TÉCNICO LISBOA

Exploring *Saccharomyces cerevisiae* to improve microbe-based production of itaconic acid

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Abstract

Microbe-based production of itaconic acid is essential for the implementation of biorefineries since this building block is considered to have a great potential to replace oil-derivatives. Although efficient, the processes implemented for production of microbial production of itaconic acid have an yield which is considered to be below the theoretical one. This low yield is attributed, in part, to the toxic effect exerted by itaconic acid in the producing cells. In this work the experimental model yeast Saccharomyces cerevisae was used for the production of itaconic acid using an optimized version of the A. terreus Cad1 gene. The results obtained show that expression of this AtCad1 gene in yeast enabled the production of itaconic acid, albeit with low yields. These results open the door to the successful exploration of yeast as a suitable microbial cell factory for the production of itaconic acid.

Keywords: Itaconic acid, AtCad1, micro-based production of itaconic acid *S. cerevisiae*

Resumo

A produção microbiana de ácido itacónico e outras "building-block molecules" é considerada um passo fundamental para a implementação de biorefinerias visto que estes compostos têm potencial para serem usados em vias biosintéticas onde são usados derivados de petróleo. Embora eficientes os processos usados hoje para a produção de ácido itacónico apresentam um rendimento inferior ao considerado possível. Este baixo rendimento é atribuído, em parte, ao efeito tóxico exercido pelo ácido sobre as células produtoras e à não optimização do metabolismo. Neste trabalho explorou-se a levedura *Saccharomyces cerevisiae* como possível hospedeiro para a produção de ácido itacónico aproveitando as suas propriedades de modelo experimental e de boa utilização para engenharia metabólica. Os resultados obtidos mostram que células de levedura transformadas com um plasmídeo contendo clonada uma versão otimizada do gene AtCad1 são capazes de excretar ácido itacónico para o meio extracelular. O rendimento obtido é, no entanto, significativamente inferior aquele que seria necessário obter para tornar este organismo como uma alternativa viável como fábrica celular. Os resultados obtidos neste trabalho abrem a porta à futura implementação da utilização da levedura como fábrica celular para a produção de ácido itacónico.

Palavras-chave: Ácido itacónico, AtCad1, produção microbiana de ácido itacónico, S. cerevisiae

Abbreviations

***A. niger*-** *Aspergillus niger*

AtCAD1-*CAD1* gene from *A. terreus*

***A. terreus*-** *Aspergillus terreus*

CAD- *cis*-aconitic acid decarboxylase

cDNA - complementary DNA

CIAP - Calf Intestinal Alkaline Phosphatase

dNTP - Deoxynucleotide Triphosphates

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic Acid

***E.coli* -** *Escherichia coli*

EDTA - Ethylenediaminetetraacetic Acid

EUROSCARF - European *Saccharomyces cerevisiae* Archive for Functional Analysis

MDR - Multidrug resistance

MFS - Major Facilitator Superfamily

MMB - Minimal growth medium

MMB -U - Minimal growth medium without uracil

MMB Gal - Minimal growth medium that has galactose as a carbon source

mRNA - Messenger RNA

OD - Optical Densitometry

PCR- Polymerase Chain Reaction

PE - Phosphatidylethanolamine

PEG - Polyethylene glycol

PI - Phosphatidylinositol

pKa - Acid dissociation constant

PMSF - Phenylmethanesulfonylfloride

RNA - Ribonucleic Acid

rpm - Rotation per minute

RT-PCR - Reverse Transcription Polymerase Chain Reaction

***S. cerevisiae* -** *Saccharomyces cerevisiae*

SDS-PAGE - Dodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TCA - Tricarboxylic acid

TLC - Thin-Layer Chromatography

YPF - Yeast Peptone Dextrose

Contents

Abstract 3

Resumo 4

1. Introduction 6

1.1 Carboxylic acids: building-block molecules of high interest 6

1.2 Microbial production of itaconic acid 8

1.3 *Saccharomyces cerevisiae* as a cell factory for the production of carboxylic acids 12

1.4 Toxicity to fungal cells imposed by carboxylic acids: lessons from *S. cerevisiae* ... 14

1.5 Thesis outline 17

2. Materials and Methods 19

2.1 Strains and growth media 19

2.1.1 *In vitro* DNA manipulations 19

2.1.2 Yeast transformation 20

2.1.3 Confirmation of the AtCAD1(opt) integration in the pGREG586 vector 21

2.1.4 Detection of itaconic acid production in *S. cerevisiae* cells transformed with the pAtCAD1(opt)::His6 plasmid 22

2.2 Assessment of the susceptibility of yeast strains to toxic concentrations of itaconic acid based on growth curves in liquid growth medium **Erro! Marcador não definido.**

2.3 Measurement of the internal accumulation of radiolabelled itaconic acid inside *S. cerevisiae* cells ..... **Erro! Marcador não definido.**

2.4 Effect of itaconic acid stress in the expression of *QDR3*, *PDR16* and *GAP1* genes ..... **Erro! Marcador não definido.**

2.3.4 Effect of itaconic acid stress in the total content of fatty acids and phospholipids recovered from whole *S. cerevisiae* BY4741 or BY4741_Δgpx1/Δgpx2/Δgpx3 cells ..... **Erro! Marcador não definido.**

3. Results 22

3.1 Heterologous expression of the optimized *AtCAD1* gene (*OptAtCAD1*) in *S. cerevisiae* 22

3.2 Role of genes involved in phospholipid biosynthesis in *S. cerevisiae* tolerance to itaconic acid ..... **Erro! Marcador não definido.**

3.3 Role of glutathione peroxidase enzymes in S. cerevisiae tolerance to itaconic acid ..... **Erro! Marcador não definido.**

3.4 Identification of *S. cerevisiae* MDR transporters that could be involved in reducing the internal accumulation of itaconic acid ..... **Erro! Marcador não definido.**

3.5 Identification of yeast transcription factors mediating tolerance to itaconic acid ..... **Erro! Marcador não definido.**

4. Discussion 26
6. References 28

List of figures

Figure 1 - Number of publications per year matching the search phrases (("acid name" or "base conjugate name" or "acid synonym(s)") and ("fermentation" or "synonyms"). [4] 7
Figure 3 - Variety itaconic acid industrial applications [10]. 8
Figure 2 - Itaconic acid structural formula [3]. 8
Figure 4 - View of *A. terreus* genome focus on the itaconate gene cluster. *CadA,* encoding the *cis*-aconitic acid decarboxylase*, MTT* encoding a putative mitochondrial tricarboxylate transporter (ATEG_09970) and an *MFS* encoding ATEG_09972 are highlighted [3]. 10
Figure 5 - Biosynthesis pathway of itaconic acid in *Aspergillus terreus* [2]. 10
Figure 6 - Overview of a biorefinery. a) Representation of the different types of feedstock (biomass and agriculture waste)that can be processed to generate sugar. Afterwards, these sugars are converted into fuels or chemicals by a biocatalyst, e.g., *S. cerevisiae*. b) Metabolic engineered strain with the ability to produce different types of products such as organic aids (e.g., succinic acid), sesquipertenes (e.g., farnesene) or biofuels (e.g. ethanol). This metabolic engineering can also enable the degradation of the polymers present in the feedstock by the expression of enzymes that degrade this polymers [24]. 12
Figure 7 - Biosynthetic pathways of several carboxylic acids through metabolic intermediates present in microorganisms. [4] 13
Figure 8 - Organic acid toxicity and tolerance mechanisms in *S. cerevisiae*. Simulation of the H^+-ATPase present in the plasma and vacuolar membranes contribute to the recovery of internal pH to more physiological values and for metabolite compartmentalization in acid-stressed cells. The reconfiguration of cell wall structure and plasma membrane lipid composition may reduce the diffusion rate of undissociated weak acids and reduce the weak acid-induced plasma membrane damaged [26]. 15
Figure 9 - Distribution of the codon usage frequency along the length of the CDS to be express in the *S. cerevisiae.* The obtained CAI value was 0.61. (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis) 18
Figure 10 - Percentage of distribution of codon in computed codon quality groups of the *AtCAD1* gene for *S. cerevisae* (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis). 18
Figure 13 - Schematic representation of the cloning engineering strategy used to clone the optimized *AtCAD1(opt)* gene in the pGREG586 plasmid. 23
Figure 14 - Gel obtained after PCR amplification of the *OptAtCAD1* gene from the pUC57_AtCad1 vector and digestion of pGREG586 with *SalI*. Lane 1: 1 kb DNA ladder; Lanes 2-5: *AtCAD1(opt)* amplified fragment; Lane 6: pGREG586 digested with the *SalI* restriction enzyme. 24
Figure 15 - Gel obtained after the digestion of plasmid DNA recovered from E. coli colonies considered to be candidates to have the pAtCad1(opt)::His6 recombinant plasmid. Lane 0: 1kb DNA ladder; Lane 1: pAtCAD1(opt)::His6 restricted with HindIII ; Lane 2: pAtCAD1(opt)::His6 restricted with EcoRI and BglII. 24
Figure 16 - UV-Vis spectrum of a standard solution (0.025mM) of itaconic acid. 25
Figure 17 - UV-Vis spectrum analysis of the collected supernatants after 24h. Green: *BY4741* transformed with pAtCAD1(opt)::His6 plasmid cultured in MMB-U with 2% galactose supplemented with CSM, vitamins and trace elements; Blue: *BY4741*

transformed with pAtCAD1(opt)::His6 plasmid cultured in MMB-U with 2% glucose supplemented with CSM, vitamins and trace elements; Pink: *BY4741* transformed with pGRER586 plasmid cultured in MMB-U with 2%galactose supplemented with CSM... 26

