Exploration of the non-conventional yeast *I.orientalis* as a host for itaconic acid production

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Abstract

In the context of biorefinery implementation, carboxylic acid production is of particular interest as these molecules are naturally derived from microbial metabolism and considered highly interesting as building block molecules able to replace catalysts derived from petrochemistry. The present work focuses on the exploration of the non-conventional yeast species *Issatchenkia orientalis* as a potential host for the production of itaconic acid. Among other interesting traits, there are reports of high organic acid tolerance by *I.orientalis*, a desirable phenotype for the production of carboxylic acids in microbial hosts. Insights into the tolerance to itaconic acid of *I.orientalis* cells were obtained in this work, suggesting that the activity of the plasma membrane proton pump is significantly higher than that observed for *S.cerevisiae* BY4741 cells, this being observed in the presence or absence of itaconic acid. Being that intracellular acidification is commonly associated with carboxylic acid induced stress, it is possible that this higher activity of the plasma membrane proton pump underlies the generalized acid-resilience of *I orientalis* cells.

Heterologous expression of the cis-aconitic decarboxylase enzyme from *A.terreus* in *I.orientalis* enabled production of itaconic acid in titers ranging 0.4 g L^{-1} (using 2% glucose as carbon source), this value representing one of the highest yields described so far for non-optimized yeast-mediated production of itaconic acid. Besides itaconic acid, during fermentation assays, *I.orientalis* was shown to accumulate citric acid in the growth medium (in titers reaching 23.5 g L^{-1}), this representing a significant diversion of the carbon provided. In order to improve the production titers, we performed the over-expression of a native cytosolic aconitase (involved in conversion of citrate into cis-acconitic acid, the precursor of itaconic acid), of the native putative itaconate exporter *MfsAIo* and the also native putative cis-aconitic mitochondrial transporter *MttAIo*. The over-expression of aconitase had no improvement in itaconic acid production and the overexpression of *MfsA* and *MttA* resulted in improvements of 275% and 325% respectively (titers ranging 1.1 and 1.3 g L^{-1}, respectively). Furthermore, the over-expression of *MfsA* and of *MttA* also resulted in complete consumption of citrate in the fermentation broth.

Resumo

No contexto da implementação estratégica de biorefinarias, a produção de ácidos carboxílicos surge como um factor estimulante dado que estas moléculas derivam do metabolismo microbiano, e assim constituem uma fonte de compostos *building block* para síntese química como alternativa aos catalisadores derivados da indústria petroquímica. O presente trabalho foca-se na exploração de *I.orientalis,* uma espécie não-convencional de levedura como potencial produtor de ácido itacónico. Entre outros traços fenotípicos de interesse a literatura reporta uma alta tolerância deste organismo a ácidos carboxílicos. Este fenótipo torna-se particularmente interessante e é altamente desejável no contexto da produção de ácidos carboxílicos em hospedeiros microbianos. Aqui corroboramos esta hipótese e verificamos indícios de que os mecanismos de resistência subjacentes a este fenótipo estarão relacionados com a actividade das bombas de protões presentes na membrana plasmática. Face a *S.cerevisiae* BY4741, *I.orientalis* demonstra uma actividade superior, quer face à toxicidade imposta pelo ácido itacónico, quer em condições controlo. Dado que o fenómeno de acidificação intracelular é comummente associado ao stress induzido por ácidos carboxílicos, é possível que esta maior actividade basal das bombas de protões esteja subjacente à alta tolerância a ácidos carboxílicos exibida pelas células de *I.orientalis*.

A expressão heteróloga da enzima cis-aconitato descarboxilase de *A.terreus* em *I.orientalis* resultou em títulos de ácidos itacónico na gama dos 0.4 g L^{-1} em MM contendo 2% de glucose. O título obtido está entre os mais altos reportados até à data no que diz respeito à produção de ácido itacónico em levedura *a priori* de qualquer optimização. Para além do ácido itacónico verificámos ainda uma acumulação de ácido cítrico no caldo de fermentação na ordem dos 23 g L^{-1}, representando um sequestro de carbono altamente relevante. Com o objectivo de aumentar os títulos efectuou-se uma abordagem de sobreexpressão da aconitase citosólica nativa (responsável pela conversão de citrato em cis-aconitato, o precursor do ácido itacónico), do exportador nativo putativo de ácido itacónico *MfsAIo* e ainda do também nativo exportador mitocondrial de cis-aconitato *MttAIo*. A sobreexpressão da aconitase não resultou num melhoramento da produção de ácido itacónico sendo que a sobreexpressão dos transportadores resultou num aumento de título em ambos os casos. Assim obtivemos títulos de 1.1 e 1.3 g L^{-1} para a sobreexpressão do *MfsA* e do *MttA*, num aumento de 275 e 325% respectivamente. Para mais, a sobreexpressão destes transportadores resultou também num consumo completo do citrato que antes havia sido acumulado no caldo de fermentação.

List of Abbreviations

AtCad- Cis-aconitate Decarboxylase from *Aspergillus terreus*

CAD – Cis-aconitate Decarboxylase

MM – Minimal Media Broth

OD_{600} – Optical density at a wavelength of 600 nm

PEG – Polyethylene glycol

PMMA- Polymethyl - metacrylic acid

rpm – rotations per minute

TCA – Tricarboxylic acid

YPD – Yeast Peptone Dextrose

dd - Doubly distilled

MLS- Mitrochondrial Location Signal

MfsA- putative transporter protein belonging to the Major Facilitaconic acidtor Superfamily A

MttA- putative transporter protein belonging to the Mitochondrial Tricarboxylate Transporter A

Aco- Aconitase protein (truncated, lacks MLS)

1 CONTEÚDO

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2 INTRODUCTION

2.1 OVERVIEW

Throughout the 20th century the petrochemical industry has flourished, not only due to its role in the transportation sector, but also to its contribution to the development of the chemical synthesis industry. Emerging in the 1950's, a great variety of synthetic polymers became available on the industrial scale. Exploration of oil and gas as fossil raw materials for the chemical industry and polymer production greatly improved cost-effectiveness and simplified manufacturing of macromolecular materials. Since then the attractive combination of low cost with facile processing and innovation represents the key feature of plastics. However, following the golden years of petrol and coal, the industries' vision shifts towards the long-term, urging the transition to more sustainable raw material sources. In this sense, bioprocess engineers look to develop new biological methods which make use of raw materials such as those derived from agricultural activities [1] or from biodiesel production [2]. In fact, such an approach brings about not only environmental but also economic benefits by valuing what is commonly perceived as waste. During the early days of polymer industry science and engineering, nearly all materials were based exclusively upon chemically modified biopolymers. Among natural sources of biopolymers one finds great potential in sugar-based cellulose, which is the major component of biomass, wood, and cotton, representing the most abundant organic compound produced by living organisms [3,4]. Following this trend, recent years have witnessed great development and interest towards bio-based production routes from raw material to commercial formulations, as an alternative to petrochemical-based synthesis. To avoid chemically-mediated compound synthesis, scientists looked to make use of the naturally existing cellular mechanisms to aid in polymer or polymer building block synthesis, effectively turning cells into living factories. This motivation allied with the advances in genome analysis and engineering gave rise to a field of research that is now called White Biotechnology, this being considered a branch of biotechnology which typically utilizes less resources and aims to explore nature's richness in search of methods to replace petroleum-based synthetics. In particular, biorefinery approaches looking to produce organic acids are of relevance, due to the interest of these molecules as high-added value and high commodity compounds. The relevance of organic acids and other molecules considered as emerging "building blocks" emerge from the very

high demand of products obtained from a highly-specialized chemical industry using processes largely based on petrochemical-based derivatives. The possibility of producing these added-value chemicals in a biorefinery setting is also essential to increase economic viability of biomass-based factories. Biorefinery conversion technologies encompass three steps: (i) converting biomass to sugar or other fermentation feedstock, (ii) fermenting these biomass intermediates using biocatalysts and (iii) processing the fermentation product to yield fuel-grade ethanol and other fuels/chemicals. While first generation biorefineries focus on the use of of natural polymers largely found present in food crops (e.g. starch, sucrose or even simple sugars), the second generation of biorefineries aims to use structural parts of the plant, especially lignocellulose which contain more complex, harder to access sugars. Although raw materials for lignocellulosic hydrolysate are largely abundant, the fact that their use is dependent on the implementation of various step (including pretreatment of the material to release the fermentable sugars) associated with the low yields of ethanol obtained from the fermentation of these substrates, creates a requirement for further process valorization [5][6]. The diversification of products that can be produced from biomass, to include other biofuels besides ethanol, as well as various added-value molecules that could be obtainable by microbial fermentation, is one of the more relevant strategies to overcome economic limitations.

Carboxylic acids can be used in the synthesis of diverse commodity assets including rubbers, paint, pharmaceutical compounds, diverse polymers, among others. As such, in 2004, these molecules have emerged in a screening undertaken by the United States Department of Energy (US-DOE) among the top added value chemical platforms to be obtained from renewable biomass and that could serve as an economic driver for the development of biorefineries[7]. The more relevant molecules identified in said study are listed in Table 1, out of the listed sugar-derived molecules, 10 are organic acids.

Table 1-Top sugar-derived building blocks – Following a second screening, those building blocks which may be obtained from a sugar fermentation setting are listed. As reported by the Department of Energy of the United States [8]

Sugar-derived chemicals	Molecular formula	Applications
1,4 diacids (succinic, fumaric and malic)	$C_4H_6O_4$	Solvents, fibers such as Lycra
2,5 furan dicarboxylic acid	$C_6H_6O_3$	Bottles, films, containers
3-hydroxy propionic acid	$C_3H_6O_3$	Contact lenses, diaper polymers, carpet fibers
Aspartic acid	$C_4H_7NO_4$	Chelating salts, sweeteners
Glucaric acid	$C_6H_{10}O_8$	Solvents, nylons
Glutamic acid	$C_5H_9NO_4$	Monomers for polyesters and polyamides
Itaconic acid	$C_5H_6O_4$	Nitrile latex, Copolymers in styrene butadiene polymers
Levulinic acid	$C_5H_8O_3$	Fuel oxygenates, solvents
3-Hydroxybutyrolactone	$C_4H_6O_3$	High-value pharmaceutical compound intermediate
Glycerol	$C_3H_8O_3$	Polyether polyols, drgus and pharmaceuticals
Sorbitol	$C_6H_{14}O_6$	Antifreeze, polymers
Xylitol/Arabinitol	$C_5H_{12}O_5$	Unsaturated polyester resins

The process used to select which chemicals would be more amenable to be used in a biorefinery setting necessaily included the criteria that these would have to be obtainable through sugar-based fermentation. Indeed, some of the organic acids identified as potential building blocks are already produced by microbial cells through the Tricarboxylic Acid Cycle or the glycolytic pathway, for

example, thus rendering these compounds of particula interest. Indeed, several organic acids are already produced at an industrial scale namely citric acid with production largely based on *Aspergillus niger* fermentation [9,10].

Filamentous fungi have long been identified to be good producers of organic acids as fermentation by products making these organisms widely explored as cell factories for the production of these compounds [11,12]. Nevertheless, their utilization in a biorefinery setting has several limitations: (i) the physiology behind secretion is still poorly understood, (ii) knowledge on improved protein secretion for one protein is not necessarily transferable to other proteins or fungal systems, (iii) limitaconic acidtion of productivity due to non-optimal macroscopic morphologies produces complications in aeration and rheology and (iv) unwanted mycotoxin production during fermentation [13]. In this sense, the exploration of *S.cerevisiae* has been considered of interest in the context of biorefineries due to the natural ability of this species to produce ethanol as well to its long track record of utilization in industrial biotechnology. However, within the specific context of biorefineries the utization of *S.cerevisiae* has been facing some challenges including the high susceptibility of this species to inhibitors that are present in lignocellulosic hydrolysate and its limited ability to use xylose which is one of the most abundant sugars in hydrolysates. In this sense, the possibility of using other non-Saccharomycetous species as potential cell factories to be used in biorefineries is being considered, either alone or in combination with *S.cerevisiae* to produce biofuels along with bulk chemicals. In this work the use of a strain of *I.orientalis* isolated from a spontaneous Louisianna rotten bagasse fermentation [14] as a potential cell factory for the production of itaconic acid is explored. Besides metabolic optimization, insights into the extreme tolerance of this species to itaconic acid at a low pH, a trait considered highly valuable for organic acid producers, was also investigated.

2.2 THE C5 BUILDING BLOCK ITACONIC ACID

The C_5 compound itaconic acid is featured as a particularly promising chemical platform due to the presence of two carboxylic acid functionalities and an α-β unsaturated double-bond which make this compound an excellent precursor for diverse chemical transformations [15]. Among the valuable properties exhibited by itaconic acid as a monomer are its methylene group and the functionalities associated to the carboxylic acid moiety which can be used in addition polymerization reactions yielding free-carboxyl group polymers. Itaconic acid can act either as a co-monomer or it may self-polymerize, the latter being relevant for the synthesis of higher molecular weight poly-itaconic polymers [16] which are then chemically functionalized to obtain the final product. Industrial applications of Itaconic acid include the polymer synthesis industry (namely industries using polyester-based with added functionalities such as UV/thermal curing and shape memory polymers [7,8]), detergents, coatings and rubbers like nitrile latex. Nevertheless, the most prominent market of itaconic acid is the conversion to methyl methacrylate, also known as Plexiglas, with a potential demand of itaconic acid up to 3.2 million tons/year [17]. Other chemicals that may be replaced by itaconic acid include acetone cyanohydrin (used in methyl methacrylate production), acrylic acid (used for synthesizing superabsorbent polymers), maleic anhydride and sodium tripolyphosphate (used in detergents). Overall, the itaconic acid market size was estimated at 75 million in 2016. Projections predict the market potential for itaconic acid to exceed USD 290 million by 2024, including a demand for poly methyl-methacrylate estimated to surpass USD 10 billion [18].

