 29 – Activity of *lxr*A with NADPH at 340 nm.

Table 4 – Activity of *lxr*A with NADPH.

***lxrA* cofactor**	**Activity**		
	(mol l^{-1} min^{-1})	**(U l^{-1})**	**(U mg^{-1})**
NADPH	1.05 x 10^{-5}	10.45	4.52

In order to ensure *lxr*A purity, SDS-PAGE was carried out. The sample was concentrated with Vivaspin 500 µl (Sartorius Stedim Biotech) to approximately 23,1 mg l^{-1} (10 times more) to have a detectable protein in the SDS-PAGE. Simultaneously, a 1/10 diluted sample was also added to the assay. From both samples it was possible to identify a single band with a molecular mass estimated of 35 kDa (Figure 30).

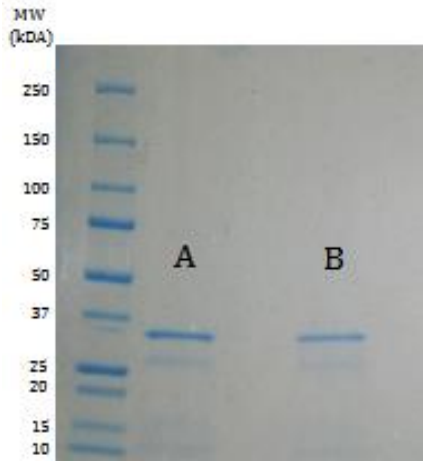


Figure 30 – SDS-PAGE of purified *lxr*A. A: *lxr*A concentrated with Vivaspin; B: *lxr*A concentrated with Vivaspin 1/10 diluted.

3.3. *In vitro* pathway test

A coupled assay was done in order to assay the activity in liver extract. *Lxr*A, GDH and liver decarboxylase were tested in a single process using L-gulonate-3-dehydrogenase, Thio-NAD^+ and NADPH at 340 nm. Firstly, a gradual addition of the enzymes was added to a sample containing L-gulonate, Thio-NAD^+ and Buffer (Tris-HCl pH 8.0) (Figure 31). A second coupled assay was done incubating L-gulonate, Tris-HCl pH 8.0, Thio-NAD^+ and GDH for 1 hour. Prior to the assay, it was added NADPH to the sample. The consumption of NADPH was measured adding the decarboxylase and *lxr*A (Figure 32).

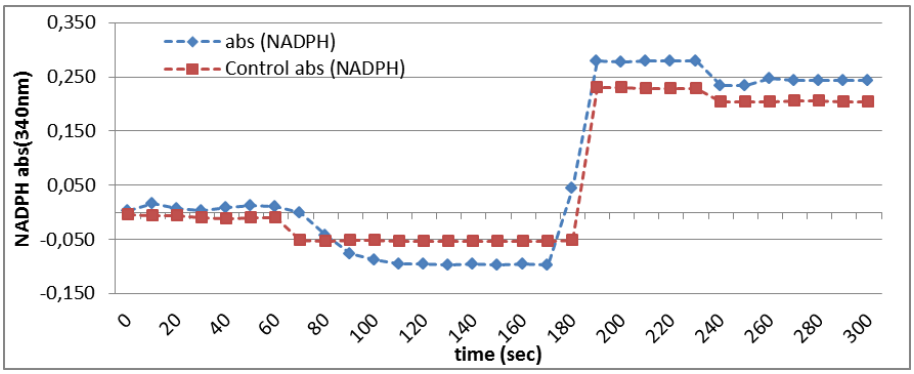


Figure 31 – Glucuronic acid pathway tested *in vitro*. Sample: L-gulonate, Tris-HCl pH 8.0 and Thio-NAD^+; GDH added after 60 seconds; liver extract added after 170 seconds; *lxr*A added after 230 seconds. Control: as sample, without L-gulonate.