List of tables

Table 1 – Examples of microbial species engineered and other natural producers for the production of itaconic acid [17]. 11
Table 2-Advantageous characteristics exhibited by *S.cerevisae* for the production of carboxylic acids. 14
Table 5 - Primers used to amplify the *AtCAD1(opt)*. A region homologous to the pGREG586 vector (corresponding to the underlined nucleotides) was included in each primer to allow *in vivo* recombination. 20
Table 6 - Mixture used in the PCR for 20
Table 7 - PCR conditions used for genes amplification. 20
Table 8 - Mixture used in the PCR reaction for gene amplification. 21
Table 9 - PCR condititions used for the gene amplification. 21
Table 15 – Titers of itaconic acid produced by *S. cerevisiae* BY4741 cells transformed with the pAtCAD1(opt)::His6 plasmid when cultured in MMB-Gal-U supplemented with CSM in maximum period of 72 hours. 26

1. Introduction

Nowadays, products as polymers, acidulants, preservatives, flavor compounds and precursors for the synthesis of pharmaceuticals are mostly produced through catalytic processing of petroleum-based precursors [3]. The US and the EU have given firm political steps for the successful implementation of biorefineries [4] establishing ambitious goals to reduce liquid petrol and industrial organic chemicals in 25-35% by 2025 [4]. However, the successful implementation of biorefineries is dependent on the identification of building block molecules that will be used in replacement of oil-derivatives [5]. Several products of microbial metabolism are recognized to have the potential to be used as building block chemicals and for this reason the exploration of microbes as cell factories for the production of numerous interesting metabolites has gained a lot of attention in the recent years. An important advantage of microbe-mediated conversion of sugars is the fact that it is carried out in a single step which simplifies the process and reduces the required energy input. Another relevant advantage is the high specificity of microbial enzymes in producing the compound of interest which reduces the formation of unwanted by-products thereby facilitating the downstream purification processing. Although advantageous, the use of microbial fermentation to produce building-blocks molecules is not without problems since this approach is limited, among other factors, by the extent at which the cells produce the compound of interest. In almost all cases extensive metabolic engineering is required to rewire metabolism into the product of interest to increase the yields of the process to value economically attractive. Furthermore, the microbial enzymes that produce the “interesting metabolites” are often not optimized to the conditions required for the development of the different industrial processes and therefore enzymatic engineering may also be required to improve the process yield[6].

1.1 Carboxylic acids: building-block molecules of high interest

The potential of carboxylic acids as alternative building-block molecules relies on its carboxyl group (COOH) that may serve as a platform for several organic reactions like acetylation, acylation, redox, among others[3, 5]. In addition to the carboxyl group, most of the carboxylic acids considered of interest also harbor in their structure another carboxyl group or an alcohol group, thus increasing the range of synthetic routes that could be performed using these molecules[3]. The US department of Energy and the European focus group BREW (Medium and long-term opportunities and risks of the biotechnological production of bulk chemicals from renewable resources) has identified seventeen carboxylic acids among the most interesting “green” molecules that could be used as building-blocks [7]. The economic

market predicted for these molecules is considered highly attractive with projected markets higher than 200,000 tons per year [5]. It is also very important to notice the increasing interest in this type of molecules regarding their bio-based production by the increasing number of publications in the last 23 years (Figure 1)[3]. Among these highly interesting acids is itaconic acid, the main focus of this thesis.

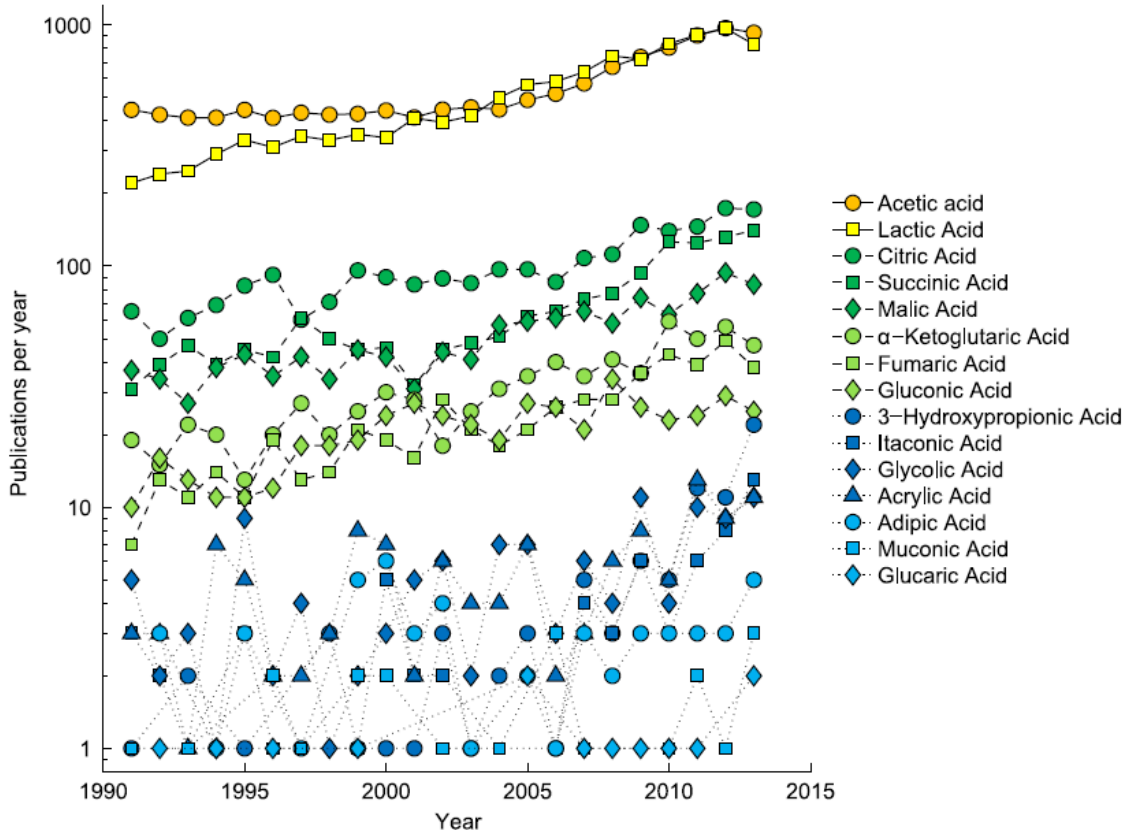

Figure 1 - Number of publications per year matching the search phrases (("acid name" or "base conjugate name" or "acid synonym(s)") and ("fermentation" or "synonyms"). [3]

Itaconic acid is a C_5 saturated dicarboxylic that contains a conjugated double bond and a carboxyl group activated by an ethyl-methyl group (Figure 2) which makes it an effective intermediate for the synthesis of complex organic compounds through several possible routes including salt formation with metals, esterification, addition reaction and polymerization. The range of products that can be produced from itaconic acid is very broad and has application in different fields as shown in Figure 3 [8].

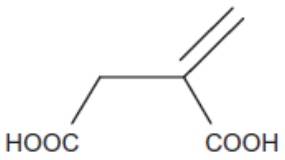

Figure 2 - Itaconic acid structural formula [2].

Figure 3 - Variety itaconic acid industrial applications [9].

Since 1990s, the applications of itaconic acid has been expanded to the biomedical field where it is used in the production of dental and ophthalmic products and, more recently, in drug delivery [10]. There are four countries reporting production of itaconic acid: China, USA, Japan and France[10]. Significantly, the Lux Research (http://www.luxresearchinc.com/), a research and advisory firm on emerging technologies, selected Itaconix (http://www.itaconix.com/) as one of the top 10 most compelling companies profiled in 2012. According to a recent report by Global Industry Analysts, Inc., the global market of itaconic acid is expected to increase up to $398,3 million (€296,0 million) in 2017[1].