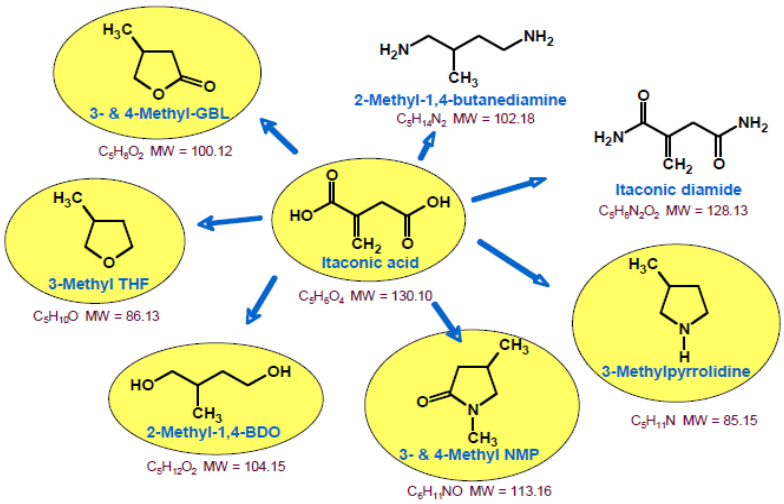

Figure 1- Itaconic acid *molecular structure along with the primary family of derivatives which may be obtained from the backbone. Reproduced from http://eng.genetika.ru/science/*itaconic-*acid/ [Accessed: 5 Aug 2017]*

2.2.1 Microbial-based production of itaconic acid

Itaconic acid is produced naturally by some organisms belonging to different species including *Aspergillus terreus* and *Ustilago maydis* as the most relevant ones, achieving the highest final titers. The itaconic acid biosynthesis pathway has been well-elucidated in *A. terreus*, as schematically represented in Figure 2: glucose enters the glycolytic pathway and one of the two pyruvate molecules produced is converted to acetyl-coA by the mitochondrial pyruvate dehydrogenase complex, while the remaining pyruvate molecule is carboxylated to yield oxaloacetate. Both these molecules enter the TCA cycle, where citrate synthase performs the conversion to citrate. The isomerization of citrate to isocitrate in the regeneration of the cycle includes an intermediate dehydration step via aconitase (*Aco*) that leads to the formation of cis-aconitate, the precursor of itaconic acid. Conversion of cis-aconitic acid into itaconic acid is mediated by the enzyme cis-aconitate decarboxylase (*CadA*), encoded by the *cad1* gene. Quantification of enzymatic activity from different cellular fractions together with $_{14}$C-labelling experiments suggest that CadA is a cytosolic enzyme, which implies one import and one export step from the mitochondria. This step is mediated by generic mitochondrial transporters generally known as Mitochondrial Tricarboxylate Transporters (*MTT*). Following cytosolic formation of itaconic acid it is then exported to the cell exterior with experimental evidence pointing towards mediation via a Major Facilitaconic acidtor Superfamily Transporter (*MFS*) (Figure 3) [19,20].

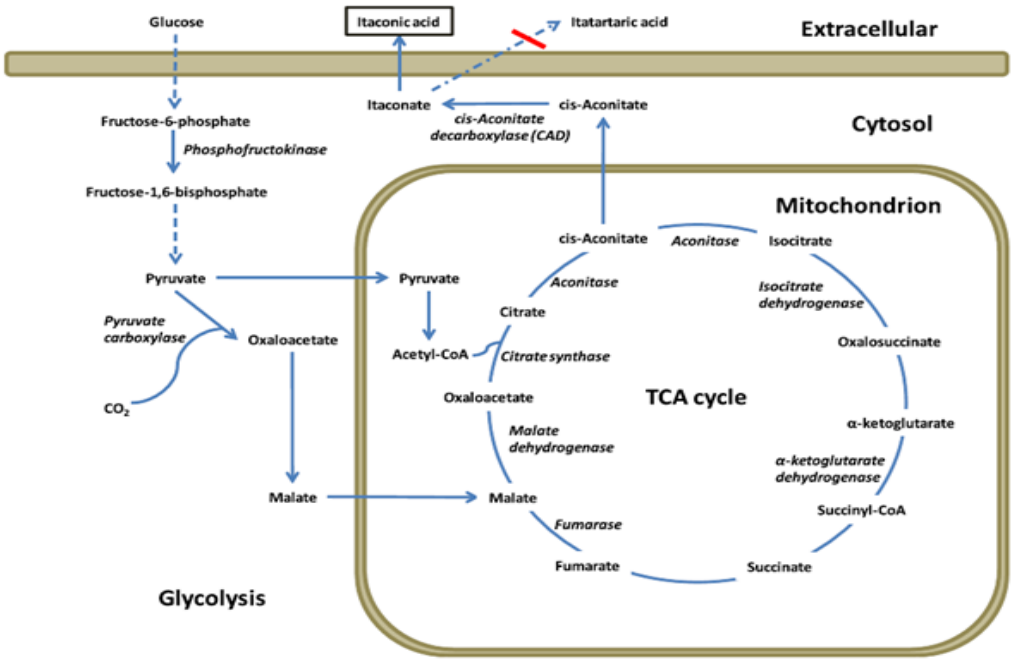

Figure 2- Itaconic acid *biosynthetic pathway in Aspergillus terreus –* itaconic *acid production depends on mitochondrial export and cytosolic cis-aconitate decarboxylase conversion. Reproduced from* [21].

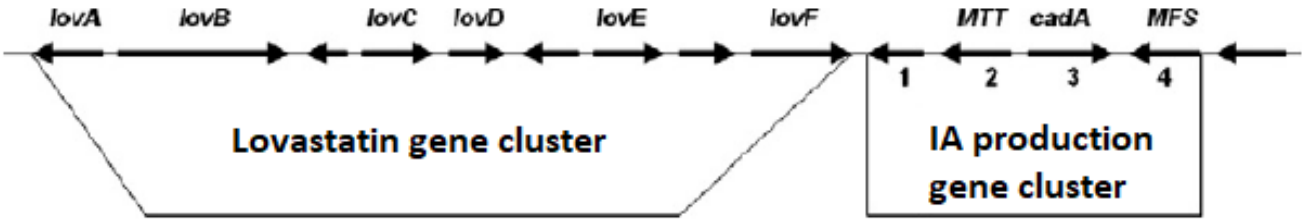

Figure 3- Itaconic *acid and lovastatin gene clusters; 1- putative regulator containing fungi specific transcription factor domains 2- Mitochondrial TricarboxylateTransporters 3- Cis-aconitate decarboxylase 4-Major Facilitator Superfamily transporter reproduced from the work of Li et al.,2011* [22].

Presently, industrial-scale itaconic acid production is performed using *Aspergillus terreus* achieving titers of up to 130 g L^{-1}, following metabolic and medium optimization approaches [23,24], however, this value is still well below the maximum theoretical yield of 240 g L^{-1} [25]. A highly favourable trait of the use of *A.terreus* for the production of itaconic acid concerns its robust growth in acidic conditions which bypasses the need of titrating the fermentation broth to recover the acid in its undissociated form, a step that can alone impair economic viability of the overall production process.

Production of itaconic acid in *U. maydis* ranges between 15 to 63 g L^{-1}, depending on the strain used and on medium composition [26,27]. The biosynthetic pathway leading to itaconic acid production in *U. maydis* is still not fully understood although evidences have been uncovered. A putative cis-aconitic decarboxylase with low identity, um06344, has been identified in the genome of *U. maydis.* The overexpression of this gene resulted in an increase in final itaconic acid titer, but its deletion did not

interrupt itaconic acid production. These observations suggested that *U.maydis* must have more than one biosynthetic route for the production of itaconic acid [27]. Posterior work revealed a new gene cluster associated with itaconic acid production encompassing um05074 (cyp3), um05076 (tad1), um11777 (itp1), um11778 (adi1), um05079 (mtt1), and um05080 (ria1) [28]. Adi1 was found to encode an isomerase catalyzing the conversion of cis-aconitic acid to trans-aconitic acid while tad1 was found to encode a trans-aconitic acid decarboxylase (Figure 4), indicating that *U.maydis* is able to produce itaconic acid both from the cis- and trans-aconitic forms, something that does not occur in *A.terreus.*

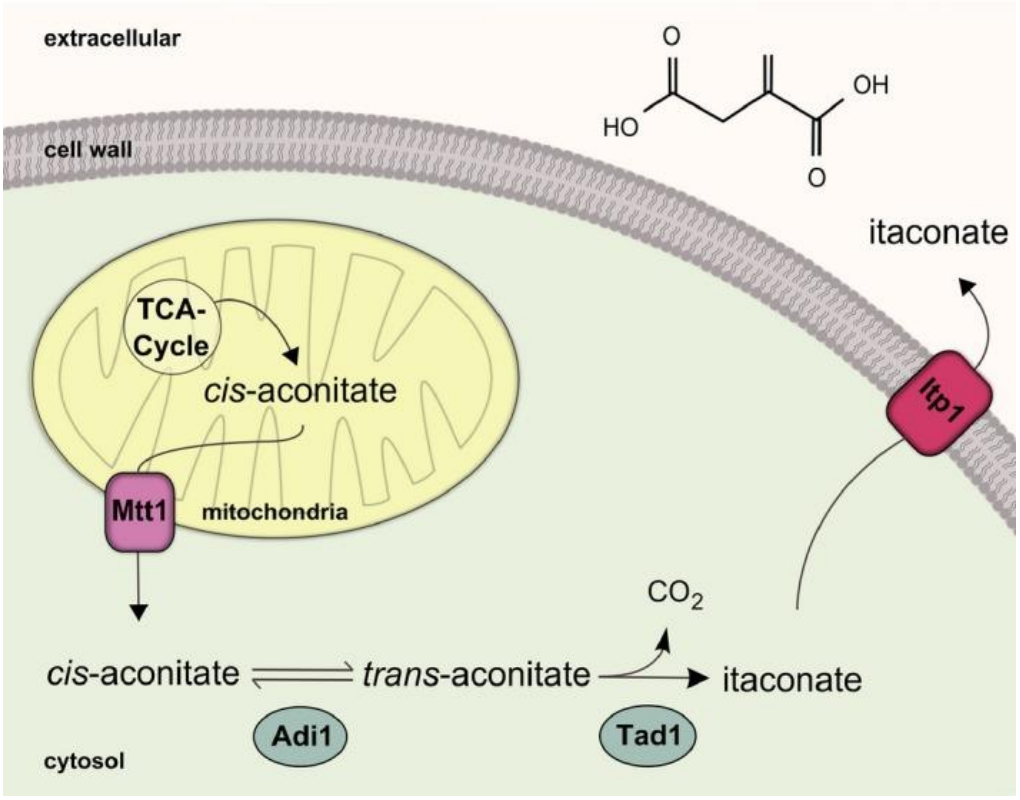

Figure 4- Proposed intracellular organization of the itaconic acid *biosynthesis pathway in U . maydis. Cis-aconitate is secreted by the mitochondrial tricarboxylate transporter Mtt1. In the cytosol cis-aconitate is converted into* itaconic acid *via the intermediate trans-aconitate. Secretion of* itaconic acid *into the medium is mediated by the major facilitator Itp1. Reproduced from Geiser iet al., [29].*

2.2.2 Metabolic engineering to improve microbe-based production of itaconic acid

Using *A. terreus* as a host

Early genetic engineering efforts in Aspergilii have focused on the improvement of glycolysis rate so as to increase carbon availability for the TCA cycle in the form of pyruvate, and consequently, itaconic acid titers. Citrate is one of the most relevant inhibitors of glycolysis, mainly by inhibiting phosphofructokinase. As such, the accumulation of citric acid as the result of a fermentative process which is observed to occur in *Aspergillus niger* is remarkable. Tevz *et a*l., have expressed the citrate-resistant *A.niger* PFK enzyme in *A.terreus* [30] this resulting in an improvement of about 2-fold the titers of itaconic acid produced in the growth medium: 45.5 g L^{-1} comparing with 21.2 g L^{-1} obtained for *A.terreus* cells expressing the wildtype *PFKA*. To design further engineering approaches, the genes which are present in the itaconic acid production cluster along with other relevant players were identified and overexpressed in an industrially established *A.terreus* strain. The overexpressed genes include *cadA*, *MfsA*, *MttA*, *gpdA*, *acoA*, *citA* and *mt-pfkA*. Based on experimental insights, one engineering overexpression of *MttA*, *MfsA* and *cadA* enhanced itaconic acid production to 81 and 82 g L^{-1} respectively for the first two. Highest titer reported is for cadA overexpression at 88 g L^{-1} with the introduction of other genes showing varied effects on itaconic acid production [31].

Another approach used to improve production of itaconic acid in *A.terreus* involved the control of fermentation pH through a lye-mediated pH shift approach [24]. In specific, it has been found that to produce itaconic acid *A.terreus* needs to be cultivated in an acidic medium [32] (around pH 3) Hevekerl *et al*., [24]. started fermentations at uncontrolled pH and then set the pH to be maintained at 3 by adding a strong base to the culture medium in the second day of cultivation and from there onwards, keeping pH stable, this strategy produced titers of 129 g L^{-1}. This work set the base for a more recent work by the same author, which combined this pH control approach with an increase in phosphate availability. At a 15 L scale, this strategy yields itaconic acid concentrations of 150 g L^{-1} [33]. This in itself is quite impressive, taking into account that the strain used in the study was a non-engineered strain.

Using A.niger as a host

Owing to its phylogenetic similarity and regulatory tendency to accumulate citric acid at concentrations of over 200 g L^{-1} *A.niger* was studied as a host for production of itaconic acid. Heterologous expression of the *CadA* enzyme from *A.terreus* enabled production of itaconic acid in concentrations ranging 0.289 g L^{-1}.This final titer was achieved by both heterologous expression of *CadA* and medium optimization [34]. Associated to *A.niger* fermentation often comes coupled the production of by-products which consume carbon that could otherwise be directed towards product formation [35]. Deletion of oxaloacetate hydrolase (oahA) and of glucose oxidase (goxC), the enzymes responsible for the synthesis of oxalate and gluconic acid, respectively, also resulted in improved production of itaconic acid to up 7.1 gL^{-1} by directing carbon to the synthesis of this acid [35]. Posterior work looking to identify where itaconic acid is synthesized in *A.niger* was pursued by targeting *CadA* to the mitochondria, the production improved to 1,1 g L^{-1} itaconic acid [36] which appears to indicate that in *A.niger*, itaconic acid is synthesized in the mitochondria. These improvements set the base for *A.niger* engineering in further work such as that performed by Hossain *et al*., [19] which reports the overexpression of transporters associated with itaconic acid synthesis in a similar approach to that employed for *A.terreus.* This yielded very positive results, as the strain expressing *AtCad+MttA+MfsA* produced final titers of 2 g L^{-1}. In the same work, authors identified through RNA-seq that a citrate synthase was overexpressed in the best performing *A.niger* strains. By overexpressing *citB* in the *AtCad+MttA+MfsA* strain, final titer improved to 26.2 g L^{-1}, contradicting previous finds which associated itaconic acid synthesis with the mitochondrial compartment.

Using *U.maydis* as a host

U.maydis iis a microorganism with a dimorphic life cycle. The haploid unicelular form divides by budding, has a yeast-like appearance (sporidia) and can be easily propagated on artificial media. The greatest advantage of industrial itaconic acid production using *U.maydis* is the fact that itaconic acid is secreted to the medium during cultivation by the yeast-like form of Ustilago and therefore handling of the biomass in large scale fermenters even in high concentrations does not bring about the difficulties that are observed while fermenting filamentous fungi. Moreover utilization of both C5 and C6 sugars by U.maydis is a fundamental industrial advantage as it allows access to lignocellulosic substrates. A first prospect using different media formulations of 68 Ustilaginaceae belonging to 13 different species,

among which *U.maydis*, reports that strain 2229 of this smut fungus is an itaconic acid producer of titers up to 15.7 g L^{-1} with 200 g L^{-1} glucose as carbon source [27]. One other work reveals once more the importance of pH control for itaconic acid production, in the work of Maassen *et al*., strain MB215 displays final titers of 33 g L^{-1} using the same amount of glucose as a carbon source. The highest titer reported for *U.maydis* is derived from a metabolic rewiring approach whereupon the deletion of the *cyp3* gene increases itaconic acid titer to 63.2 g L^{-1} [19]. This gene encodes the production of cytochrome P450 monooxigenase which catalyzes the conversion of itaconic acid to 2-hydroxyparaconate, a pathway step that has only been described in *U.maydis*.