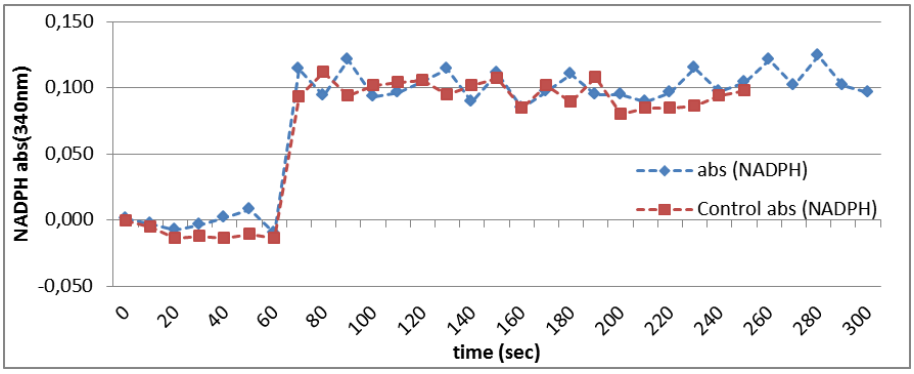


Figure 32 – Glucuronic acid pathway tested in vitro. Sample: incubation of L-gulonate, GDH and Thio-NAD+ during 1 hour; NADPH was added after incubation; liver extract added after 60 seconds; *lxr*A added after 180 seconds. Control: as sample, without L-gulonate.

4. Discussion

The His-tagged L-gulonate-3-dehydrogenase was successfully purified, being able to convert L-gulonate to 3-keto-L-gulonate. It was able to use both NAD^+ and Thio-NAD^+ but had about 16-fold higher activity with NAD^+ compared to Thio-NAD^+.

The NADPH-dependent *lxr*A showed also to be active and pure. The enzyme had 38-fold higher activity than GDH using Thio-NAD^+. By SDS-PAGE it was possible to confirm that the His-tagged *lxr*A was pure, as it contained a single dark band.

The first coupled assay was done by gradually adding its components (Figure 31). The low activity of GDH with Thio-NAD^+ was thought to limit the experiment, as the production of 3-keto-L-gulonate was slow and $NADP^+$ formation was not detected in the first experiment (Figure 31). In order to provide 3-keto-L-gulonate formation, GDH was incubated with L-gulonate and Thio-NAD^+. Despite the variations seen on the colorimetric assay, no activity was found when NADPH, the decarboxylase and *lxr*A were added (Figure 32). The experiment proved that the pathway was not able to produce xylitol, indicating that no decarboxylase was purified or an inactive decarboxylase was used for the assay.

The use of a bovine liver that was got from a butcher shop might be the explanation for a purification of an inactive decarboxylase: the purification was done by following Winkelman and Ashwell's protocol, where a decarboxylase was successfully purified from guinea pig acetone powder. Therefore, the purification process might not be the reason for ineffective coupled assay. In addition, the liver that was used to purify the decarboxylase could have had denatured proteins and a fresher bovine liver could have avoided that condition.

III. Conclusion

The recently completed D-galacturonic acid pathway of *Aspergillus niger* could now be applied to the Biorefinery (Kuivanen, et al., 2012). In this study it was demonstrated that citrus wastes can be converted to high-value compounds in a consolidated process, using this filamentous fungus. Most importantly, the process can be scaled up using both solid-state and submerged-state fermentation, which is crucial in industry. For the orange peel fermentation, the conditions were chosen according to a literature review. It is believed that the optimization of some variables as orange peel size, spore suspension concentration, aeration and downstream process would result in higher L-galactonate concentrations, increasing the potential of this process.

L-xylulose reductase of *Aspergillus niger* and L-gulonate-3-dehydrogenase were successfully engineered and purified to active enzymes. However, the main aim was not achieved, as the coupled assay did not provide an activity of a 3-keto-L-gulonate decarboxylase from the bovine liver. It is believed that a coupled-enzyme assay can be useful for this experiment and that simple adjustments might already give some results.

Regardless the final results achieved in the eukaryotic D-glucuronic acid pathway project, this project represented a great personal and research challenge requiring the application of advanced metabolic engineering techniques.

Once again, Biotechnology showed that has the potential to be a new solution for new problems. In health and in industry, it is creating remarkable effects in research and this work is the proof of that.

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