1.2 Microbial production of itaconic acid

Itaconic acid results from decarboxylation of *cis*-aconitic acid, a by-product of the Krebs cycle, in a rection that is mediated by the Cad1 enzyme (Figure 5). Until recently

natural production of itaconic acid has only been reported in a very limited number of filamentous Fungi including *Aspergillus terreus* and *Ustilago medyis* [11]. It is now known that mammalian cells also produce itaconic acid naturally as a response against bacterial infection. This production occurs trough the enzyme encoded in the in the immunoresponsive gene 1 (*Irg1*), identified as a *cis*-acotinate decarboxylase highly expressed in macrophages when exposed to bacterial lipopolysaccharyde (LPS) [12-14]. It is thought that the production of itaconic acid decreases bacterial growth by inhibiting the function of isocitrate lyase, an enzyme that is essential for bacterial survival inside the macrophages [12]. Production of itaconic acid at an industrial scale is mostly performed by fermentation carried out by *A. terreus* TN-484-M1 strain (an isolate that was obtained in a survey for fungi exhibiting resistance to high itaconic acid concentrations [1, 5]) or by *A. niger* strains engineered to synthesize the Cad1 enzyme. Production of itaconic acid via chemical synthesis is also possible, however, it is a multi-step reaction which increases the cost of the process to prohibitive values. The yields obtained for production of itaconic acid mediated by *A. terreus* or *A. niger* is around 90 g/L (using glucose as a carbon source) [8], this value being significantly below the theoretical yield which is estimated in 240 g/L[10]. This low yield is, in part, attributed to the fact that the metabolism is not optimized for the production of itaconic acid. The fact that glucose is the carbon source leading to the highest levels of itaconic acid is something very problematic for the economical viability of this process since the cost of the sugar represents more than 25% of the total costs [10]. As a solution for this problem feedstocks have been considered to be used as the carbon source, however this implies bringing to the broth unwelcome compounds or additional phosphate or nitrogen sources that divert cell metabolism from the itaconic acid production pathway [1]. Another significant problem comes from the accumulation of toxic concentrations of itaconic acid in the fermentation broth, specially at the later stages of the fermentation when the pH of the growth medium is very acidic (around 2.5)[10].

The *A. terreus* Cad1 enzyme is encoded by the *cadA* gene which is localized in a genomic region that also includes a putative regulator of the zinc-finger family, a putative mitochondrial tricarboxylate transporter (*MTT*), and a putative Major Facilitator Superfamily (*MFS*) transporter (Figure 4)[2]. *MTT* is suggested to function in the transport of tricarboxylic acids from mitochondria to cytosol and/or vice versa [2]. The clustered location of *MTT* together with the *CAD* gene suggested that this transporter may be involved in the export of itaconate from the mitochondria to the cytosol[2]. This point is supported by the findings described in van der Straat *et. al* [15] in which it is shown that the combined expression of *cadA* along with *MTT* increased itaconid acid production [15]. Surprisingly, the expression of *mfsA* along with *cadA* had no effect in

the production of the acid suggesting that it remain to be identified plasma membrane transporters that could function in the export of itaconate from the cytosol to the cell exterior[15].

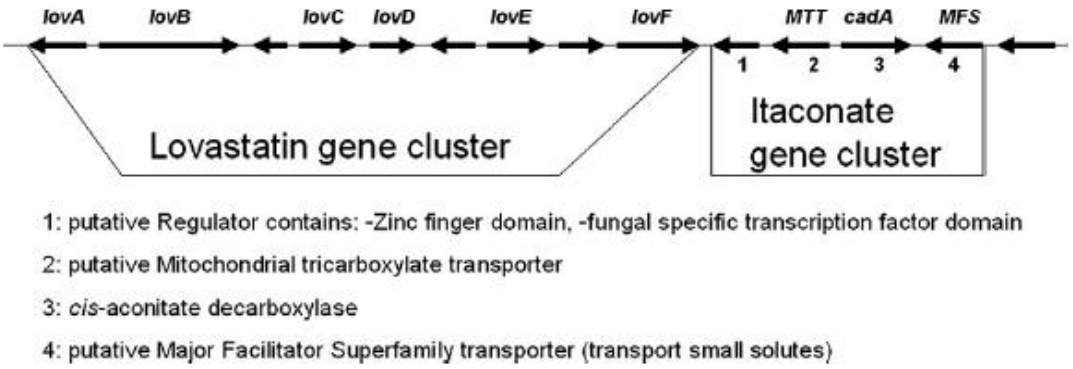

Figure 4 - View of *A. terreus* genome focus on the itaconate gene cluster. *CadA*, encoding the *cis*-aconitic acid decarboxylase, *MTT* encoding a putative mitochondrial tricarboxylate transporter (ATEG_09970) and an *MFS* encoding ATEG_09972 are highlighted [2].

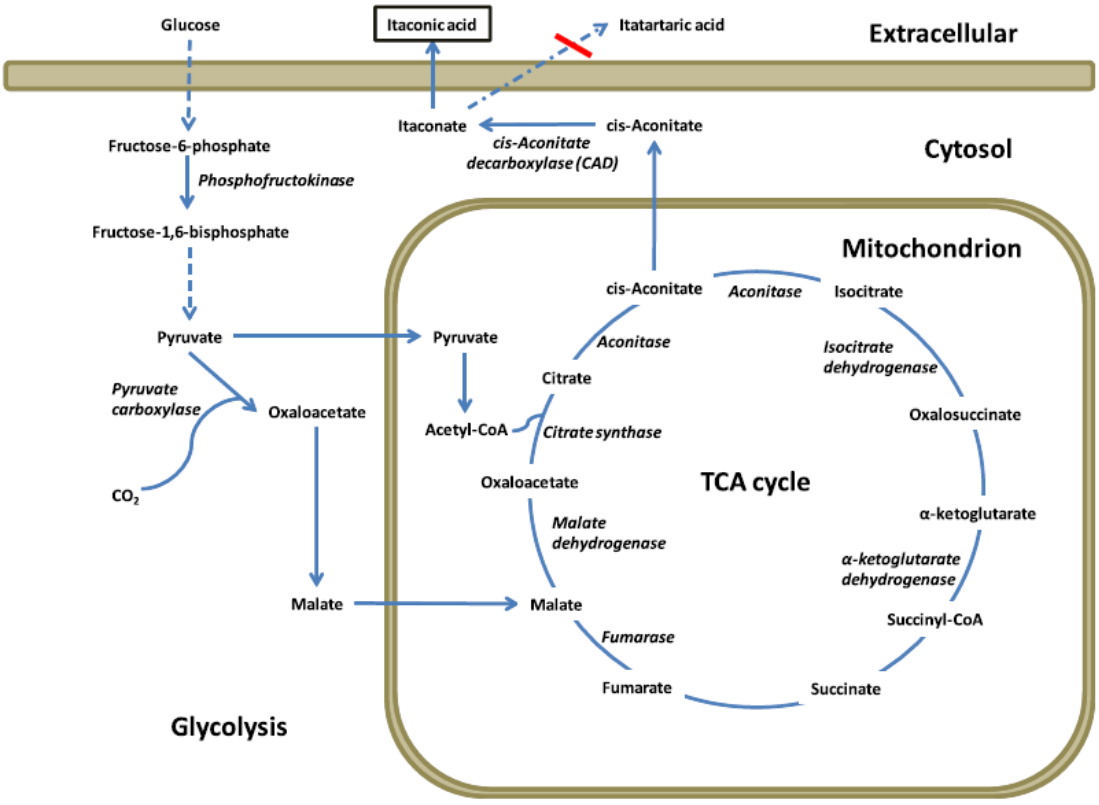

Figure 5 - Biosynthesis pathway of itaconic acid in *Aspergillus terreus* [1].

In the last years, there have been published several studies exploring the production of itaconic acid in different hosts (Table 1). Although successful, the yield of these processes is still well below the yields obtained with *A. terreus*.

Table 1 – Examples of microbial species engineered and other natural producers for the production of itaconic acid [16].

Host	Genetic modifications	IA production
E. coli pET-9915 [2]	CAD (optimized for *E. coli* codon usage)	<1 g/L
E. coli BW25133	CAD	4,1 g/L
Aspergillus niger AB 1.13	CAD	0,6 g/L
Aspergillus niger AB 1.13	CAD + MTT (transporter)	2.2 mg IA/g wet weight
Aspergillus terreus [17]	Modified PFK1	45.5 g/l (vs 21,1 g/L wildtype strain)
Potato cells	CAD (optimized codon usage)	9 mg IA/ wet weight
Yarrow lipolytica	CAD, aconitase	2,6 g/L
Ustilago maydis [18]		25 g/L
S. cerevisiae [19]	CAD1 with synthetic hybrid promoter	24 mg/L
S. cerevisiae [19]	CAD1 with synthetic hybrid promoter expressed in a *Δade3*/*Δbna2*/*Δtes1* background	168 mg/L
Pseudozyma antarctica NRRL Y-7808		30 g/L

A key factor for the production of itaconic acid is the medium composition, which has been suffering optimizations over the last 40 years. The required medium components are: high concentration of glucose (7.5-15%), magnesium sulfate, low nitrogen and phosphorous, low but adequate levels of zinc, copper [20] and iron and limited magnesium (approximately 10 ppb)[9, 20]. The presence of these minerals and nutrients are fundamental for the production, however the amount in which they are present must be highly controlled because if the values are outside of the optimum value the yield is negatively influenced[9]. This tight control over nutrient composition is problematic if the process is to be carried out more complex medium, besides the presence of potentially harmful chemicals formed during the pre-treatment or that occur

naturally [9]. For instance, the presence of nitrogen and trace amounts of zinc and iron ions improve the production but phosphate has to be limited once mycelial growth is established to prevent carbon diversion into further mycelia production[9].

1.3 *Saccharomyces cerevisiae* as a cell factory for the production of carboxylic acids

S. cerevisiae is widely used for years by the food industry and, more recently, by industries aiming the production of bioethanol either from crops or, more recently, from lignocellulosic feedstocks. *S. cerevisiae* is also considered an interest system for the production of lignocellulosic bioethanol, since is fairly resistant to inhibitors present in biomass hydrolysates (e.g. acetic acid, vanillin or furfural) and is able to grow anaerobically [21]. These two reasons drove the increasing exploration of *S. cerevisiae* in the so called, industrial "white" biotechnology that is focused on the fermentative production of industrially relevant biochemicals, such as glycerol, propanediol, organic acids, sugar alcohols, L-glycerol-3-phosphate (L- G3P), steroids and isoprenoids (Figure 6) [21]. These substances are either directly used in the pharmaceutical or chemical industry or represent building blocks or precursors for further chemical or enzymatic syntheses[21] [22].