Other microbial hosts

While *A.terreus*, *A.niger* and *U.maydis* are taken as highly established producers of itaconic acid, there are some complications associated with filamentous fungi in a bioprocess, namely, maintaining proper aeration and complications in the downstream processing. In specific, the complications associated with fungal fermentations relate to the impact that hyphae growth produces on medium rheology: an increase in medium viscosity makes it so that aeration is more complicated, energy requirements are higher and fermentation results are less reproducible when compared to yeast-based fermentation [37,38] . Yeasts tend to have many beneficial and industrially attractive traits including growth to high cell densities on a wide variety of carbon sources, lower energy requirements, ability to perform post-translational modifications, potential to compartmentalize reactions in organelles, high secretion capacity and lower susceptibility to infectious agents like bacteriophages. While filamentous fungi also share many of these advantageous characteristics, they are often more difficult to transform with exogenous DNA [39] and less amenable to simple bioreactor cultivation. Also, the utilization of *A.terreus* in many countires requires safety facilities of level 2, which is not compatible with most industrial settings; and in some cases the utilization of this fungal species is even forbidden. In this sense, studies have been implementing the production of itaconic acid in different microbial systems, also with varying degress of success as shown by the compilation made in Table 2.

Table 2- Summary of the most recent efforts made to produce itaconic acid *in different hosts by* itaconic acid *biosynthesis cluster heterologous expression and metabolic optimization strategies*

Microorganism	Modification	Itaconic acid concentration	Source
Ustilago maydis M215 Δcyp3	Deletion of cytochrome P450 monooxygenase encoding CYP3	63 g L^{-1} (fed-batch cultivation)	[40]
E.coli	itaconic acid23: MG1655 ΔaceA ΔsucCD ΔpykA ΔpykF Δpta ΔPicd::cam_BBa_J23115 pCadCS	32 g L^{-1}	[42]
Aspergillus niger AB 1.13	Overexpression of *citB+MfsA+MttA in AtCad transformant strain*	26.2 g L^{-1}	[19]
Solanum tuberosum	CAD (optimised codon usage)	9 mg IA/ g wet weight	[43]
Yarrowia lipolytica	CAD, CS (*E.coli)*, aconitase (*E.coli*)	2.6 g L^{-1}	[44]
Saccharomyces cerevisiae BY4741	CAD (optimised for *S.cerevisiae* codon usage)	168 mg L^{-1}	[45]

Being the workhorse in industrial biotechnology and owing to the central role this species plays as an experimental model system, *S.cerevisae* has also been considered as a host for production of itaconic acid. However, the titers obtained until so far are around 160 mg/L [45,46] which is considerably below those reported for other fungal systems indicating that there is still much work for optimizing yeast-based production of itaconic acid. Among the identified difficulties, the need to bypass the production of ethanol to prevent huge diversion of the carbon source, is particularly challenging as observed for other organic acids [46,38].

2.3 EXPLORATION OF NON-*SACCHAROMYCES* YEASTS AS CELL FACTORIES

Taking advantage of the vast wealth of knowledge and data made available through works on *S.cerevisiae*, there are several reported non-conventional yeast species considered interesting to be used in industrial bioprocesses [47,48,49], some of which are already in implementation. These non-conventional yeast species are typically isolated from spontaneous fermentations occurring in the waste of a given industry such as cocoa-bean fermentation, cheese and bioethanol production byproducts, lignocellulosic hydrolysates, among others [49]. These fermentation settings are characterized for their harshness thereby posing a strong selective pressure that results in naturally highly resilient strains, displaying traits of interest for a biorefinery setting. In specific, among the desirable traits exhibited by non-conventional yeasts is their extreme thermo-tolerance, which is relevant in the context of the high temperatures that are used in the pre-treatments of lignocellulosic substrates [50]. *Saccharomyces cerevisiae* optimum fermentation range is in the 25-37ºC while some non-conventional yeasts can ferment at temperatures of more than 45ºC [51].

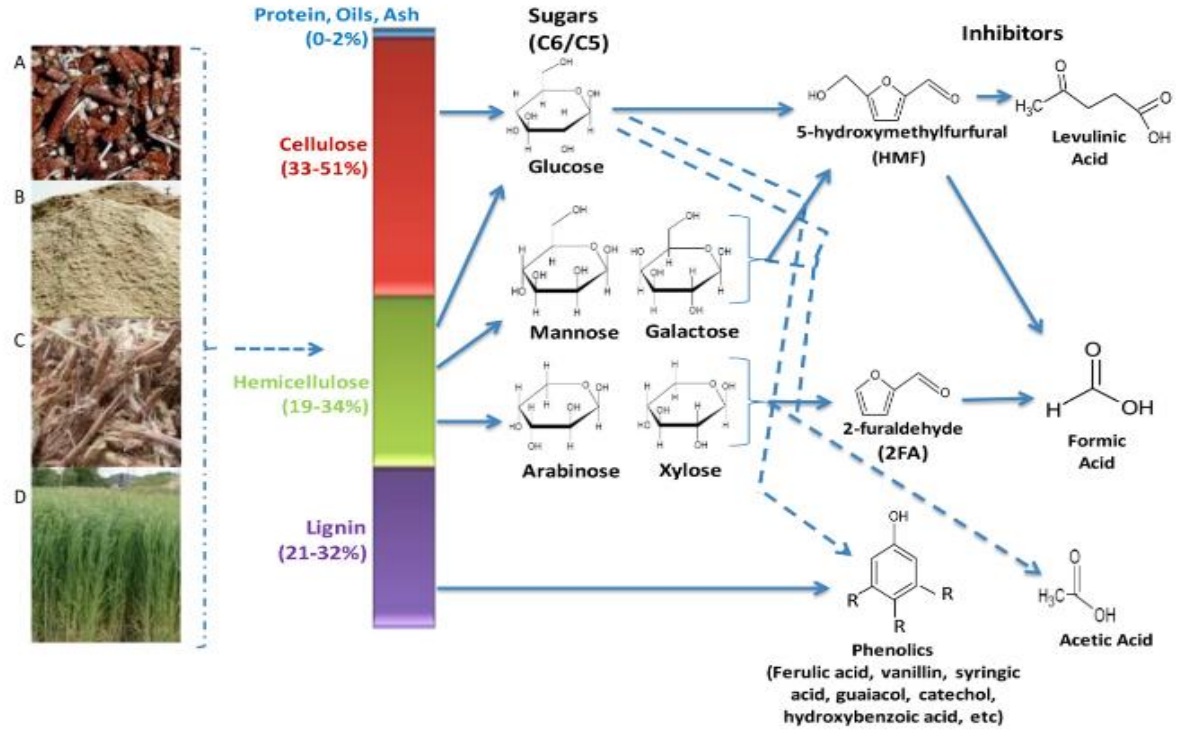

Figure 5- Main hydrolytic components of lignocellulose biomasses and generated inhibitory compounds. Biomasses are generated from wastes such as **(A)** maize cobs **(B)** saw dust **(C)** sugar cane bagasse **(D)** fast growing grasses. Pre-treatment processes releases the sugars (C6/C5) and lignin, however these processes also cause the breakdown of lignin and dehydration of the sugars, producing the inhibitory compounds that greatly reduce the overall efficiencies of fermentation.Reproduced from [6].

Additionally, the pre-treatment chemical hydrolysis of lignocellulose produces quite complex

hydrolysates which contain not only a diversity of sugars but also a variety of potentially toxic compounds derived from the chemicals used to treat the biomass. These toxic compounds are known collectively as pre-treatment derived inhibitors, which greatly hinder productivity and yield. At high temperature and pressure, C5 and C6 sugars are degraded to hydroxymethylfurfural (HMF) and furfural, respectively, due to dehydration [52] . HMF and furfural are known to have damaging effects on RNA, DNA, proteins and membranes even at low concentration [53,54]. Acetic acid is the most abundant weak acid generated following lignocellulosic biomass treatment, with concentrations ranging between 5 and 10 g L^{-1} [55,56]. Regardless of the source or preparation methods used, three main inhibitory compounds are produced during the pre-treatment and hydrolysis steps, namely organic acids, furan derivatives and phenolic compounds to which *S.cerevisiae* has proven susceptible [57–59].

Table 3-Summary of stress resistance traits for notable non-conventional yeast species. The phenotypic landscape of non-conventional yeasts is rich in extreme tolerance adaptations which render these species interesting for bioprocess exploration. Information collected and adapted from [47,49,60].

Species	Glucose (w/v)	Salt	Temperature	Ethanol	5-HMF	Acetic acid
Zygosaccharomyces rouxii	90%	3 mM NaCl				
Kluyveromyces marxianus			52ºC			
Ogataea polymorpha			50ºC			
Debaryomyces nepalensis		1500 mM NaCl			5 g L^{-1}	
Dekkera bruxellensis			>35ºC	10-16%		
Issatchenkia orientalis	48%	0,85 mM NaCl	<45ºC		7 g L^{-1}	
Candida ethanolica			37ºC	7%	5 g L^{-1}	
Zygosaccharomyces bailii	60%		40ºC			24 g L^{-1}
Yarrowia lipolytica	50%					

The table above intends to provide information relative to the biodiversity and untapped potential that characterize non-conventional yeasts. Many of these non-conventional yeast species exhibit industrially relevant traits such as the ability to utilize complex substrates as nutrients, extreme tolerance against stress and fermentation inhibitors. These organisms developed specific mechanisms

to survive under extreme environmental conditions. *Yarrowia lipolytica* is herein represented not necessarily for the organisms tolerance traits, but because this is one of the most prevalent examples of successful non-conventional yeast implementation in bioprocesses. This species is notorious for its oleaginous phenotype, a regulatory tendency in *de novo* lipid synthesis which results in the accumulation of lipids to an extent which caught the attention of the lipid and biofuel production industries [61,62]. In recent work, a 60-fold increase over the parental strain in lipid accumulation was obtained via expressing combinations of different lipogenesis targets in conjunction with phenotypic induction, to a titer of over 25 g L^{-1} of lipids [63]. Differences in metabolic regulation such as this are worthy of study and point out that the evolution of most of these species is independent of that of *S.cerevisiae*, diverging from the lineage prior to the genomic duplication event which characterizes the world famous hemiascomycetous yeast [64]. Therefore, there is wide speculation that most of these species may possess mechanisms that are not present in the model yeast. To date, most non-conventional yeasts have been characterized as spoilage yeast due to their frequent isolation from contaminated foods and beverages.

2.4 FOCUSING ON THE NON CONVENTIONAL YEAST *I.ORIENTALIS*

I.orientalis is frequently found in spontaneous fermentation settings having been isolated from food and fruit sources, such as sourdoughs, fermented butter-like products, the starter culture of Tanzanian fermented togwa, the African fermented cassava lafun, a Ghanaian fermented cocoa bean heap, fermented pineapple juice, orange juice, and grape [49]. There have been some reformulations as to the classification of this organism which may originate some confusion: *I.orientalis* is the designation given to a specific stage of *Candida krusei* development, also designated as *Pichia kudriavzevii.* Organisms may be classified on basis of sexual development stage, giving essentially the same organism, two different names. According to this criterion, two designations are considered, namely teleomorph and anamorph, referring to the sexual and asexual stages of development, respectively. The asexual name *Candida kefyr* and the sexual name *Kluyveromyces marxianus* refer to the same organism. In the same sense, *I.orientalis* is the sexual form, also called the teleomorph of *Candida krusei* [65]. Very recent research dating from July of the present year reports the isolation and sequencing of a clinical isolate of *Candida krusei*, as the first high-quality genome sequencing and assembly project for a clinical isolate [66]. This species differs from other *Candida* species such as

Candida albicans in the sense that it is not included in the CUG clade whereas CTG is translated as serine rather than leucine [67]. *Candida krusei* is an opportunistic fungal pathogen implied in immunocompromised patient infection. In the clinical setting, the most relevant trait of this species relates to its innate resistance to the most commonly used antifungal drug, fluconazole. Studies have shown that *C. krusei* Erg11p, the drug target, is significantly less susceptible to fluconazole inhibition than most other fungal Erg11p proteins [68], and that efflux pumps such as Abc1p are at least partially responsible for the innate fluconazole resistance of *C. krusei* [69]. The strain used in this study was first isolated from rotting Louisiana bagasse at the Carver Biotechnology Center belonging to the University of Illinois at Urbana-Champaign. In this work from 2014 which reports the isolation of said strain, genome sequencing and assembly was also performed, revealing 5,093 predicted genes, 7,107 proteins and an average GC content of 38.5% [70]. This same effort reports that out of the 5,093 genes predicted, 85% have homologs in *S.cerevisiae* which is relevant for the rational design of genome/metabolic engineering approaches. Furthermore, the aforementioned work reports the engineering of a uracil auxotroph, of paramount importance for heterologous expression approaches.

Table 4- Summary of gathered knowledge on resistance traits and genomic resources for the exploration of I.orientalis (syn. Pichia kudriavzevii). Sequences deposited at NCBI, Whole-Genome Sequencing projects access codes provided below.

Basic ploidy level	**Genome size**	**Most recent full nuclear genome sequences**				
Diploid	7-12 Mb among sequenced strains	*Pichia kudriavzevii* strain 129 *Pichia kudriavzevii* strain KMBL5774 *Pichia kudriavzevii* strain Ckrusei653 *Pichia kudriavzevii* strain SD108			WGS: MQVM01 WGS:MPBH01 WGS: NHMM01 WGS: JQFK01	
Carbon source usage	**Furan derivative tolerance**	**Organic acid tolerance**	**pH tolerance**	**Sugar tolerance**	**Salt tolerance**	**Ethanol tolerance**
Glucose Fructose Xylose Mannose	Up to 7 g L^{-1}	High resistance acetic/formic acid	Growth and production at pH 2	40% w/v Glucose	5% w/v NaCl	12-15% v/v

I.orientalis has been found to display several traits which make it interesting for exploration in a biorefinery context incuding utilization of multiple carbon sources, high resistance to furan derivatives and carboxylic acids, among others. As an example, while *I.orientalis* tolerates furan derivatives in concentrations up to 3 g L^{-1}, showing some sensitivity between 3-5 g L^{-1} and being inhibited above 7 g L^{-1} [71]1 [72], tolerance of *S.cerevisiae* is in the range of 0.02 g L^{-1} [73]. In this context, several works have been dedicated to exploring this species as a platform for second-generation bioethanol production or biorefinery settings [74,75,49]. *I.orientalis* has also been engineered to produce D-xylonic acid, through the heterologous expression of a D-xylose dehydrogenase from *Calobacter crescentus*, enabling production of 146 g L^{-1} D-xylonic acid at 1.2 g L^{-1} h^{-1} and a pH of 3 [76].

