Exploration of *Issatchenkia orientalis* as a host for the production of itaconic acid

The high commodity chemicals industry relies on fossil fuel derived molecules to be used as precursors in chemical synthesis. Itaconic acid is a C5 dicarboxylic acid which can be used as a building block for the synthesis of added value chemicals when produced as a bulk chemical. This molecule is originated in microbial metabolism, deriving from cis-aconitate produced in the TCA cycle, rendering it a much greener alternative to fossil fuels. This work reports the exploration and establishment of the non-conventional yeast *I.orientalis* as a platform for the production of itaconic acid through heterologous expression of *A.terreus CAD1* (cis-aconitate decarboxylase). Upon transformation with the plasmid-borne *AtCad* (CAD1) gene, a final titer of itaconic acid of 0.4 g L^{-1} was obtained in 2% glucose MM. An increase in carbon source doubled itaconate titer to 0.9 g L^{-1}. The non-conventional yeast *I.orientalis* was found to display a natural tendency to accumulate citric acid up to 23 g L^{-1} in 2% MM. By separately overexpressing native genes, mitochondrial transporter *MttAIo* and the plasma membrane transporter *mfsAIo* in the *AtCad* transformant strain, itaconic acid titer was increased, with greater relevance for *MttA*, which reached a final titer of 1.3 g L^{-1} in 2% MM. For either overexpression strategy, the pool of citrate was completely depleted.

Key words: biorefineries, non-conventional yeast, itaconic acid, heterologous expression

INTRODUCTION

Recent years have raised awareness towards bio-based production routes starting from raw material to commercial goods, as an alternative to petrochemical derivates chemical synthesis. Looking towards the achievement of ecological and economic sustainability it is crucial to take advantage of lignocellulosic feedstocks for biomass production, as these derive from what is commonly conceived as waste. In particular, organic acid production has taken the centre stage relative to the broad applicability of organic acids for usage as polymer building blocks, as well as for commodity chemical synthesis. In 2004, the United States Department of Energy (US-DOE) produced a report on the top twelve added value chemical platforms to be obtained from renewable biomass as the result of an extensive screening effort, eight of which are identified as organic acids [1].

In 2015, a more specific study was conducted relating to the synthesis and application of organic acids as chemical building blocks, listing the seven market-share wise most promising organic acids which may be obtained in a biorefinery setting [2]. Among these, the C5 itaconic acid is featured as a promising chemical platform due to the presence of two carboxylic acid functionalities and an α-β unsaturated double-bond which make it a relevant precursor for diverse chemical transformations [3] Specifically, itaconic acid is of great interest for the bio-based polymer synthesis industry, namely polyester-based ones with added functionalities such as UV/thermal curing and shape memory polymers. Other applications include detergents, coatings and rubber such as nitrile latex. The most promising application relates to the conversion of IA to methyl methacrylate, also known as Plexiglas, with a potential market of up to 3.2 million tons/year [4].

Presently, itaconic acid synthesis is performed by the fungus *Aspergillus terreus* with highest reported concentration of 86.2 g L^{-1} [5] 150 g L^{-1} [6] for the shake flask and 15 L bioreactor fermentation settings, respectively. In *A. terreus*, the itaconic acid pathway involves the transport of the TCA cycle intermediate cis-aconitate from the TCA cycle to the cytosol, where the cis-aconitate decarboxylase enzyme (*cadA*) catalyses the conversion to itaconic acid. One other relevant itaconic acid producer is *A.niger* which due to its citrate accumulating phenotype has been extensively explored as cell factory for the production of itaconic acid [7–9]. Most recent efforts report a itaconic acid titer of 26.2 g L^{-1} in a heavily engineered strain [9] Itaconic acid production is presently established in fungi, however the process requires further optimization looking to achieve higher final concentrations, identify suitable non-filamentous microorganisms and also to allow the use of low cost sustainable raw materials as a carbon source. Other concerns relate to *A.terreus* associated mycotoxin production, fungal morphology growth related complications in broth rheology and nutrient distribution as well as poor genome tractability of fungi [10]

Looking to overcome these limitations we turned to the model yeast S.cerevisiae with its well described tolerance to low pH's, simple growth morphology and associated available genetic edition tools [11] the suitability of this organism to produce itaconic acid has been the target of different studies performed in the laboratory. Preliminary results revealed the metabolic background of *S.cerevisiae* relevant for itaconic acid production as well as identifying genes involved in determining itaconic acid resistance [12]. Posterior work performed by Vila-Santa *et al.,* [13] reports the successful establishment of *S.cerevisiae* via heterologous expression of *AtCad* and *in silico* guided genetic edition to titers of up to 25 mg L^{-1}. This work also resulted in a list of OptKnock suggested genes for deletion to improve itaconic acid titer which was later explored by Santos *et al.*,[14] resulting in an increase to 68 mg L^{-1}.