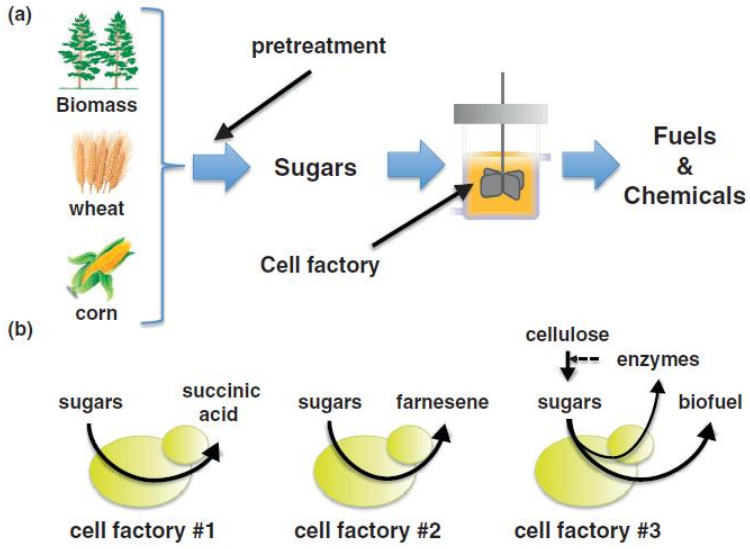

Figure 6 - Overview of a biorefinery. a) Representation of the different types of feedstock (biomass and agriculture waste)that can be processed to generate sugar. Afterwards, these sugars are converted into fuels or chemicals by a biocatalyst, e.g., *S. cerevisiae*. b) Metabolic engineered strain with the ability to produce different types of products such as organic aids (e.g., succinic acid), sesquipertenes (e.g., farnesene) or biofuels (e.g. ethanol). This metabolic engineering can also enable the degradation of the polymers present in the feedstock by the expression of enzymes that degrade this polymers [23].

However the yeast is not able to metabolize pentoses, the carbon source more abundant in lignocellulosic hydrolysates, which ultimately is a disadvantage[3]. The extensive knowledge that has been gathered on the physiology of this microbe together with the numerous tools available for genetic and metabolic engineering have turned the yeast very attractive platform for the production of a number of add-value compounds [3, 5], including several organic acids (summarized in Figure 7). Compared to classical methods of genetic strain improvement (selection, mutagenesis, mating, and hybridization), metabolic engineering-based methods brings two improvements [21], (i) allows a direct modification of the yeast strains without the need of creating/accumulating unfavorable mutations, (ii) it turns possible to introduce genes from foreign organisms thereby introduce novel traits and open the door to unforeseen applications for this microbe. The last is particularly important for industrial biotechnology since it provides new pathways that increase the variety of industrial "waist" that can be used as growth media (e.g., lignocellulosic biomass) and/or to produce compounds not naturally produced by *S. cerevisiae* [21], in a synthetic biology perspective.

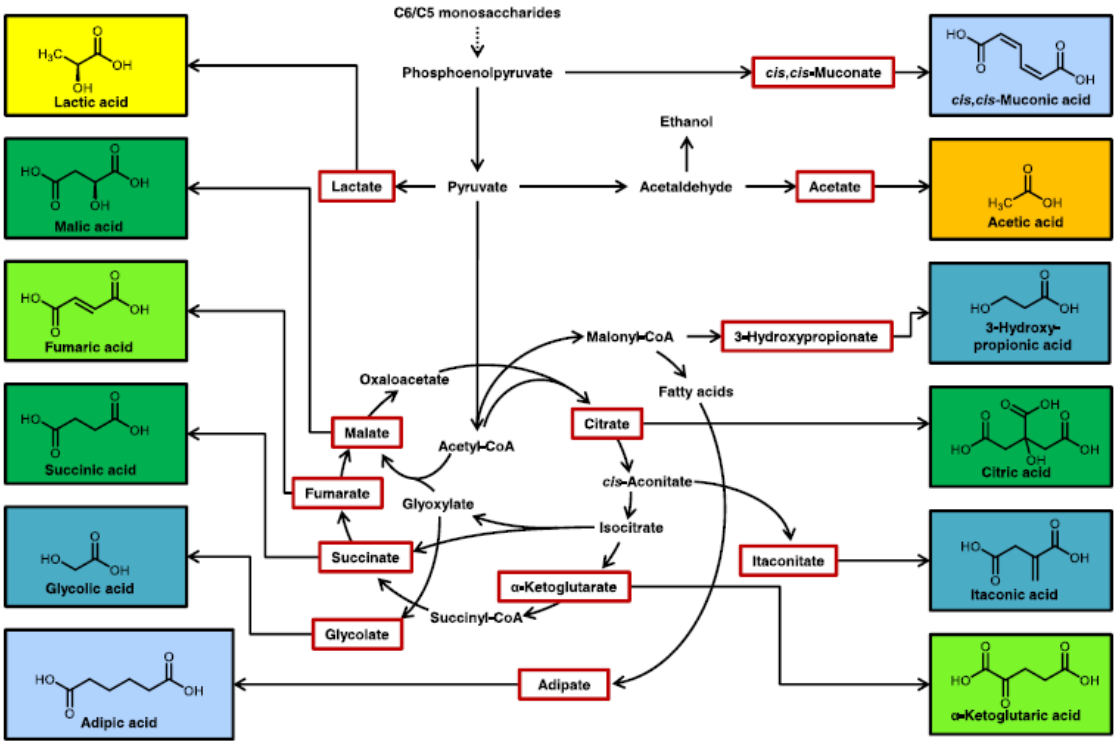

Figure 7 - Biosynthetic pathways of several carboxylic acids through metabolic intermediates present in microorganisms. [3]

One of the most important phenotypic advantages that *S. cerevisiae* has for the production of carboxylic acids is the fact that it grows well at acidic pHs which is an important trait since these molecules should be produced in their undissociated form in order to avoid the need of having to use huge amounts of base to neutralize the acid anion with a concomitant increase in cost and difficulties in the downstream processing.

In the particular case of carboxylic acids the advantages of *S. cerevisiae* as a host system are described in Table 2.

Table 2-Advantageous characteristics exhibited by *S.cerevisae* for the production of carboxylic acids.

Characteristic	Advantage
Growth at low pH	Production of acids in its undissociated forms (avoiding the use of neutralizing agents that complicate the downstream processing) [5]
Not filamentous	Avoids problems such as variability in fermentation performance, increase in the complexity of the broth rheology and difficulties in mixing and aeration of the cultures [5]
Amenable for *in silico* metabolic engineering	Available a genome-scale metabolic model, available collections of mutant strains that lack (or that over-express) genes of interest allowing high-throughput scanners[24]

1.4 Toxicity to fungal cells imposed by carboxylic acids: lessons from *S. cerevisiae*

As previously mentioned, the toxic effect exerted by the accumulation of high concentrations of itaconic acid in the fermentation broth is believed to be one of the main causes for the reduced yields that are obtained in microbial fermentations carried out by *A. terreus* or *A. niger*. The molecular mechanisms by which carboxylic acids exert toxicity in fungal cells have mainly elucidated in *S. cerevisiae*. At an external pH below the weak acid pKa value, the lipophilic undissociated form of the acid (RCOOH) predominates and may permeate the plasma membrane by simple diffusion (Figure 8). In the case of itaconic acid (pKa 3.85) it is expected that the 95% of the toxic undissociated form should be present in the growth medium considering a pH of the fermentation of 2.5, which is within the described pH ranges that can go down up to 2.

Once in the near-neutral cytosol, chemical dissociation of the carboxylic acid occurs leading to the release of protons (H^+) and of the respective counter-ion ($RCOO^-$). Due to their electric charge, these ions are not able to cross the hydrophobic lipid plasma membrane bilayer and are therefore expected to accumulate in the cell interior (Figure 8).