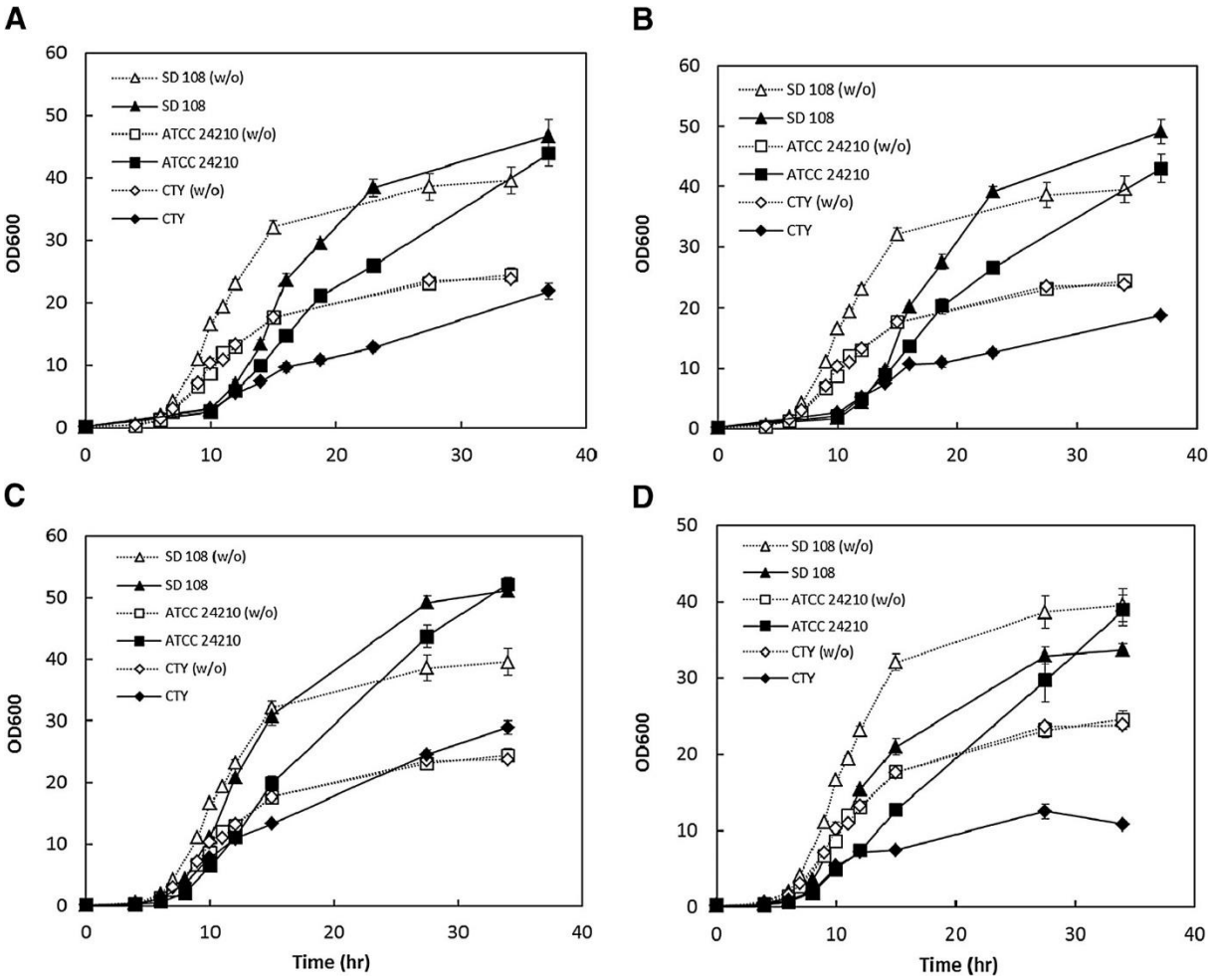

Figure 6- Cell growth of the strains in SC medium containing different organic acids. All the initial pH values were 5.6. SD 108, *I.orientalis* SD 108; ATCC24210, *I.orientalis* ATCC 24210; CTY, Classic Distiller's Turbo Yeast; w/o, without any organic acid added. (A) 30 g L^{-1} of succinic acid; (B) 30 g L^{-1} of itaconic acid; (C) 23 g L^{-1} of adipic acid; (D) 20 g L^{-1} of acetic acid. Reproduced from [70]

Work performed by Xiao and collaborators demonstrates that the *I.orientalis* SD108 strain is tolerant to different organic acids including succinic, adipic, acetic and itaconic acid [14], specially when compared

with *S.cerevisiae* (Fig.6). However, it should be noted that the experiments depicted in Figure 6 were conducted at pH 5.6, a pH at which most of the tested acids will be in their dissociated non-toxic form (succinic pK_{a1} = 4.2 pK_{a2} = 5.6; adipic pK_{a1} = 4.43 pK_{a2} = 5.41; acetic acid pK_a=4.76; itaconic pK_{a1} = 3.85 pK_{a2} = 5.55). In this sense, a proper assessment of *I.orientalis* to organic acid- imposed toxicity and, in particular, to itaconic acid is lacking. Much of the knowledge gathered on the molecular mechanisms of yeasts' tolerance to carboxylic acid stress has been gathered in *S.cerevisae* [77]. It is known that the toxic form of the carboxylic acid is the charge neutral undissociated form which can permeate the plasma membrane by simple diffusion. Once in the cytosol, the near neutral pH leads to chemical dissociation of the acid and, consequently, protons accumulate with multiple detrimental effects in cell viability [78,79]. The resulting cytoplasmic acidification induces the activation of Pma1p, a plasma membrane ATPase that pumps protons out of the cell, and also of the vacuolar ATPase, which pumps the exceeding protons to the vacuole lumen [80]. The accumulation of the negatively charged counter-ion also exerts a number of deleterious effects in yeast cells including oxidative stress, increase in turgor pressure, perturbation of the plasma membrane structure (more evident in the case of the more lipophilic organic acids), among others [79,81]. Within this context, several transporters have had their relevance described, either belonging to the Major Facilitator Superfamily or to the ATP-binding cassette, in contributing for maximal *S.cerevisiae* tolerance to different weak acids.

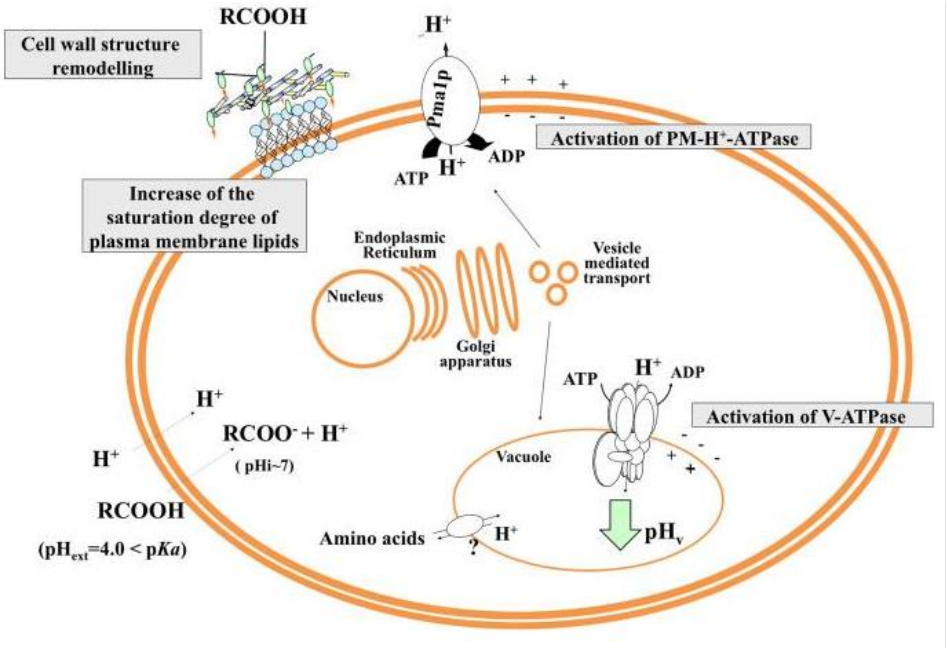

Figure 7- Organic acid toxicity and tolerance mechanisms in S.cerevisiae. Stimulation of the H^+-ATPase present both in plasma and vacuolar membranes contributes to counteracting the effects of intracelular pH acidification. Additionally, the reconfiguration of cell wall structure and plasma membrane also plays a role in reducing cell permeability to undissociated weak acids. Adapted from the work of Mira et al., [82].

Concerning non-Saccharomycetous species, the issue of tolerance to carboxylic acids has not been much explored, with the exception of the yeast species *Zygossacharomyces bailii*, a highly problematic spoilage yeast. In this case, the extreme tolerance of *Z. bailii* to acetic and benzoic acids has been linked to the ability of these cells to co-consume the acids while growing in the presence of glucose [83,84], a feature that does not occur in the Crabtree positive *S.cerevisiae* due to the well-known repressive effect exerted by the presence of glucose in the metabolization of alternative carbon sources [85,86].

2.5 THESIS OUTLINE

Previous works undertaken at the iBB laboratory enabled the production of itaconic acid in S.cerevisiae through the heterologous expression of the AtCad1 enzyme under the control of a galactose-inducible promoter [87,88]. The initial titers were around 25 mg L-1 [87] but with further strain improvement the titers increased to 165 mg L^{-1} [46]. In spite of this improvement the titers obtained were still low, compared with those achieved by other fungal hosts. In this work the non-conventional yeast I.orientalis SD108 was explored as a host for the production of itaconic acid at a low pH, taking into account the previously described favourable traits of this species. The study of *Issatchenkia orientalis* as a potential cell factory is being developed in partnership with the Institute for Genomic Biology based in the University of Illinois at Urbana-Champaign, USA, collaborating with the research group led by Prof.Doctor Zengyi Shao. In addition to the heterologous expression of *AtCad1* to enable itaconic acid production, metabolic engineering modifications and growth medium optimizations were performed aiming to reroute the metabolism of this yeast in the direction of itaconic acid biosynthesis. The susceptibility of this strain to itaconic acid and to other carboxylic acids of economical interest was also examined, in comparison with the tolerance exhibited by *S.cerevisae*. Differently from the work previously performed by Xiao *et al.,* [69] the assessment of susceptibility of *I.orientalis* to itaconic acid was tested at an acidic pH (pH 3.5) to favour the presence of the toxic undissociated form. Several general traits underlying tolerance to carboxylic acids, such as the activity of the plasma membrane proton pump, as well as the internal accumulation of the acid upon sudden challenge with an inhibitory concentration, were also compared in *S.cerevisiae* and in *I.orientalis*.

Successful expression of *AtCad1* enzyme, using the *AtCad* gene from *A. terreus* C-terminally fused to a 6-histidine tail and put under control of the galactose-inducible promoter Gal1 in *S.cerevisiae* was previously performed at the laboratory [45]. This heterologous expression strategy yielded itaconic acid concentrations of up to 165 mg L^{-1}. Upon the conduction of fermentation assays in MM medium, SD108 *I.orientalis* shows no tendency to produce itaconic acid. Following plasmid transformation with a glucose-inducible promoter, the heterologous expression of *AtCad* was achieved yielding up to 900 mg L^{-1}, prior to any strain optimization procedures.

3 MATERIALS AND METHODS

3.1 STRAINS, GROWTH MEDIA AND PLASMIDS

Strains used in this study are listed below. *I.orientalis* uracil auxotroph SD108 was kindly provided by Prof.Dr.Zengyi Shao of the Biorenewables Research Laboratory at Iowa State University.

Table 5-S.cerevisiae and I.orientalis strains used in this study

Strain	Genotype	Reference or source
I.orientalis SD108	ura3Δ	[14]
S.cerevisiae BY4741	MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0	Euroscarf collection

Strains were maintained in YPD growth medium which contains 20 g L^{-1} glucose (Merck), 20 g L^{-1} yeast extract (Difco) and 10 g L^{-1} bactopeptone (Difco), this undefined rich media allows strain storage in solid plates at 4ºC. *I.orientalis* SD108 was grown in synthetic MM medium containing 20 g L^{-1} glucose (Merck), 1.7 g L^{-1} Yeast Nitrogen Base without amino acids nor ammonium sulphate (Difco), 2.65 g L^{-1} ammonium sulphate (Merck) and 50 mg L^{-1} of Synthetic Complete aminoacid solution (see Table 7 for detailed composition). For selected fermentation assays, this MM medium was further supplemented with a trace elements and vitamins solution with detailed composition indicated in Table 8, along with the final concentration of each nutrient in the growth medium. *S.cerevisiae* BY4741 was cultivated in MM growth medium supplemented with Ura (20 mg L^{-1}) Met (10 mg L^{-1}) His (10 mg L^{-1}) and Leu (120 mg L^{-1}).

Table 6- Discriminated composition of Synthetic Culture aminoacid solution for medium supplementation

Component	Concentration in medium (mg L^{-1})	Component	Concentration in medium (mg L^{-1})
Adenine	7,5	Leucine	150
Alanine	30	Lysine	30
Arginine	30	Methionine	30
Asparagine	30	p-aminobenzoic acid	30
Aspartic acid	30	Phenylalanine	30
Cysteine	30	Proline	30
Glutamine	30	Serine	30
Glutamic acid	30	Threonine	30
Glycine	30	Tryptophan	30
Histidine	30	Tyrosine	30
Inositol	30	Uracil	60
Isoleucine	30	Valine	30

Table 7– Discriminated Trace Element and Vitamin medium supplementation solution composition

Vitaconic acidmins mg L^{-1}		Trace elements mg L^{-1}	
D-biotin	0,05	Na_2EDTA	1.5
Panthothenic acid calcium salt	1	$ZnSO_4 \cdot 7H_2O$	0.45
Nicotinic acid	1	$MnCl_2 \cdot 2H_2O$	0.1
Myo-inositol	25	$CuSO_4 \cdot 5H_2O$	0.03
Thiamine chloride hydrochloride	1	$Na_2MoO_4 \cdot 2H_2O$	0.03
Pyridixol hydrochloride	1	$CaCl_2 \cdot 2H_2O$	0.45
4-Aminobenzoic acid	0,2	$FeSO_4 \cdot 7H_2O$	0.30
		H_3BO_3	0.1

The plasmids used in this study are listed below in Table 9. Plasmids obtained in collaboration from Shao Biorenewables Laboratory at the Carver Biotechnology Center, ISU, United States of America.

Table 8-: Plasmids used in the present study.

Plasmid	Description
pWS-Io-Ura	Backbone plasmid used for cloning of the *A. terreus AtCADA* gene; Ura 3 selection marker
pWS-Io-Ura-IA	Allows the expression of A. terreus *AtCadA* gene (codon optimized for *I.orientalis*) under control of the *TDH3* promoter;
pWS-Io-Ura-IA-*MfsA*	Allows the expression of *A. terreus AtCadA* gene (codon optimized for *I.orientalis*) under the control of the *TDH3* promoter and of the *Issatchenkia orientalis MfsA* gene under control of the *Tef1* promoter
pWS-Io-Ura-IA-*MttA*	Allows the expression of *A. terreus AtCadA* gene (codon optimized for *I.orientalis*) under the control of the *TDH3* promoter and of the *Issatchenkia orientalis MttA* gene under control of the *Tef1* promoter
pWS-Io-Ura-IA-Aco	Allows the expression of *A. terreus AtCadA* gene (codon optimized for *I.orientalis*) under the control of the *TDH3* promoter and of the *Issatchenkia orientalis Aco* gene under control of the PGK promoter

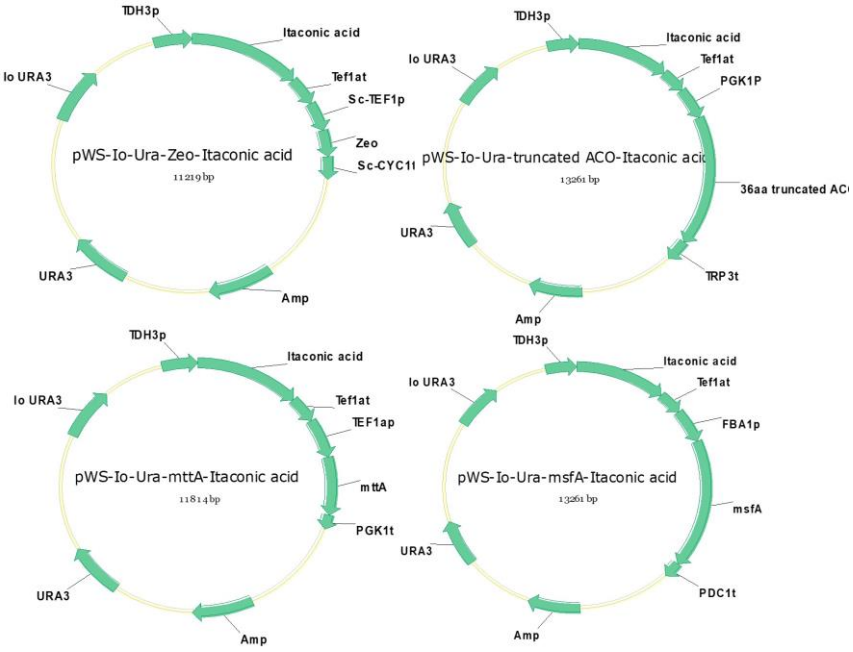

Figure 8- Plasmid maps for the plasmids used in this study based on pWS backbone. Each plasmid possesses selection markers for yeast and bacteria.

3.2 *I.ORIENTALIS* TRANSFORMATION

The different plasmids were transformed into *I.orientalis* SD108 using the Alkali-Cation TM Yeast Transformation kit (MP Biomedicals). For this, cells were cultivated until mid-exponential phase ($OD_{600\ nm}$=0.6) in 50 mL of YPD and then centrifuged for 5 minutes at 6000 rpm and 4ºC. The pellet obtained was resuspended in 4.5 mL TE (pH 7.5) and centrifuged again in the same conditions. The pellet was resuspended in 2.5 mL of Lithium/Cesium Acetate Solution and the obtained suspension was then incubated for 30 minutes at 30ºC and 100 rpm. Cells were once more centrifuged and finally resuspended in 500 µL of TE (pH 7.5) to obtain competent cells. The transformation mix is composed of 100 µL of competent cells, 10 µL of purified plasmid DNA, 5 µL of carrier DNA and 5 µL of histamine solution. This mix was then incubated at room temperature for 15 minutes. After this, 0.2 ml of TE/Cation Mix and 0.8 mL of PEG were added to each reaction and these were incubated for another 10 minutes at 30ºC. Cells were then heat shocked for 10 minutes at 42ºC and subsequently cooled to 30ºC in the growth room. Finally, the mix was centrifuged at 8000 rpms for 3 minutes and cells were resuspended in 100 µL of YPD and plated on MM plates without uracil to allow for selection by mark complementation. Transformants were selected in solid MM plates without uracil.

3.3 ITACONIC ACID-PRODUCING FERMENTATIONS

I.orientalis SD108 was batch-cultured at 30ºC in MM growth medium or in modified versions of this medium as indicated in Table 10. For the fermentations *I.orientalis* cells were cultivated (30ºC, 250 rpm) over-night in shake-flasks (100 mL capacity) containing 30 mL MM medium. On the following day, an appropriate volume of the pre-inoculum was used to inoculate (at an $OD_{600\ nm}$ of 0.15 ± 0.05) 100mL Erlenmeyer flasks containing 30 mL of fresh MM medium (pH=3.5). Fermentations were performed during 7 days. Growth was followed based on the increase of $OD_{600\ nm}$. 1 mL sample was taken from each shake flask every 24 hours. Samples were centrifuged and the supernatants separated on an Aminex HPX-87H® column using H_2SO_4 0.005 M as the eluent (at a flow rate of 0.6 mL min^{-1}). A UV detector set at 210 nm was used for detection of organic acids while detection of sugars, ethanol and glycerol was performed using an RI detector. The HPLC analysis method used was developed in [88] indicating the following retention times for each compound: citric acid - 8.2 min; pyruvic acid - 9.2 min; malic acid - 9.5 min; itaconic acid- 12.5 min; glucose - 8.7 min.

Table 9-Detailed medium composition for initial assessment of medium composition influence on itaconic acid production by I.orientalis SD108.

	Glucose	Ammonium sulphate ($(NH_4)2SO_4$)	Yeast Nitrogen Base	Aminoacid solution SC Ura^-	Vitaconic acidmin solution	Trace Elements
	g L^{-1}			mL L^{-1}		
2% MM	20	2.67	1.7	50	-	-
4% MM	40	2.67	1.7	50	-	-
2% MM ½$NH_4$2SO_4	20	1.34	1.7	50	-	-
2% MM ¼$NH_4$2SO_4	20	0.67	1.7	50	-	-
2% MM + Vitamins	20	2.67	1.7	50	1	-
2% MM + TE +Vitamins	20	2.67	1.7	50	1	1

3.4 ITACONIC ACID SUSCEPTIBILITY ASSAYS

To assess growth of *I.orientalis* SD108 in the presence inhibitory concentrations of itaconic acid, the cells were cultivated overnight at 30ºC in controlled pH=3.5 MM at 250 rpm. In the following morning, a designated volume of the pre-inoculum was used to inoculate (at an $OD_{600\ nm}$ of 0.1 ± 0.05) fresh MM medium either or not supplemented with inhibitory concentrations of itaconic acid. The range of concentrations used is indicated in the table below. Growth was monitored based on the increase in OD_{600nm} of the culture. In this experiment the pH of the growth medium was adjusted (using HCl as the acidulant agent) to 3.5 in the assays performed with itaconic acid. The stock solution of itaconic acid used to supplement the growth medium was also adjusted to pH 3.5. As a control experiment the susceptibility of *S.cerevisiae* BY4741 to the same concentrations of itaconic acid was also tested.

Table 10--Summary of organic acids used to evaluate I.orientalis' tolerance; range of concentrations at which the acids were applied and corresponding medium pH.

Stressor	Stock Solution (M)	Tested molarity (mM)	Medium pH
Itaconic acid	2	400, 500 and 600	3.5 ± 0.05

3.5 IN VIVO ATPASE ACTIVITY DETERMINATION

To estimate the *in vivo* activity of the plasma membrane proton pump upon organic acid challenge in *I.orientalis* cells were grown overnight in MM flask at pH 3.5. The following morning, an appropriate amount of this pre-culture was used to inoculate fresh MM medium (pH= 3.5) and grow cells to mid-exponential phase ($OD_{600\ nm}$ = 0.8 ± 0.05). After growth, cells were harvested by filtration (0.2 µm filters, Whatman) twice washed with distilled water and incubated at 30ºC in a 20 g L^{-1} sorbitol solution at pH 3.5 for 30 minutes. The incubation in sorbitol aims to inhibit plasma membrane proton pump activity. After this time, cells were filtrated, washed with dd H_2O at pH 3.5 to remove sorbitol residues and resuspended in 10 mL dd H_2O to obtain a dense cell suspension ($OD_{600\ nm}$= 20 ± 0.05). This 10mL dense cell solution was divided in 1mL aliquots to be used for the assay. Each assessment of *I.orientalis* SD108 and BY4741 PM-H^+ ATPase activity was conducted in a temperature controlled water jacketed cell (capacity 5 mL) at 30ºC with agitation, coupled to a continuous pH reader. For each assessment, 1 mL of cell suspension is added to 3.0 mL of dd H_2O at pH 3.5 and, when required, 1 mL of 2, 4 or 8 mM itaconic acid. Stock solutions for itaconic acid were prepared individually and controlled at pH 3.5. After mixing, the assay pH was quickly adjusted to 3.5 ± 0.05 using stock NaOH and HCl solutions. Each assay is then initiated with the addition of 1 mL of glucose 100 g L^{-1} at pH 3.5, this reactivates PM-H^+ ATPase and pH variations were measured for 10 minutes at each 10s interval by potentiometry using a pH microelectrode attached to a pH meter (Metrohm 691). The same procedure was performed for *S.cerevisiae* BY4741 for comparison purposes.

3.6 ITACONIC ACID INTRACELLULAR ACCUMULATION ASSAYS

The accumulation ratio of [H^3]-itaconic acid (taken as the ratio between the intracellular and extracellular concentrations of radiolabelled acid) was compared in *I.orientalis* and in *S.cerevisiae* BY4741. For this, cells were cultivated in MM medium (at pH 3.5) for about 8 hours after which they were re-inoculated in fresh MM medium. The inoculation was performed aiming to collect the cells on the next morning in their exponential phase (OD_{600nm} ~1). In the morning of the assay, cells were harvested by filtration (25 mm; Filter-LAB, MFV3) and then resuspended in 5 mL of MM medium with 350 mM of cold itaconic acid and 3µl of radiolabelled [H^3]-itaconic acid (American Radiolabeled

Chemicals, inc.) (15 Ci/mmol) to obtain a dense cell suspension (OD_{600nm} = 0.7± 0.05). This 5 mL culture was then incubated at 30°C in a water bath with orbital agitation (150 rev/min). Culture samples were taken at 1, 5, 10, 15, 20 and 30 minutes of incubation in the presence of itaconic acid. For quantification of intracellular [H^3]-itaconic acid, a 200 µl culture sample was filtered through pre-wetted glass microfiber filters (Whatman GF/C) and washed with cold water. V_i refers to a cells' internal volume, A_e stands for specific activity of [H^3]-itaconic acid and cpm is a measure of radioactivity.

$$A_i^T(mM) = \frac{cpm_i}{A_e(\frac{cpm}{mmol})} \times \frac{1}{V_i(\mu L)}$$

In order to measure extracellular [H^3]-itaconic acid, a 100 µl culture sample was collected and the supernatant was recovered by centrifugation in a tabletop centrifuge (14000 rpm, 30 seconds).

$$A_e^T(mM) = \frac{cpm_e}{A_e(\frac{cpm}{mmol})} \times \frac{1}{100\ (\mu L)}$$

The supernatant used for measurement of extracellular itaconic acid or the filters containing the cells used to measure the intracellular concentration of the acid, were added to 7 ml of scintillation liquid (Ultima GoldTM - Beckman) and their radioactivity was measured in a Beckman LS 5000TD scintillation counter. The same procedure was used to compare the levels of itaconic acid accumulated inside *I.orientalis* and *S.cerevisiae* cells. The intracellular volume used for *S.cerevisae* cells was 2.5 µL/mg dry biomass [89]. In the case of *I.orientalis* this V_i factor had to be independently calculated. To assess this parameter, exponential phase *I.orientalis* cells cultivated in MM medium (at pH 3.5) were observed using a bright field microscope (Zeiss-Axioplan) in connection with a CoolSnap fx camera (Photometrics) under a magnification lens of 100x. Images were captured and stored with the aid of image capture and edition software (Metamorph Imaging system 4.6.9-Universal Imaging Corporation). With recourse to this same software the volume of *I.orientalis* cells was calculated by applying the formula for determining the volume of ellipsoidal structures, $V = (\pi/6) \times b2a$, where *b* corresponds to the length of the cells and *a* to its width. This aproach has been previously used by Arango et al., to determine the celular volume of *Candida* tropicalis cells [90]. Prior to image capture the software was calibrated to have its measure unit pixel (1px) correspond to 0.065 µm in the

aforementioned amplification. Across measurements made for 30 independente cells, an estimated average volume for *I.orientalis* of 120.93 µm3 was estimated. This individual volume was used (V_i) to calculate the volume of the entire set of cells used in the 200 µL of cells that were used to measure the internal accumulation. For this it we worked under the assumption that OD_{600nm} =1 corresponding to 10^7 cells.

4 RESULTS

4.1 *I.ORIENTALIS IS HIGHLY TOLERANT TO* ITACONIC *ACID AT A LOW PH*

To assess the tolerance of *I.orientalis* to itaconic acid at a low pH, the cells were cultivated in MM medium supplemented with 400 and 500 mM at pH 3.5, which is a pH value below the two pKas of itaconic acid. These concentrations of itaconic acid were selected to be within the range of concentrations expected to be present in the broth of fermentations used to produce this molecule [91]. As a comparison the susceptibility of *S.cerevisiae* BY4741 cells to the same concentrations of itaconic acid was tested.

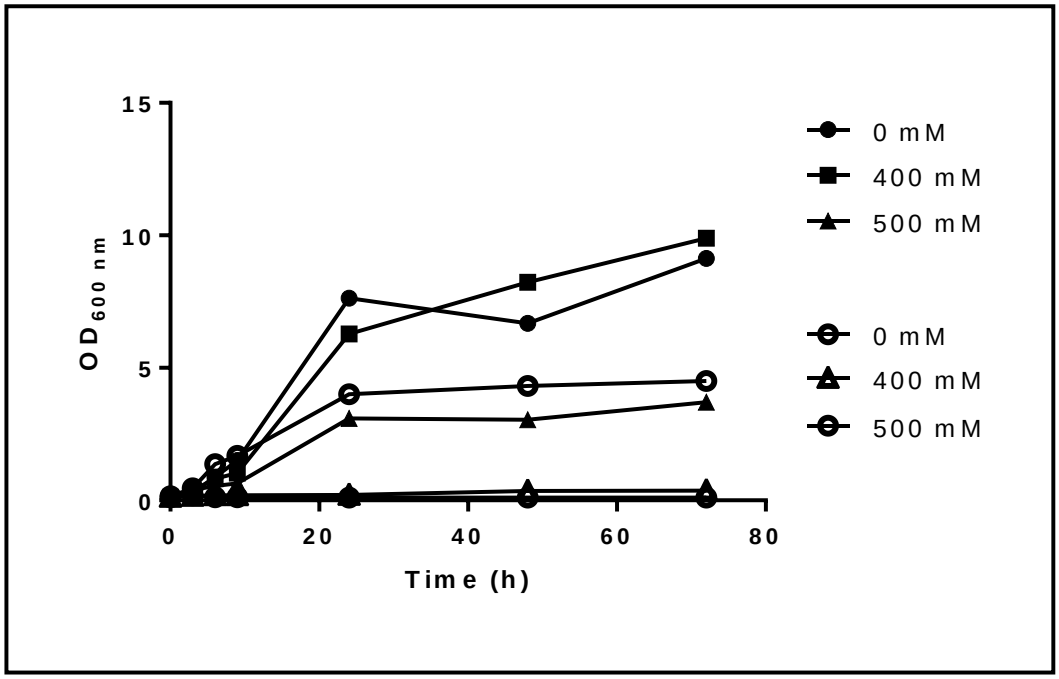

Figure 9- Growth curve comparison of I.orientalis SD108 strain (full symbols) and S.cerevisiae BY4741 strain (hollow symbols) under exposure to itaconic acid *stress in varying concentrations. Each datapoint represents the mean value of three independent replicates. Assay conducted at a pH of 3.5.*

The results obtained clearly show the higher resilience of *I.orientalis* cells to itaconic acid stress, this being particularly visible for the 400 mM concentration where growth was almost identical to the growth of control cells. A small, but detectable, lag phase (of about 2 h) is observed in MM growth medium and also in this medium supplemented with itaconic acid, this being attributable to an eventual dilution effect of the cells in the fresh medium. Despite this, the higher resilience of *I.orientalis* cells to itaconic acid is evident, especially at 400 mM of itaconic acid, which fully abrogated growth of *S.cerevisae* but only mildly affected growth of *I.orientalis.*

Table 11- Summary of growth evaluation parameters across diferent itaconic acid supplementations in MMB medium at controlled pH = 3.5.