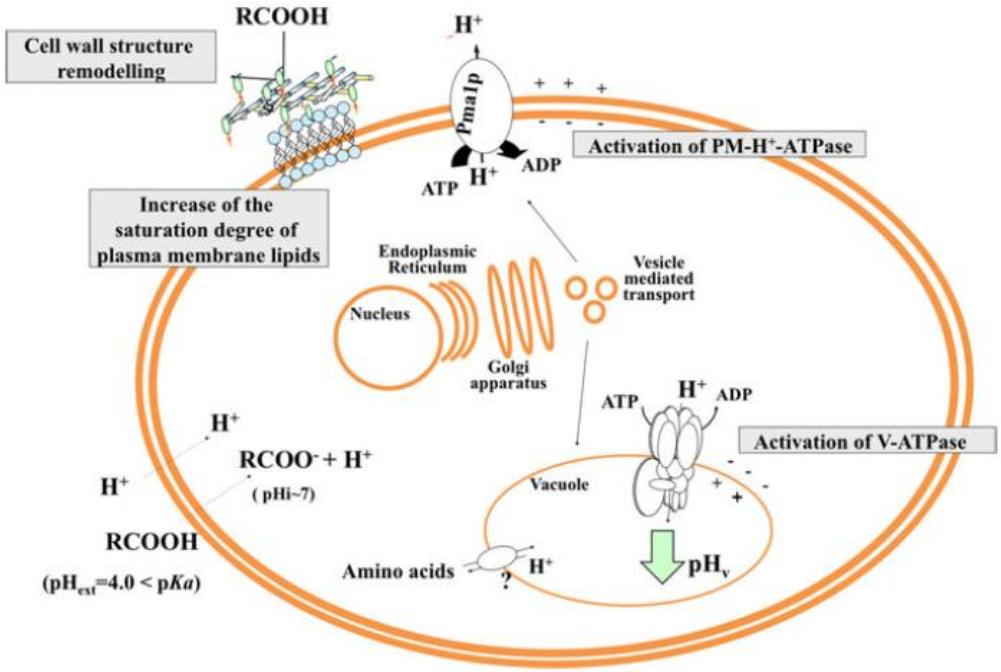

Figure 8 - Organic acid toxicity and tolerance mechanisms in *S. cerevisiae*. Simulation of the H^+-ATPase present in the plasma and vacuolar membranes contribute to the recovery of internal pH to more physiological values and for metabolite compartmentalization in acid-stressed cells. The reconfiguration of cell wall structure and plasma membrane lipid composition may reduce the diffusion rate of undissociated weak acids and reduce the weak acid-induced plasma membrane damaged [25].

To compensate this increased protons flux, resulting from dissociation of the acid and from membrane permeabilization, *S. cerevisiae* cells rely on the activity of two proton pumps, one located in the plasma membrane, the PM-H^+-ATPase protein, encoded by the *PMA1* gene, and the other located in the vacuolar membrane, the V-ATPase (Figure 8). The Pma1p excretes the exceeding protons to the cell exterior while V-ATPases catalyzes their efflux to the lumen of the vacuole. The activity of these two proton pumps counter-acts the dissipation of the plasma and vacuolar membrane, respectively, also contributing for the maintenance of intracellular pH within physiological values. Both these two proton pumps have been described to have a role in yeast response and resistance to weak acids such as acetic and lactic acids [26, 27].

Due to its electric charge, the resulting counter-ions ($RCOO^-$) are not able to cross the hydrophobic lipid plasma membrane bilayer, consequently accumulating inside the cell.

This accumulation can exert several different deleterious effects as the increase in turgor pressure, oxidative stress, protein aggregation, lipid peroxidation, inhibition of membrane trafficking. In the presence of more lipophilic acids there is also a disturbance of the organization and function of cellular membranes which leads to a subsequent increased cell permeability to protons (aggravating the reduction of internal pH) and to the dissipation of the electrochemical potential maintained across the membrane, driving force for the secondary transport (Mira, Teixeira [25]). To reduce the internal accumulation of acid counter-ions *S. cerevisiae* cells rely on the activity of specific inducible transporters. Several transporters of the Major Facilitator Superfamily (MFS) involved in multidrug resistance (MDR) have been implicated in *S. cerevisiae*, being one of them the ABC transporter Pdr12p which has been found to be play a fundamental role in yeast to tolerate moderately lipophylic acids such as propionic, benzoic and sorbic acids [28]. Although these MFS-MDR transporters are described as drug efflux pumps, evidences support that these must have a natural substrate, being the drug transport only an opportunistic event [29].

Weak acids also inhibit the activity of glycolytic enzymes as a result of the internal acidification [27, 30, 31], which in combination with the activation of energy-consuming defense mechanisms (ATP consuming processes such as PM-H^+-ATPase, V-H^+-ATPase, and ABC drug pumps) deplete cells of energy [32]. The active expulsion of weak acid anions from the cell interior would be energetically expensive and futile if the undissociated acid could reenter the cells at a similar rate. Consequently, one of the mechanisms proposed to reduce the diffusion rate of weak acids is the reinforcement of cell wall structure to decrease its porosity [33, 34] (Figure 8).

A very important tool in the assessment for identification of determinant resistance gene against different weak acids is the use of large-phenotypic screenings, comprising non-essential yeast genes. This tool has allowed the identification of other functions relevant to the increase tolerance against weak acids such as vacuolar acidification, intracellular trafficking, ergosterol biosynthesis and genes involved in response to energy depletion [35]. This tool has been used in a previous study carried out by our laboratory to identify *S. cerevisiae* 430 genes that are determinants of resistance to itaconic acid [36]. Clustering based on physiological function of these itaconic acid-tolerance genes revealed an enrichment of those involved in “carbohydrate metabolism”, “phospholipid metabolism” and “transcriptional regulation”.

1.5 Thesis outline

The work developed in this thesis had two major objectives: i) establish *S. cerevisiae* as a host system for the production of itaconic acid; ii) explore the indicatives gathered by the previously performed chemogenomic analysis to get insights into the molecular mechanisms by which itaconic acid exerts toxicity in yeast cells. It is expected that the identification of genes and biological processes relevant for tolerance to itaconic acid in *S. cerevisae* could provide an interesting set of candidate genes that could be subsequently manipulated aiming to obtain strains more tolerant to itaconic acid, and thereby, more robust to be used in industrial processes aiming the large-scale production of this chemical. Furthermore, the knowledge gathered in *S. cerevisiae* regarding itaconic acid-tolerance mechanisms may also be extrapolated for *A. niger* and *A. terreus*, considering the close phylogenetic distance that separates this fungi; thus contributing to improve the performance of already established industrial processes of itaconic acid production based on microbial fermentation.

Regarding the first objective it had already been attempted in our laboratory the heterologous expression of *A. terreus* Cad1 enzyme in yeast cells [36]. In this previous work the AtCad1 gene had been cloned under the control of a galactose-inducible promoter and using an N-terminal–histidine tag. Although it had been demonstrated that upon galactose induction, the *AtCAD1* gene was strongly over-expressed, it was never possible to confirm the expression of AtCad1 protein using Western Blots. This absence of protein expression was attributed to an eventual impairment of translation due to the existence of several non-optimal codons. In the first part of the work performed in this Master theses the AtCad1 gene was studied using bioinformatics approaches. In specific, the CAI (Codon Adaptation Index) value of the gene was calculated using appropriate bioinformatics tools (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis). CAI evaluates the deviation of a given protein coding gene sequence basing on a reference set of highly expressed genes from species that will be used as a host[37]. With this analysis it was possible to verify that the gene had a CAI of 0.61 (Figure 9), which is below the level considered acceptable (0.8) for good protein expression in yeast cells. Other properties of the *AtCad1* gene were also examined including its GC content and its Codon Frequency Distribution, that calculates the distribution of codons (in %) in computed codon quality groups of that organism (Figures 9 and 10). A value of CDI of 100 corresponds to codons having the highest usage frequency for a given amino acid in yeast while codons with values lower than 30 are likely to hamper the expression efficiency. The GC content of the AtCad1

gene was of 53.35% which is within the ideal range percentage (30%-70%). The percentage of low frequency codons in yeast found in the AtCAD1 gene was of 7% (Figure 10) indicating this gene employs tandem rare codons that can reduce the efficiency of translation or even disengage the translational machinery.

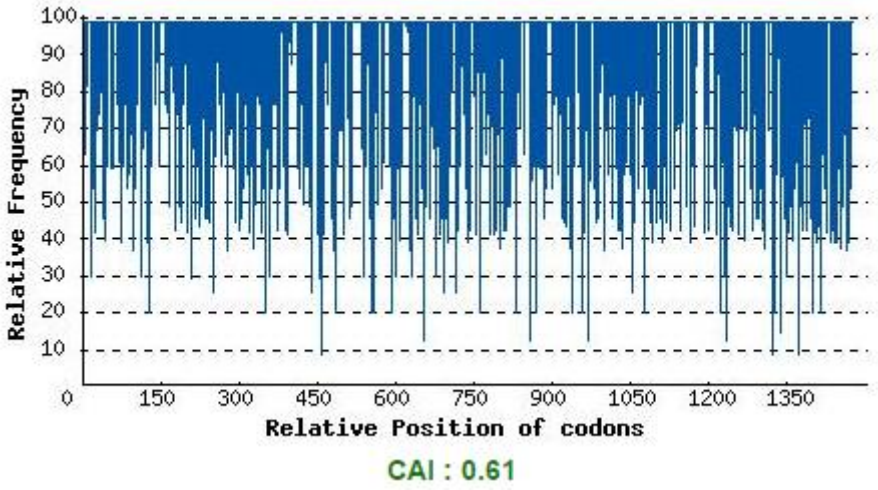

Figure 9 - Distribution of the codon usage frequency along the length of the CDS to be express in the *S. cerevisiae.* The obtained CAI value was 0.61. (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis)

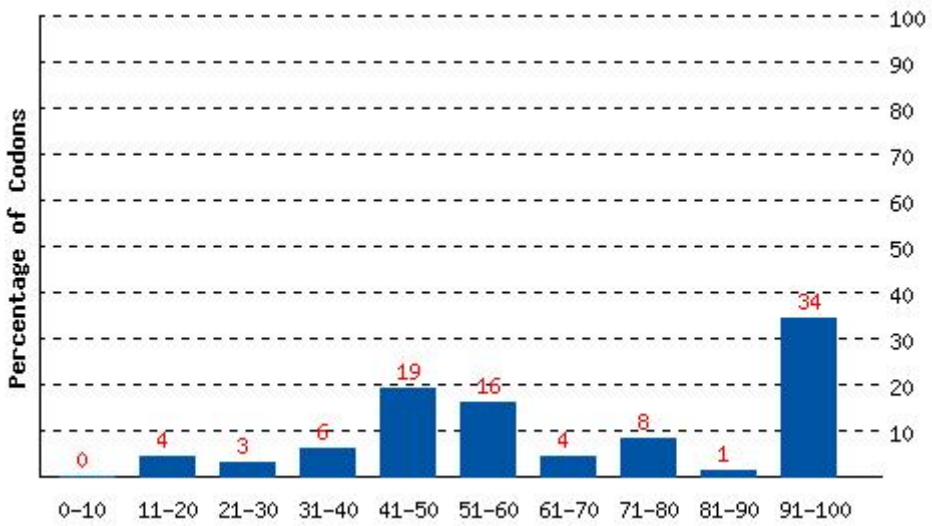

Figure 10 - Percentage of distribution of codon in computed codon quality groups of the *AtCAD1* gene for *S. cerevisae* (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis).