	I.orientalis SD108			***Saccharomyces cerevisiae BY4741***		
Itaconic *acid concentration (mM)*	μ h^{-1}	lag phase (h)	doubling time	μ h^{-1}	lag phase (h)	doubling time
0	0.51	2	1.35	0.21	2.5	3.3
400	0.34	2	2.03	0.075	-	9.24
500	0.26	2.5	2.66	0.025	-	25.67

The high resilience of *I.orientalis* cells to itaconic acid prompted the search for traits underlying this phenotype. In specific our attention was focused on the ability of *I.orientalis* cells to reduce the internal accumulation of itaconic acid as well as on this microbe's ability to counter-act the acid-induced internal acidification, two key aspects that have been found to modulate stress induced by carboxylic acids in different yeast species [78,82]. As such, we determined the internal accumulation of radiolabelled itaconic acid (3 μM) during cultivation of *I.orientalis* in the presence of an inhibitory concentration of cold itaconic acid (350 mM, at pH 3.5). Under the experimental setting used, these radiolabelled accumulation assays mimicked the experimental setting used in the growth curves shown in Fig 9. The results obtained indicate that, at least upon sudden itaconic acid challenge, no significant differences were observed between the ammount of itaconic acid that accumulated inside *I.orientalis* or *S.cerevisiae* cells during the first 30 minutes where this was monitored. It could be relevant to monitor this parameter for longer periods of incubation, where the difference in the ability of these two yeasts to extrude itaconic acid could actually be more visible.

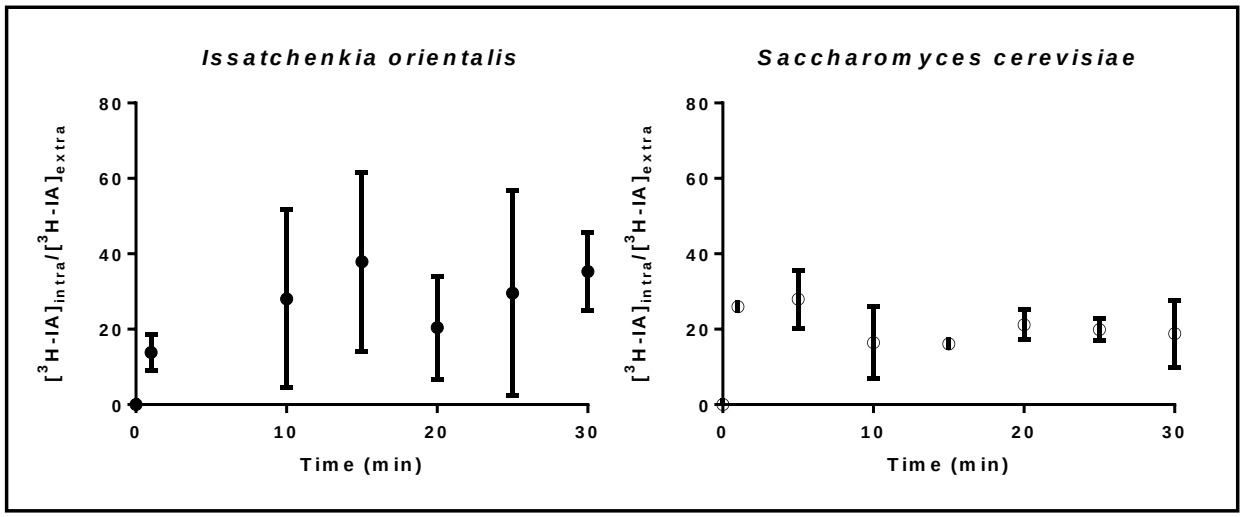

*Figure 10-- Graphic representation of the accumulation ratio of [H³]-*itaconic acid *in I.orientalis and S.cerevisiae BY4741 during growth in MMB ph=3.5 supplemented with 350 mM cold* itaconic acid*. The accumulation ratios displayed in each datapoint are the mean value of 5 independent assays*

To monitor the ability of *I.orientalis* and *S.cerevisiae* cells to counter-act the expected intracellular acidification caused by itaconic acid we examined the ability of de-energized *I.orientalis* cells to acidify the external surrounding, either in the presence or absence of the acid, upon a pulse of glucose. The results obtained, displayed in Fig.11, show that *I.orientalis* has a higher ability of acidifying the medium suggesting a higher basal proton pump activity, comparing with the one exhibited by *S.cerevisiae* BY4741 cells (Fig.11 and Table 12). This is made evident by the calculated rate of acidification and also in the final pH of the assay, both measured across the initial 80 seconds (Table 12). In the presence of the lower concentrations of itaconic acid, the rate of acidification exhibited by *I.orientalis* and also *S.cerevisiae* cells slightly increased, comparing to the one registered in control conditions (Table 12). Proton pump inhibition is only verifiable at the highest used concentration of 8 mM, visible either in *I.orientalis* and in *S.cerevisae* cells, albeit with a very wide gap in the extent of the inhibition, the acidification rate of *I.orientalis* was only marginally affected while *S.cerevisae* cells show very diminished activity.

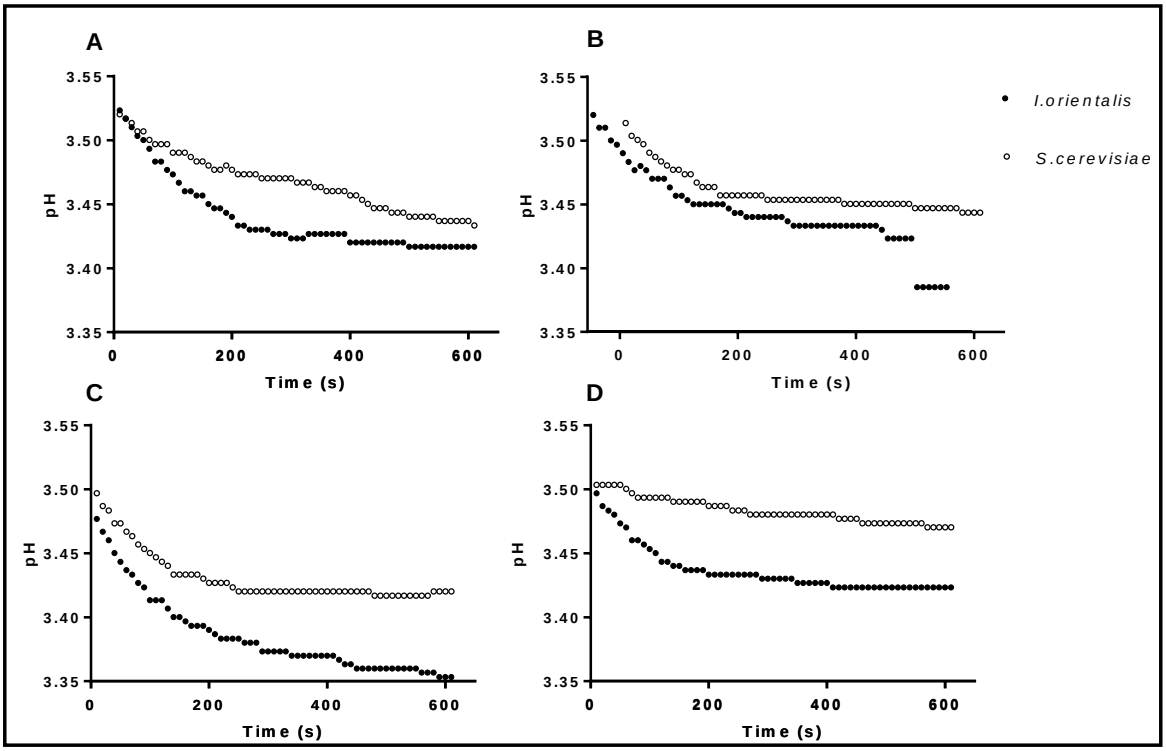

Figure 11-- Graphic representation of pH variation over time for I.orientalis and S.cerevisiae during cultivation in MMB pH=3.5 under varying supplementations with itaconic acid ***A****- 0 mM* ***B****- 2 mM* ***C****- 4 mM* ***D****-8 mM. Each data point displayed results from the mean value of 3 independent assays*

Table 12- in vivo ATPase assay slope measurement as proxy for proton pump activity and response to the presence of stressor agent itaconic acid at varying concentrations. Values in parenthesis represent % (percent) variation relative to the control condition. Final pH represents the pH value after 80 seconds and is a measure of proton pump response to immediate acid challenge.

	Itaconic acid concentration							
Organism	0 mM	Final pH	2 mM	Final pH	4 mM	Final pH	8 mM	Final pH
S.cerevisiae BY4741	$-3.33E^{-4}$ ±0.0001 (100%)	3.49	$-4.5E^{-4}$ ±0.0002 (135%)	3.46	$-5.17E^{-4}$ ±0.0002 (155%)	3.48	$-1.27E^{-4}$ ±0.0001 (38%)	3.49
Issatchenkia orientalis	$-5.17E^{-4}$ ±0.0001 (100%)	3.47	$-5.5E^{-4}$ ±0.0002 (106%)	3.39	$-7.5E^{-4}$ ±0.0002 (145%)	3.42	$-5.00E^{-4}$ ±0.0003 (96%)	3.46

4.2 *EXPLORATION OF I.ORIENTALIS* AS A CELL FACTORY FOR THE PRODUCTION OF ITACONIC ACID

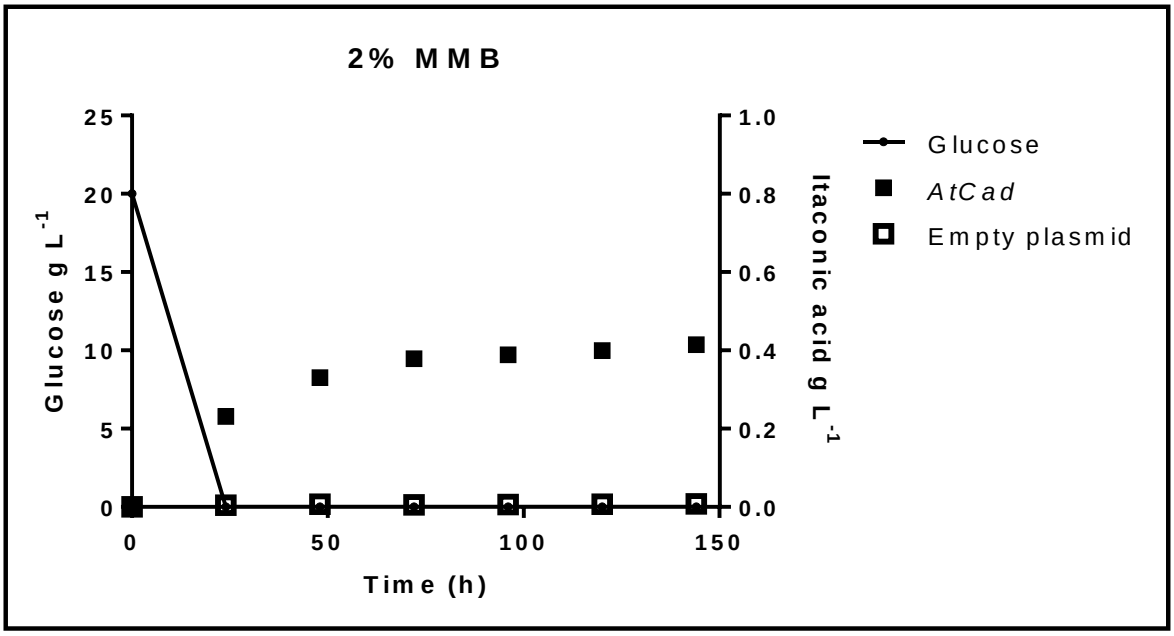

Figure 12- Concentration of glucose (circles) and of itaconic acid (squares) present in the culture supernatant of I.orientalis cells transformed with empty plasmid (closed symbols) or with the plasmid that drives AtCad expression (open symbols). Each data point displayed results from the mean value of 3 biological replicates.

Following successful transformation of *I.orientalis* with the plasmid-borne cis-aconitic acid decarboxylase (AtCad) from *A. terreus*, itaconic acid production was tested in MM medium containing 20 g L^{-1} of glucose as carbon source. The maximum itaconic acid titer in the broth was 0.41 g L^{-1} which accounts for about 2% yield (g itaconic acid produced/g glucose consumed) on glucose. All the available glucose was consumed in 24 hours, either in cells transformed with the empty plasmid strain or with the plasmid that drives *AtCad1* expression. Further analysis of the chromatrograms obtained upon separation of the samples of the culture broth allowed us to monitor over time the concentration of other compounds that were also being secreted. In particular, it was possible to observe two distintct peaks, one having a retention time of 6 minutes and another eluting at 8 minutes. This last peak was identified as citrate, as observed by the increase in the peak area when the sample is supplemented with citric acid. In Fig.13 the profile of citrate accumulation in the culture medium is displayed in comparison with the one of itaconic acid.