In view of these results an optimized version of the *AtCAD1 gene* (obtained by chemical gene synthesis) was cloned in the pGREG586 cloning vector under the regulation of the galactose-inducible promoter. The strategy used for cloning of optimized AtCad1 in yeast as well as the results obtained regarding protein expression and titers of itaconic acid produced will be presented and discussed in the results section.

2. Materials and Methods

2.1 Strains and growth media

The *S. cerevisiae* strain BY4741 (MATa, *his3*Δ1, *leu*2C0, *met15*Δ0, *ura3*Δ0) was used as wild-type along this work.

The rich medium YPD contains, per liter, 20g glucose (Merck), 20g yeast extract (Difco) and 10g bactopeptone (Difco). The synthetic medium MMB contains, per liter, 20g glucose, 1.7g yeast nitrogen base without amino acids (Difco), 2.65g $(NH_4)_2SO_4$). The MMB growth medium was also supplemented with 20mg/L histidine, 60mg/L leucine, 20 mg/L methionine (all acquired from Sigma) to supplement the auxotrophies of the BY4741 strain. The composition of MMGal growth medium is the same of MMB medium with the exception that galactose (final percentage of 2%) was used as carbon sources in replacement of glucose. Fermentations for production of itaconic acid in *S. cerevisiae* cells ectopically expressing AtCad1 were performed in MMGal growth medium supplemented with 50 mL of CSM (Complete Supplement Mixture). CSM is a mixture of amino acids composed of 10 mg/L adenine, 50 mg/L Arginine HCl, 80 mg/L L-Aspartic Acid, 20/L mg L-Histidine HCl, 100 mg/L L-Leucine, 50 mg/L L-Lysine HCl, 50 mg/L L-Phenylalanine, 100 mg/L L-Threonine, 50 mg/L L-Tryptophane, 50 mg/L L-Tyrosine and 14 mg/L L-Valine.

Escherichia coli XL1-Blue strain was used for common molecular biology procedures and its maintenance and cultivation was performed in LB growth medium. For plasmid selection the LB medium was supplemented with 150 mg. mL^{-1} of ampicillin. Solid growth media was obtained by supplementing the corresponding liquid growth medium with 2% agar.

2.1.1 *In vitro* DNA manipulations

The pGREG586 plasmid (2 µg) was digested with 20 U of SalI restriction enzyme (Takara) and the mixture was incubated at 37˚C for 3 hours. The AtCAD1(opt) region to be cloned in the pGREG586 plasmid was obtained by PCR amplification using the pUC57_AtCad1 plasmid as a template. The pUC57_AtCad1, obtained from GeneSpring, was the plasmid in which it was cloned the optimized version of the *CAD1* gene (*AtCAD1(opt)*). PCR reactions were performed using Phusion® high-fidelity polymerase (Thermo Scientific) and a set of primers that were specifically designed for the effect and whose sequences are indicated in Table 5. The mixture and the experimental setup used for the PCR reaction are described in Tables 6 and 7, respectively. Confirmation of the amplification of *AtCAD1(opt)* region was performed by

running the product of amplification in an agarose gel (0.8%). The DNA band corresponding to the *AtCAD1(opt)* gene was excised from the gel and further purified using the JETQUICK Gel Extraction Spin kit (Genomed).

Table 3 - Primers used to amplify the *AtCAD1(opt)*. A region homologous to the pGREG586 vector (corresponding to the underlined nucleotides) was included in each primer to allow *in vivo* recombination.

Gene	Primer	Sequence
AtCAD1(opt)	Forward	**5' - <u>GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC A</u>**AT GAC AAA ACA ATC TGC CG - **3'**
	Reverse	**5' - <u>GCG TGA CAT AAC TAA TTA CAT GAC TCG AGG TCG AC</u>**T CAG ACC ATT GGG GAT TTT AC **- 3'**

Table 4 - Mixture used in the PCR for genes amplification.

Solution	Volume (µL)
HF buffer	10
dNTPs	1.0
Primer forward	1.0
Primer reverse	1.0
DNA template	2.0
MgCl	2.0
DMSO	1.5
Taq phusion	0.5
H2O	31
Total	50

Table 5 - PCR conditions used for genes amplification.

Temperature (ºC)	Time	
98	3''	
98	10''	x30
56	20''	
72	1'	
72	7'	

2.1.2 Yeast transformation

The PCR-amplified *AtCAD1(opt)* fragment and the SalI-digested pGREG586 vector were used to transform *S. cerevisiae* BY4741 cells using the Alkali-Cation™ Yeast Transformation kit (MP Biomedicals). *S. cerevisiae* BY4741 cells (50 mL) cultivated until mid-exponential phase (OD_{600nm}=0.4) in YPD growth were harvested by centrifugation for 5 minutes at 6500 rpm, at 4°C, and the supernatant was discarded. The cell pellet was resuspended in 9 ml of TE (at pH 7.5) and centrifuged in the same conditions. The supernatant was discarded and the cells were resuspended in 2.5 mL of a Lithium/Cesium Acetate Solution. The cell suspension was incubated at 30°C for 25 minutes with 100 rpm agitation. After that time cells were centrifuged again in the same previous conditions and gently resuspended in 500µL of TE at pH 7.5. 100µL of competent yeast cells were taken for each transformation. Each transformation mixture contained: 10µL the *AtCAD1(opt)* fragment, 5µL of carrier DNA, 5µL of histamine solution and 1.5µL of the SalI-digested pGREG586 plasmid. The mixture was incubated at room temperature for 15 minutes and after that 0.2 ml of TE/Cation solution and 0.8 ml of PEG were added. This step was followed by two incubations: the

first incubation lasted 10 minutes and was performed at 30°C and the second lasted 10 minutes and was performed at 42°C. After these two incubation steps the reaction was cooled to 30°C, centrifuged for 5 seconds and the supernatant was discarded. Cells were resuspended in 100µL of YPD medium and plated on top of agar selective MMB media without uracil (MMB-U) and incubated at 30°C until visible cell growth. The transformants obtained were streaked in MMB medium without uracil.

2.1.3 Confirmation of the AtCAD1(opt) integration in the pGREG586 vector

Confirmation of the integration of the *AtCAD1(opt)* gene in the pGREG586 plasmid was performed, in a first stage, by colony PCR. A loop of cells from each colony recovered after the transformation step was resuspended in 10 µl of 20 mM of NAOH solution in an 1.5 ml eppendorf and then incubated at 100°C for 10 minutes. After this, the samples centrifuged at 13000*g* for 1 minute to separate cell debris from soluble material which is expected to include the plasmid DNA. The primers used for amplification of *AtCAD1(opt)* gene were used for the PCR reaction which was carried out as described in Tables 8 and 9, respectively.

Table 6 - Mixture used in the PCR reaction for gene amplification.

Solution	Volume (µL)
Taq buffer	2
dNTPs	0.4
Primer forward	0.4
Primer reverse	0.4
DNA template	2.0
MgCl	1.2
Taq Med	0.2
H_2O	13.4
Total	20

Table 7 - PCR condititions used for the gene amplification.

Temperature (ºC)	Time	
94	1'	
94	30'	x30
56	1'	
72	2'	
72	10'	

Plasmid DNA was recovered from colonies confirmed to harbor the *AtCAD1(opt)* in the pGREG586 vector (that is, those colonies that had produced a band of amplification after the colony PCR reaction) using the QIAprep® Spin Miniprep Kit (Quiagen). The DNA recovered was used to transform *E. coli* XL1-Blue cells by classical transformation. For this purpose a mixture of 15 µL of the total plasmidic DNA (obtained from each positive yeast candidate), 150 µL of *E. coli* competent cells and 50 µL of a TCM solution (10mM CaCl2, 10mM MgCl2 and 10 mM Tris-HCl pH 7.5). The mixture was incubated on ice for 15 minutes, followed by a heat shock at 42 ºC for 3 minutes and another incubation on ice for 5 minutes. 800 µL of liquid LB medium was added to the mixture and the cells were incubated at 37ºC with orbital agitation at 250 rpm. Cells were centrifuged (8000 rpm for 3 minutes), plated in agar LB medium with ampicillin (150 µL/mL) and incubated at 37ºC overnight. Plasmid DNA was recovered from the *E. coli* transformants obtained using the QIAprep® Spin Miniprep Kit

(Quiagen). The concentration of DNA obtained at the end of the extraction protocol was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Confirmation of the correct integration of the fragment in the pGREG586 vector, herein designated pAtCAD1(opt)::His6 (Figure 14), was confirmed by performing the restriction map of the recovered plasmids with BglII, HIndII and EcoRI enzymes and, later on, by DNA sequencing.