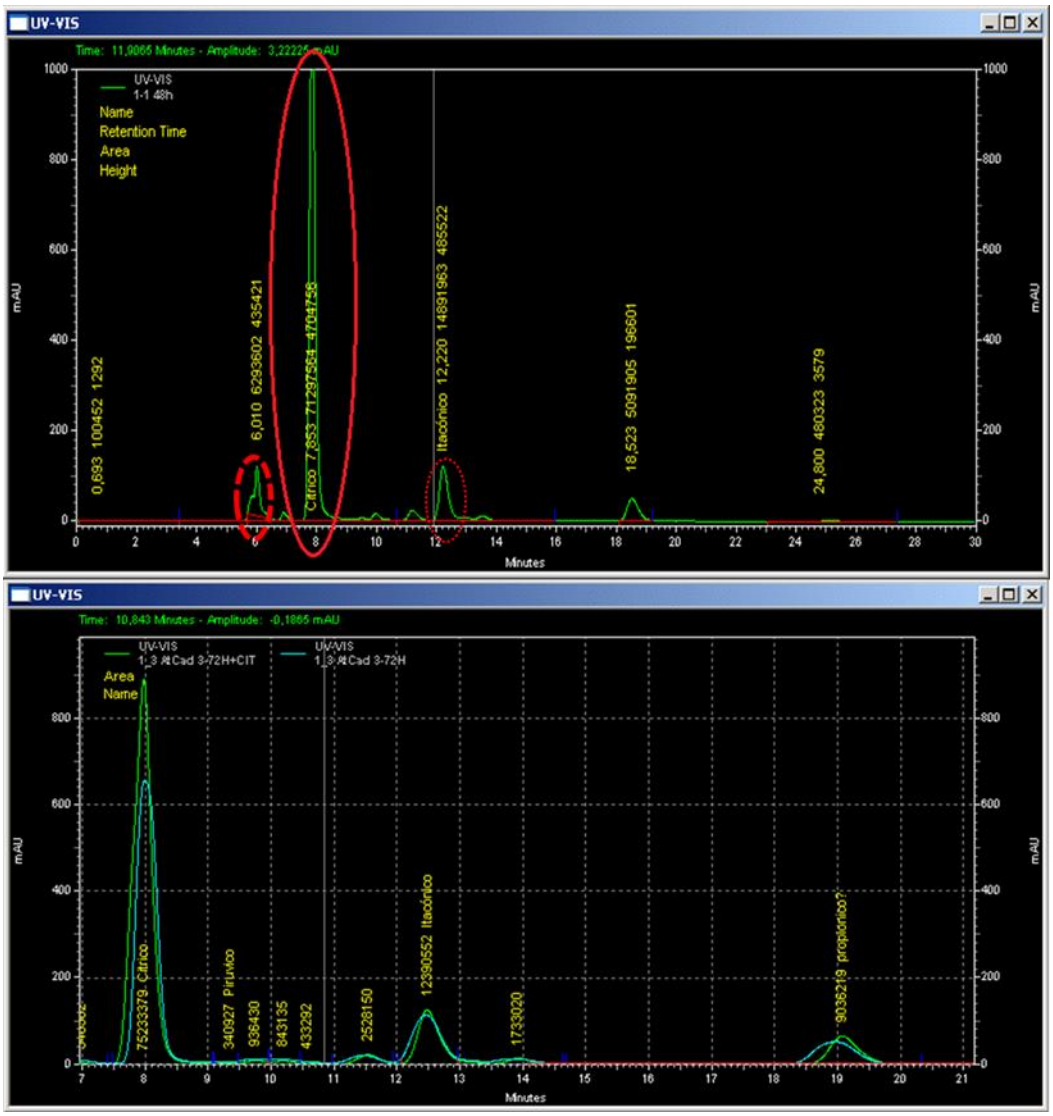

Figure 13- Chromatogram analysis of AtCad transformants fermentation medium supernatant. Upper: accumulation of two C-compounds, one identified as citric acid (line in full) and the other not being recognized by HPLC method calibration (dashed line). Itaconic acid corresponding peak is verified indicating successful production (dotted line). Lower: by supplementing HPLC vials for sample analysis with citric acid, the nature of this metabolite is confirmed as overaccumulating in I.orientalis.

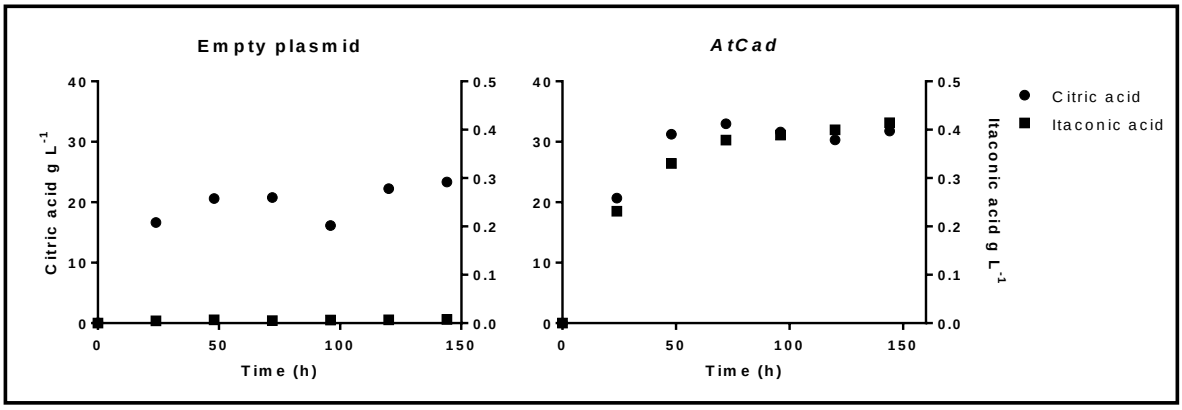

Figure 14- Concentration of citric acid (circles) and itaconic *acid (squares) in I.orientalis transformants with the empty plasmid or with the plasmid that drives AtCad expression. Citric acid accumulation is verifiable in both transformants fermentation broth.*

Upon successful establishment of *I.orientalis* as a platform for the production of itaconic acid, the next step was to optimize the growth medium composition to improve production of the acid. In this sense, fermentations were performed to verify the influence of carbon amount, nitrogen source availability and also cofactor supplementation as, in literature, variations in the aforementioned factors show impact in *A. terreus'* ability to produce and accumulate itaconic acid [92,93]. Oxygen availability was also tested for impact on growth and fermentative profile since aeration rate was also found to play a role in determining production of itaconic acid by other hosts [35]. The results obtained showed that only the increase of glucose in the growth medium (from 2 to 4%) increased the final titer of itaconic acid produced (0.9, compared to 0.4). Despite the improvement, the 4% of glucose were consumed in 24h, similar to what was observed in the medium that contained only 2% of glucose. Limitation of N led to a slight improvement (0.5 g L^{-1} itaconic acid compared to 0.42 g L^{-1} obtained in normal MM medium), most likely due to the observed reduction in growth. The reduction in O_2 availability reduced the production of itaconic acid, again due to the observed reduction in growth (0.43 growth rate, comparing with 0.86).

Table 13-Summary of I.orientalis' fermentations in tested MM medium variation growth parameters, concentration of itaconic acid and *citric acid found in broth,* itaconic *acid productivity, glucose consumption rate and* itaconic *acid yield (g* itaconic acid */ g glucose).*

	Growth Rate μ (h^{-1})	**Final OD_{600}**	**Final ITA titer (g L^{-1})**	**Final CIT titer (g L^{-1})**	**ITA productivity (mg L^{-1} h^{-1})**	**Glucose consumption rate (g h^{-1})**	**% Yield (g ITA / g glucose)**
2% MM	0.86	27.6	0.43	31.77	2.98	0.85	2
2% MM ½ $NH_4 2SO_4$	0.71	23.6	0.42	30.06	2.92	0.81	2
2% MM ¼ $NH_4 2SO_4$	0.68	26.2	0.50	28.03	3.47	0.80	2.5
4% MM	0.74	38.1	0.91	31.20	6.32	0.80	2.25
2% MM Limited O_2	0.43	16	0.33	22.44	2.29	0.80	1.8
2% MM + Vitamins	0.76	24.9	0.51	36.62	3.40	0.85	2.5
2% MM + Vitamins + Trace Elements	0.89	27.1	0.44	28.12	3.05	0.93	2

4.3 EFFECT OF THE OVER-EXPRESSION OF AT*MFSA*, IOACOA AND AT*MTTA* IN THE ABILITY OF *I.ORIENTALIS* TO PRODUCE ITACONIC ACID

The accumulation of citrate in the fermentation broth suggested that biosynthesis of itaconic acid could be limited due to limited production of cis-aconitic acid. As such, it was decided to test the effect of over-expressing the aconitase enzyme as a means to increase the availability of cis-aconitic acid. Since *A.terreus CadA* is a cytosolic enzyme [36], it was decided to truncate the *I.orientalis* native aconitase by removing the predicted mitochondrial localization signal (comprised of the first 33 amino acids of the protein). Besides this modification, we also examined the effect of over-expressing *MfsA*, the putative plasma membrane exporter of itaconic acid; and of *MttA*, the mitochondrial transporter involved in the export of cis-aconitic acid from the mitochondria to the cytosol. Previous work performed in *A.niger* shows that overexpression of either the mitochondrial carrier or the plasma membrane carrier leads to an increased itaconic acid production [94]. A similar approach in *A.*terreus also yielded positive results [31]. The overexpression of cytosolic *Aco* did not improve the titer of itaconic acid produced, being observed even a slight decrease (0.19 g L^{-1} in comparison with 0.4 gL^{-1} obtained in sole *AtCad* expression) (Table 16). The amount of citrate accumulated in the fermentation broth was not affected by the overexpression of *Aco*. The overexpression of *MttA* increased the final titer of itaconic acid to 1.3 g L^{-1}, representing an increase of 325% relative to sole *AtCad* expression (Table 14). *MfsA* overexpression also yielded positive results with final itaconic acid titer increasing to about 1.1 g L^{-1}, representing an increase of 275% relative to sole *AtCad* expression. A remarkable effect of the overexpression of *MttA* and *MfsA* is concerned with the decreased observed in citric acid accumulation in the fermentation broth.

Table 14- Summary of I.orientalis different transformants fermentation assays. Depicts growth parameters, final itaconic acid production titer, itaconic acid productivity, glucose consumption rate and carbon % yield on glucose as a substrate.

	Growth rate μ (h^{-1})	Final itaconic acid titer ($g\ L^{-1}$)	Itaconic acid productivity ($mg\ L^{-1}\ h^{-1}$)	Final citric acid titer ($g\ L^{-1}$)	Glucose consumption rate ($g\ h^{-1}$)	% Yield (g ITA / g glucose)
AtCad	0.40	0.36	2.5	28.12	0.8	1.8
Empty vector	0.40	0.00	--	23.35	0.8	--
AtCad + mTTA	0.33	1.31	9.1	0.13	0.8	6.55
AtCad + mfsA	0.38	1.08	7.5	0.0	0.8	5.4
AtCad + Aco	0.40	0.19	1.3	32.1	0.8	0.95

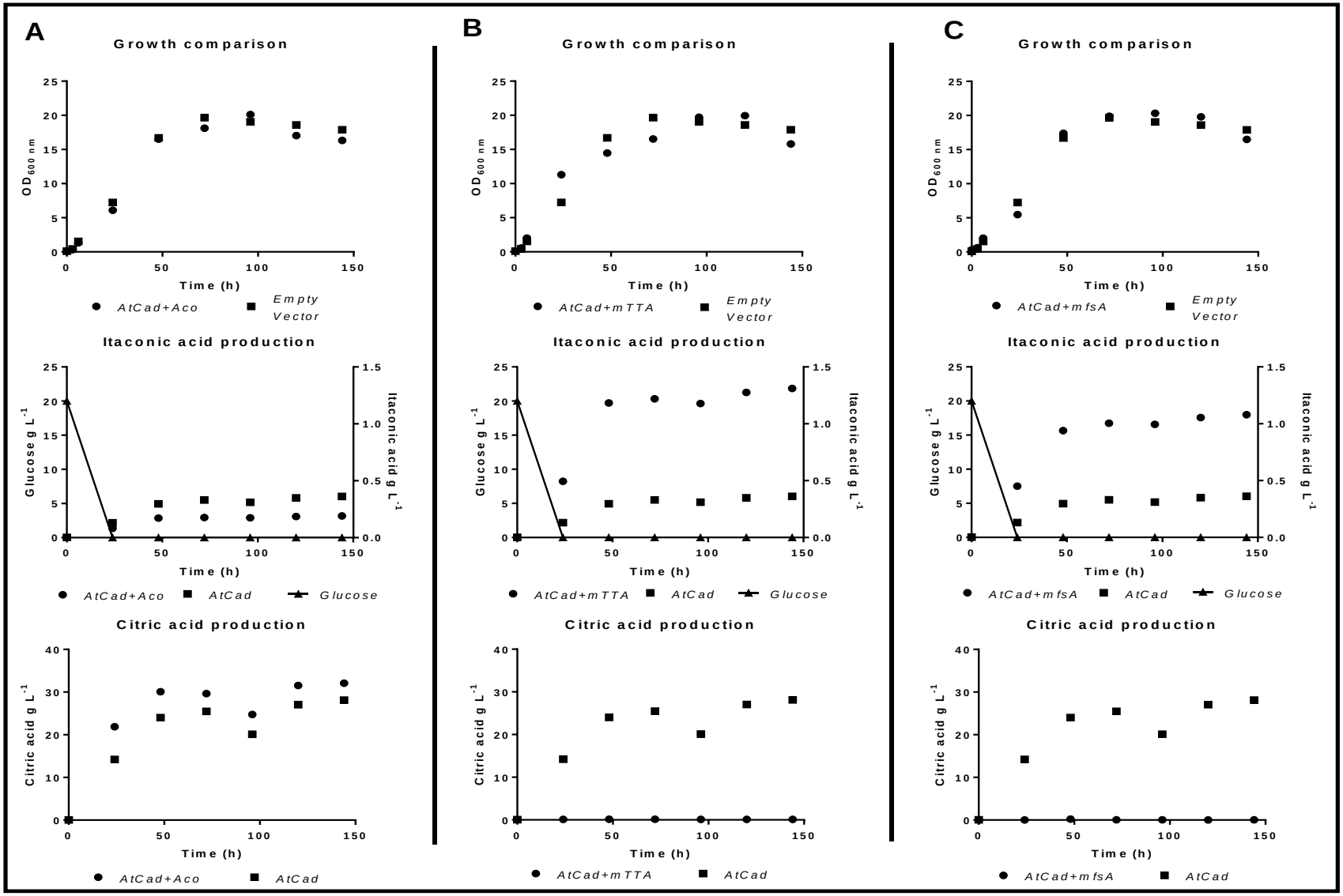

*Figure 15- P*anel. **A-** AtCad+Aco. Panel **B-** AtCad+*MttA* Panel **C-**AtCad+*MfsA* Transformant I.orientalis growth curves and fermentation profiles**.** For each panel, graphics depict OD_{600} of cell culture plotted as a function of time, concentration of itaconic acid (right Y axis) versus concentration of glucose (left Y axis) in g L^{-1} and lastly depicts concentration of itaconic acid (right Y axis) versus concentration of citric acid (left Y axis) in g L^{-1}. All displayed datapoints result from the mean value of 3 individual biological replicates.