2.1.4 Detection of itaconic acid production in *S. cerevisiae* cells transformed with the pAtCAD1(opt)::His6 plasmid

S. cerevisiae cells transformed with the pAtCAD1(opt)::His6 plasmid were tested for their ability to produce itaconic acid in MM Gal growth medium. For this *S. cerevisiae* BY4741 cells transformed with the pAtCAD1(opt)::His6 plasmid were cultivated in MMB-U growth medium supplemented with CSM until the stationary phase and then re-inoculated (at an OD_{600nm} of 0.1) into MM Gal-U or MMB-U growth media. This last growth medium was used as a control since glucose represses the activity of the *GAL1* promoter which is regulating expression of AtCad1 (Figure 14). Cells were cultivated at 30°C with orbital agitation (250 rpm) and growth was followed by accompanying the increase in OD_{600nm} . Samples of culture supernatants were taken after 24, 48 and 72 hours of fermentation and then 10 µL were separated on an Aminex HPX-87H column (Biorad), eluted at room temperature with 0.005 M H_2SO_4 at a flow-rate of 0.6 ml/min during 30 minutes, using a refractive-index detector for detection of galactose and a UV detector for detection of itaconic acid. Under the experimental conditions used itaconic acid had a retention time of 13 minutes and galactose 8.3 minutes. Reproducibility and linearity of the method were tested and concentrations of itaconic acid and galactose were estimated based on appropriate calibration curves.

3. Results

3.1 Heterologous expression of the optimized *AtCAD1* gene (*OptAtCAD1*) in *S. cerevisiae*

To be expressed in yeast cells the optimized *A. terreus AtCad1* gene was cloned in the pGREG586 plasmid, one of the vectors of the “drag and drop” system[38]. A schematic representation of the cloning strategy used is shown in Figure 13. The engineered plasmid, named pAtCad1(opt)::His6, drives expression of *AtCAD1* N-terminally tagged with a six-histidine tail and under the transcriptional regulation of the galactose-inducible promoter *GAL1* [38] (Figure 13). The *OptAtCAD1* gene (having a predicted

size of 1500 pb) was amplified by PCR using the pUC57_AtCad1 plasmid as a template (this plasmid was acquired from GeneScript and it has cloned the synthesized version of the *AtCAD1* gene). After the PCR reaction the product of amplification was run in an agarose gel and the result obtained is shown in Figure 15. The size of the amplified product corresponds to the predicted size of the optimized *AtCAD1* gene confirming the success of the amplification reaction (Figure 14). The pGREG586 vector was digested with the *SalI* restriction enzyme and the result of that digestion was also confirmed by running the digested plasmid in an agarose gel (Figure 14 lane 6). The PCR-amplified *AtCAD1* gene was cloned in the *SalI*-digested pGREG586 vector by *in vivo* gap repair in *S. cerevisiae* cells.

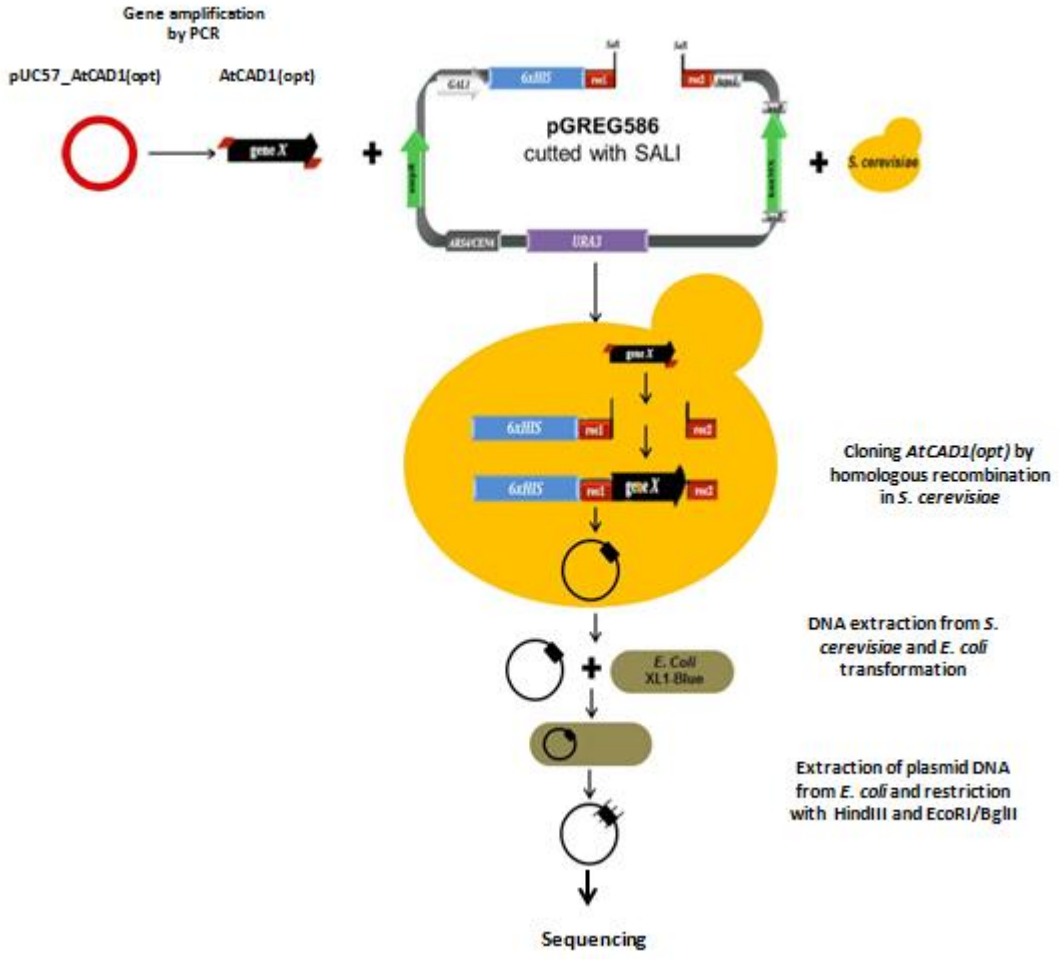

Figure 11 - Schematic representation of the cloning engineering strategy used to clone the optimized *AtCAD1(opt)* gene in the pGREG586 plasmid.

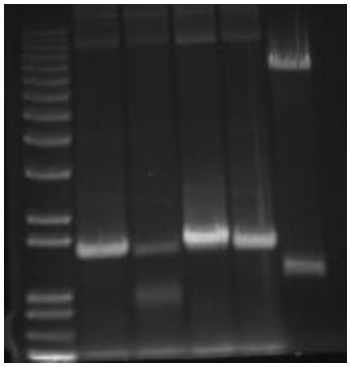

Figure 12 - Gel obtained after PCR amplification of the *OptAtCAD1* gene from the pUC57_AtCad1 vector and digestion of pGREG586 with *SalI*. Lane 1: 1 kb DNA ladder; Lanes 2-5: *AtCAD1(opt)* amplified fragment; Lane 6: pGREG586 digested with the *SalI* restriction enzyme.

After transformation of yeast cells with the *OptAtCAD1* gene and with the Sal*I*-digested pGREG586 vector around 100 transformants were obtained. To confirm that the transformants obtained harbored the pAtCAD1(opt)::His6 plasmid a colony PCR was carried out using 40 transformants. The same primers used for amplification of the optimized *AtCAD1* gene were used in this colony PCR. Only 4 of the PCR reactions performed produced a product of amplification with a size of 1.5 kb, these candidates being selected for further work. Total DNA was extracted from the positive yeast candidates and used to transform *E. coli* XL1-Blue cells. Plasmid DNA was recovered from 12 *E. coli* ampicillin-resistant transformants and then subjected to digestion with *HindII* and *EcoRI/BglII* enzymes (Figure 15).

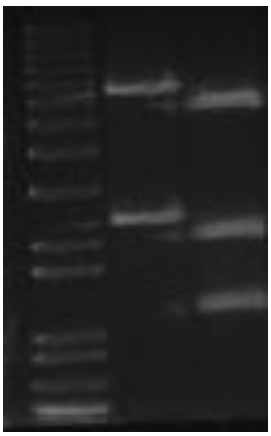

Figure 13 - Gel obtained after the digestion of plasmid DNA recovered from E. coli colonies considered to be candidates to have the pAtCad1(opt)::His6 recombinant plasmid. Lane 0: 1kb DNA ladder; Lane 1: pAtCAD1(opt)::His6 restricted with HindIII ; Lane 2: pAtCAD1(opt)::His6 restricted with EcoRI and BglII.

The pattern of bands obtained after digestion of the plasmid DNA recovered from the *E. coli* candidates with the different restriction enzymes is consistent with these clones harboring the pAtCad1(opt)::His6 recombinant plasmid. The HindIII enzyme has two recognition sites in the pGREG586 vector located in the surroundings of the cloned gene (see restriction map in annex) and therefore digestion with this enzyme is expected to generate a fragment that has the approximate size of the insert, which in

this case should be close to 1.5 kb. *EcoRI* has two cut sites in the pGREG586 vector, one located a few nucleotides upstream of the insert start codon, and one site in position 1124 of the AtCad1 gene. A band with approximately 1200 bp is obtained when the plasmid DNA of the clones was digested with the EcoRI/BglII mixture (Figure 15). The other bands obtained in the EcoRI/BglII double digestion also have the size that was expected for the pAtCad1(opt)::His6 vector. Correct insertion of the AtCad1 optimized gene in the pGREG586 vector was confirmed by DNA sequencing.

To confirm expression of AtCad1 in *S. cerevisiae* cells transformed with the pAtCad1(opt)::His6 vector it was assessed the ability of these cells to produce itaconic acid, a compound that is not naturally produced by this yeast species. For this *S. cerevisiae* BY4741 transformed with the pGREG586 cloning vector or with the pAtCad1(opt)::His6 plasmid were cultivated in MM Gal growth medium (containing 2% galactose) or in MMB (containing 2% glucose) supplemented with the CSM amino acid mixture. The decision to supplement the growth medium with CSM was based on previous indicatives obtained in a recently published paper that has also explored *S. cerevisiae* as a host system for the production of itaconic acid [19]. The supernatants collected from the different cultures after 24, 48 and 72 h of fermentation was tested for the presence of itaconic acid by HPLC. Under the experimental conditions used in the HPLC, itaconic acid had a retention time of approximately 13 minutes, as confirmed when a standard solution of the acid was used (Figure 16).

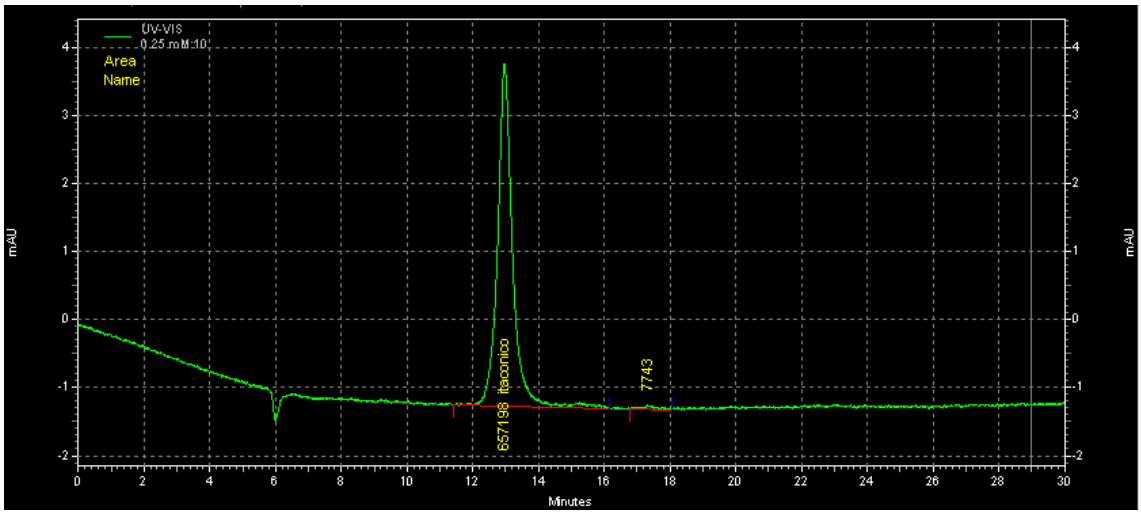

Figure 14 - UV-Vis spectrum of a standard solution (0.025mM) of itaconic acid.

No itaconic acid was found in the supernatant of *BY4741* cultures harboring the pGREG586 plasmid in any of the conditions tested. Differently, supernatants taken from cultures of *BY4741* cells harboring the pAtCAD1(opt)::His6 plasmid show a clear production of itaconic acid. As expected this production of itaconic acid was only

observed when cells cultivated in the presence of galactose. Altogether these results demonstrate that under the conditions used *S. cerevisiae* is producing itaconic acid from the pAtCAD1(opt)::His6 plasmid (Figure 17).

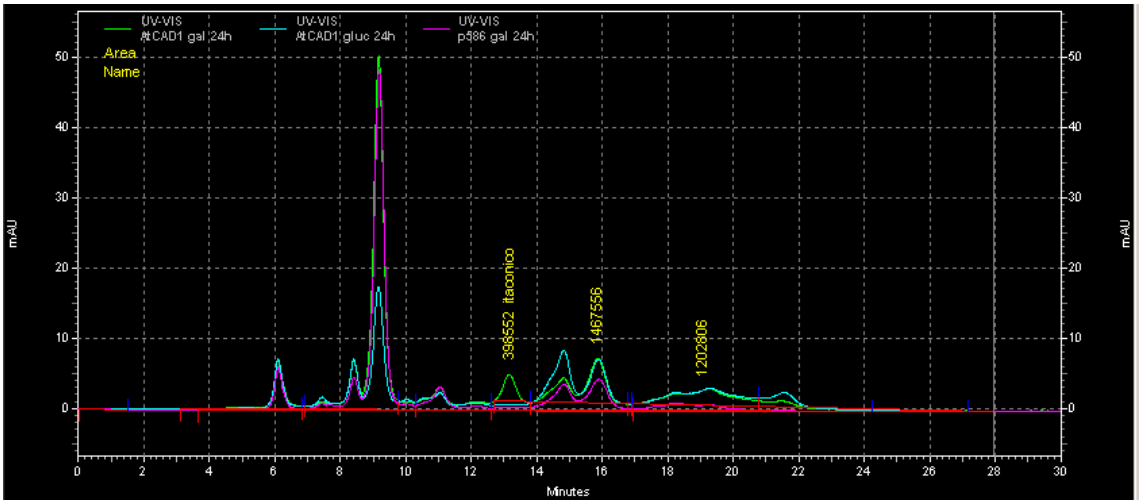

Figure 15 - UV-Vis spectrum analysis of the collected supernatants after 24h. Green: *BY4741* transformed with pAtCAD1(opt)::His6 plasmid cultured in MMB-U with 2% galactose supplemented with CSM, vitamins and trace elements; Blue: *BY4741* transformed with pAtCAD1(opt)::His6 plasmid cultured in MMB-U with 2% glucose supplemented with CSM, vitamins and trace elements; Pink: *BY4741* transformed with pGRER586 plasmid cultured in MMB-U with 2%galactose supplemented with CSM.

The results obtained show that the amount of itaconic acid increased along time, as expected, achieving the highest approximately 25 mg/L after 72h of cultivation in 2% galactose medium (Table 15).

Table 8 – Titers of itaconic acid produced by *S. cerevisiae* BY4741 cells transformed with the pAtCAD1(opt)::His6 plasmid when cultured in MMB-Gal-U supplemented with CSM in maximum period of 72 hours.

Time of growth (h)	24	48	72
Amount of itaconic acid produced (mg/L)	2,04	8,65	25,12

4. Discussion

In the first part of this thesis the work presented concerns the heterologous expression of an optimized version of the *AtCAD1* in *S. cerevisiae*. The approach utilized has allowed the expression of AtCad1 protein from a galactose-inducible promoter which was confirmed by the detection of itaconic acid in the supernatant of cultures harboring the pAtCad1(opt)::His6 plasmid. 25 mg/L of itaconic acid were obtained after 72h of the

plasmid induction with galactose. This production was close to the one reported by Blazeck *et.al* (approximately 24 mg/L) using the BY4741 strain and glucose as the carbon source. Glucose and galactose are metabolized through similar metabolic pathways (only the initial steps of glycolysis diverge) and therefore a similar yield in the acid produced from the two sugars was expected. The work described in this thesis set the foundations for the development of metabolic engineering strategies to be undertaken in our laboratory aiming to optimize productivity of itaconic acid. Such improvement is absolutely required to setup yeast as a valuable cell factory for the production of itaconic acid since the yields produced by *A. terreus* reach up to 90 g/L [8]. The HLPC analysis performed showed that yeast cells produced a significant amount of ethanol together with itaconic acid, meaning that the cells mainly fermented galactose through alcoholic fermentation instead of channeling it to the Krebs Cycle where the *cis*-aconitate is transformed in itaconate. In order to bypass this issue it could be used a pyruvate-decarboxylase negative (*Pdc*$^{-}$) strain since this strain not perform the alcoholic fermentation process. In this work the only carbon source explored was galactose, which is not a very interesting substrate for large-scale production because of its price. The ideal scenario would be to use compounds that are considered waste in biorefineries such acetate, glicerol or xylose, as these compounds are highly abundant in lignocellulosic feedstocks. In this context besides the metabolic engineering it will also be required to assess the effect of having AtCad1 protein being expressed from other promoters more active when yeast cells are growing in the above-referred carbon sources.

6. References

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