4.4 DISCUSSION

The results obtained in this thesis confirm the very high resilience of *I.orientalis* SD108 cells to itaconic acid stress at a low pH, thereby reinforcing previous indicatives [14]. This trait does not seem specific to itaconic acid challenge as *I.orientalis* SD108 cells have also been found to be highly tolerant to acetic and propionic acids (structurally unrelated to itaconic acid) (results not shown) thereby suggesting a generalized increased tolerance of the strain to carboxylic acid-induced stress. The higher resilience could not be correlated with a lower internal accumulation of itaconic acid inside *I.orientalis* cells upon sudden exposure to inhibitory concentrations of the acid, since this accumulation was identical to the accumulation observed in the much more susceptible *S.cerevisiae* cells. This indicates that *I.orientalis* are not *a priori* less permeable to the entry of undissociated itaconic acid molecules, a trait that, having been verified, could explain the higher resilience of this species and which has been observed in other acid-tolerant non-Saccharomyces species [95]. The time-frame used in the accumulation assays was relatively short to elicit cellular mechanism adaptation (30 minutes) and therefore it would be beneficial to perform the assays in a longer timeframe in order to see wether or not *I.orientalis* cells show increased capacity to reduce the internal accumulation of the acid through improved export of the itaconic acid anion. A previous study has demonstrated that the *S.cerevisiae* multi-drug resistance transporter ScQDR3 is essential for tolerance of this yeast species to itaconic acid at a low pH, this being correlated with an involvement of this transporter in reducing the internal accumulation of the acid inside the cells [87]. A BLASTP analysis revealed the presence of putative orthologues of ScQDR3 in *I.orientalis* (Hypothetical protein JL09_g1657) although the identity associated to the alignment was modest (41%). The fact that *I.orientalis* cells are able to excrete itaconic acid to the broth (as herein observed in the fermentation assays performed) also shows that this yeast is equipped with proteins that are able to promote the export of the anion to the external environment. An observation worthy of remark relates to the fact that *I.orientalis* cells seem to exhibit a higher activity (in the range of 5-fold) of the plasma membrane proton pump, in comparison to *S.cerevisiae* BY4741. In the absence of itaconic acid this higher activity is measured in order of 1.5 fold. Exposure to mild concentrations of itaconic acid stimulated the activity of the plasma membrane proton pump in S. cerevisae and in *I.orientalis*, in line with the response described to occur in response to other carboxylic acids [81,96]. For

the highest tested concentration of itaconic acid the *S.cerevisae* plasma membrane proton pump appeared to be much more inhibited than the one of *I.orientalis*. As in *S.cerevisiae*, the genome of *I.orientalis* appears to encode two plasma membrane proton pumps (IoATPase 1 and Hypothetical protein JL09_g1657, sharing 81 and 82% identity with ScPMA1 and ScPMA2, respectively). The apparent higher activity of the proton pump in *I.orientalis* even in unstressed cells could reflect a higher intrinsic activity of these proteins, a higher expression of the encoding genes or a combination of these two factors. Nonetheless, the higher activity of the plasma membrane proton pump exhibited by *I.orientalis* cells provides these cells with a greater capacity to counteract the expected itaconic acid-induced intracellular acidification preventing the associated deleterious effects. Given that intracellular acidification is transversal to carboxylic acid induced stress, the higher activity of the plasma membrane proton pump in *I.orientalis* could underlie the observed resilience of these species to multiple organic acids.

In this work we also demonstrated the potential of *I.orientalis* SD108 a promising organism for the production of itaconic acid in acidic conditions, a trait highly desirable by reducing the costs associated with downstream processing. The titers obtained with non-optimized *I.orientalis* cells (around 400 mg/L) are well above those reported for other yeast species including *S.cerevisiae* BY4741 (reporting up to 59 mg L-1 itaconic acid production [45]) or *Yarrowia lipolytica* (33 mg L^{-1} [97]). Interestingly, during fermentations *I.orientalis* cells did not produced ethanol but rather accumulated citrate (in the range of 23 g L^{-1} when 20 g L^{-1} glucose was provided, this representing an important diversion of the carbon provided. It is not clear why citrate accumulates in the fermentation broth, although this has been largely described in filamentous fungi, namely *A.niger*, the workhorse for industrial production of citric acid [98–100]. Up to now it is not fully understood the reasons why *A.niger* accumulates citrate in the fermentation broth, although it had been suggested that it could result from not being active the usual citrate-mediated inhibition of phosphofructokinase, which means that the accumulation of citrate does not lead to a reduced glycolytic flux [101,102]. In support of this hypothesis, Tevz *et al.* [30] cloned the presumed “citrate-resistant” *A.niger* PFK in *A.terreus* resulting in great improvement of itaconic acid production (45.5 g L^{-1} compared with 21.2 g L^{-1} that were obtained with *A.terreus* cells expressing the wildtype *pfkA*). BLASTP analysis revealed that the predicted PFK encoded by *I.orientalis* is quite dissimilar from the one of *S.cerevisiae* (only 56% identity observed with 723 aminoacid aligned),

however, it is also not similar to the one encoded by *A.niger* (57% identity observed with 710 aminoaid aligned) .

More studies are required to understand this citrate accumulation phenotype, nevertheless, it seems that *I.orientalis* could also be an interesting host for the production of citric acid itself. To improve the production of itaconic acid in *I.orientalis* the over-expression of the putative plasma membrane itaconic acid exporter *MfsA* was devised, as well as that of the mitochondrial transporter *MttA*, which drives transport of cis-acconitic acid from the mitochondria to the cytosol. Both strategies improved production of itaconic acid, although with *MttA* the improvement was considerably higher. These observations strongly support the idea that the itaconic acid biosynthetic pathway in *I.orientalis* is compartmentalized between the mitochondria (where cis-acconitic acid is formed) and the cytosol. Notably, in both cases the improvement in itaconic acid production was accompanied of a drastic decrease in the amount of citrate that was accumulated in the fermentation broth. The increased flux through the itaconic acid biosynthetic pathway may prevent citrate accumulation since the break-up of the TCA cycle (which occurs when cis-acconitic acid is exported from the mitochondria to the cytosol) may result in an increased utilization of the citrate that was accumulating in the cytosol (which afterwards would be secreted to the fermentation broth). A similar effect of reduction in citrate accumulation upon increased itaconic acid biosynthesis was also observed in *A.niger* [19]. One possible avenue of further exploration could be to overexpress both *MfsA* and *MttA*, although previous studies showed that this strategy does not improve upon the titers obtained with the over-expression of *MttA* alone[31,94]. With the experimental setting that was used it seems that itaconic acid production is limited by the amount of carbon source provided in the medium and therefore a next step could be to perform carbon-limited fed-batch fermentations. In summary, this work strengthens the hypothesis of focusing efforts on non-conventional yeast as these exhibit industry relevant traits which still are not mechanistically comprehended or described. This exploration establishes *I.orientalis* as a platform for the production of itaconic acid on basis of its exreme tolerance to this acid in a low pH environment and also its naturally optimized metabolism for the production of citric acid, a precursor of itaconic acid.

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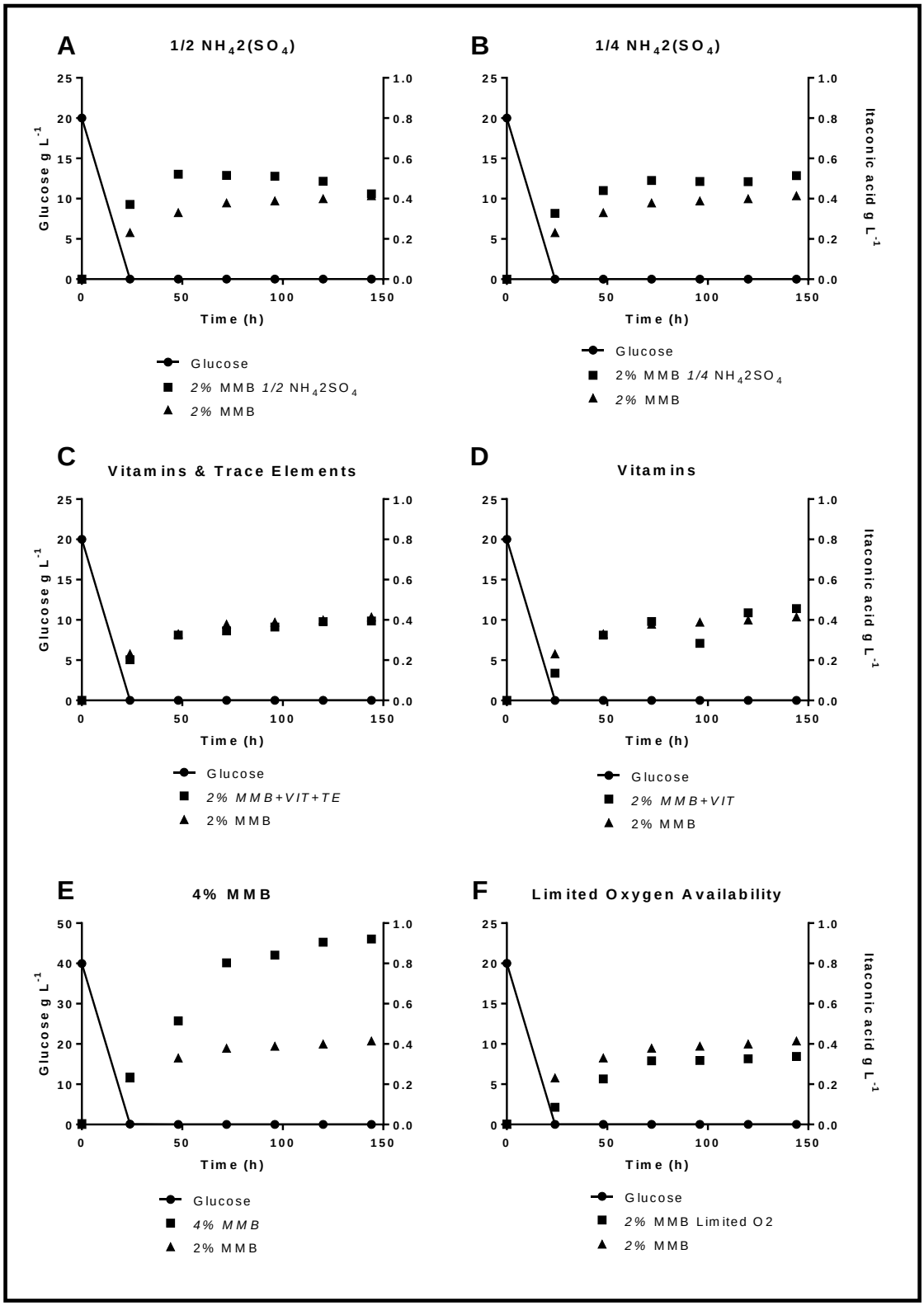
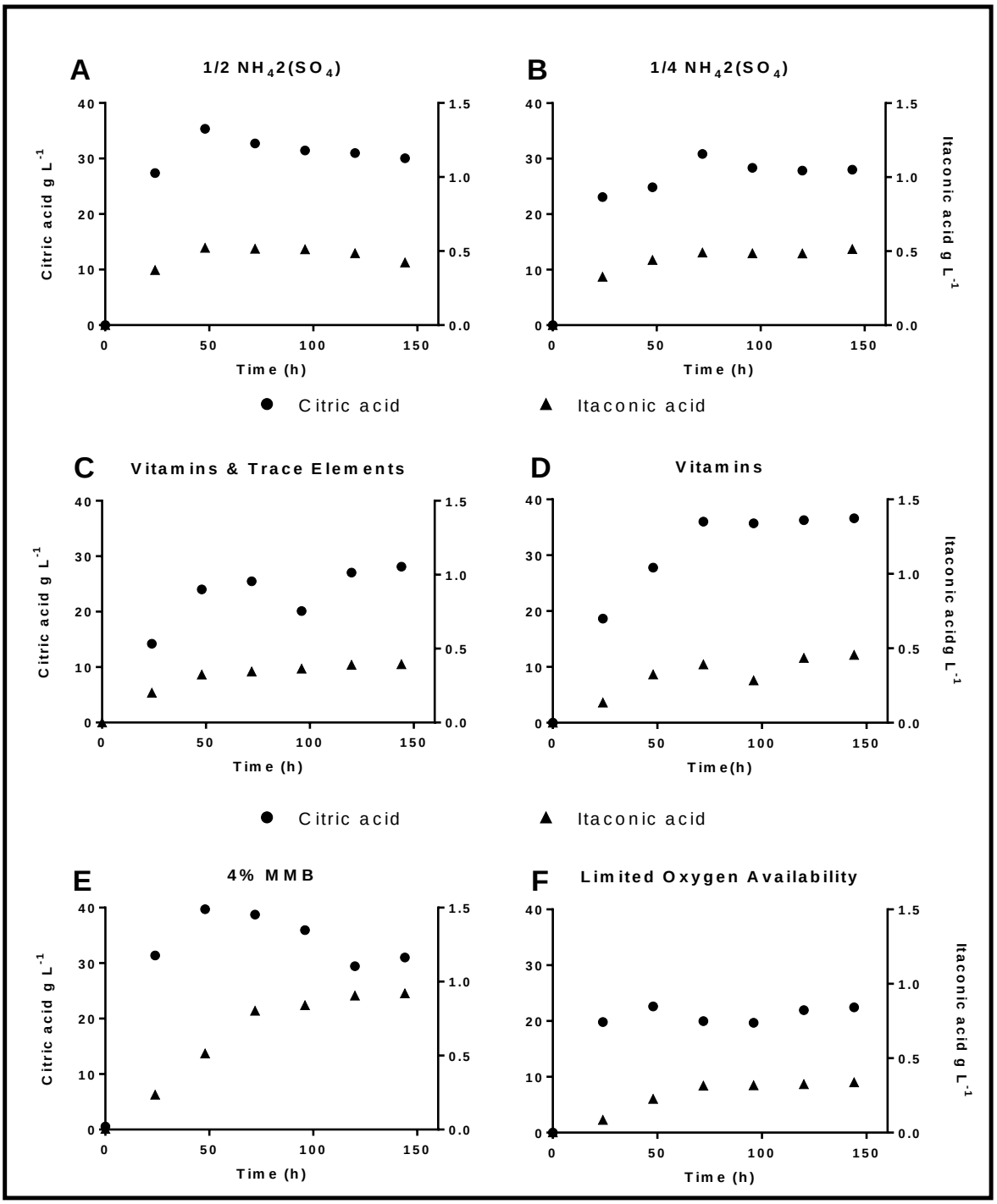
4.6 ANNEXES

Annex 1-Concentration of glucose (circles) versus concentration of itaconic acid in the fermentation broth of I.orientalis harbouring the plasmid which drives AtCad expression using different medium supplementations (squares) in comparison to standard MMB formulation (triangles).



Annex 2- Concentration of citric acid (circles) versus concentration of itaconic acid (triangles) in the fermentation broth of I.orientalis harbouring the plasmid which drives AtCad expression using different medium supplementations (squares).



Annex 3- Table representing the resulting BLASTP pairwise alignments of S.cerevisiae PFK1 and A.niger PFK agains the NCBI deposited Issatchenkia orientalis genomes (taxID:4909)

ScPFK1

	Max score	Total score	Query cover	e-value	Identity
Hypothetical protein JL09_g3803 [*Pichia kudriavzevii*]	1056	1056	98%	0.0	56%

AnPFK

	Max score	Total score	Query cover	e-value	Identity
Hypothetical protein JLG_g3803 [*Pichia kudriavzevii*]	826	826	88%	0.0	57%