Production of organic acids subjects the microorganisms to acidic stress at characteristically low pH's, which may hinder metabolism, in this sense, process-robust strains are required, this coupled with the need of moving to more sustainable substrates has been bringing non-conventional yeasts to the spotlight as these organisms are known for having developed extreme tolerance strategies and ability to consume pentose sugars [15–17] . The novel yeast *Issatchenkia orientalis* has been reported to grow at low pH's [18,19] as well as displaying resistances to several fermentation inhibiting stress factors [16] A work from 2014 reports the usage of this same yeast in the production of succinic acid, while simultaneously establishing the hardy nature of this potential microbial cell factory [20]. The present work reports the successful inclusion of the plasmid-borne *Aspergilus terreus CadA* gene in a uracil auxotroph of the nonconventional yeast *Issatchenkia orientalis* SD108 and assays were conducted to find the optimal MM medium formulation for itaconic acid production. A transporter overexpression strategy was also applied looking to increase production and gain insights on metabolic pathways and compartmentalization. Furthermore, the physiology and traits of this non-conventional yeast have been explored looking to understand the mechanisms underlying *I.orientalis'* high organic acid tolerance.

RESULTS

I.orientalis is higly tolerant to itaconic acid at a low pH.

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 2.5 h) (Table 1) is observed in MM growth medium and also in this medium supplemented with itaconic acid, this being attributable to physiological features of the SD108 strain. Despite this, the higher resilience of *I.orientalis* cells to itaconic acid is evident, this being especially visible at 400 mM of itaconic acid which fully abrogated growth of *S. cerevisae* but only mildly affected growth of *I.orientalis.*

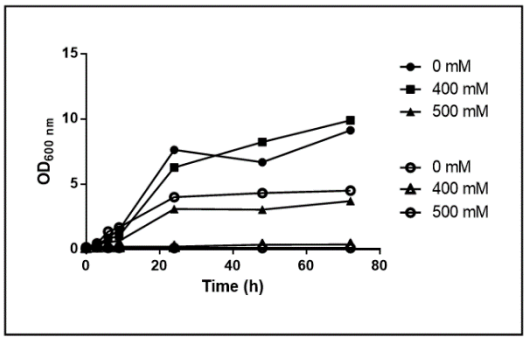

Figure 1. Growth curve comparison of *I.orientalis* SD108 strain (full symbols) and *S.cerevisiae* BY4741 strain (hollow symbols) under exposure to itaconic acid stress in varying concentrations. Each datapoint represents the mean value of three independent replicates. Assay conducted at a pH of 3.5.

The high resilience of *I.orientalis* cells towards 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 [21,22] . 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) (Figure 2),. Under the experimental setting used, these radiolabelled accumulation assays mimicked the experimental setting used in the growth curves shown in Fig 1. The results obtained indicate that, at least upon sudden itaconic acid challenge, no significant differences were observed between the amount of itaconic acid that accumulated inside *I.orientalis* or *S.cerevisiae* cells, at least 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.

Table 1. 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

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.3, 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.3 and Table 2). 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 2). 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 2). 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.cerevisiae* cells show very diminished activity.

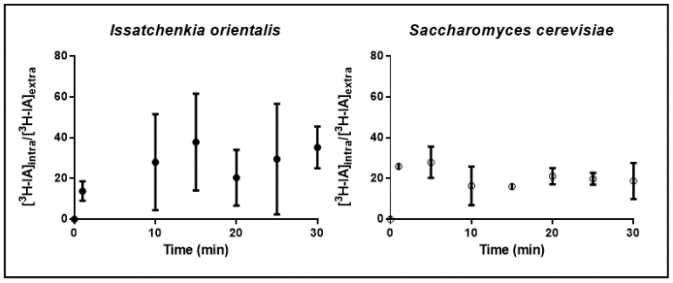

Figure 2. Graphic representation of the accumulation ratio of [H^3]-itaconic acid in *I.orientalis* and S.cerevisiae BY4741 during growth in MMB ph=3.5 supplemented with 350 mM cold itaconic acid. The accumulation ratios displayed in each datapoint are the mean value of 5 independent assays

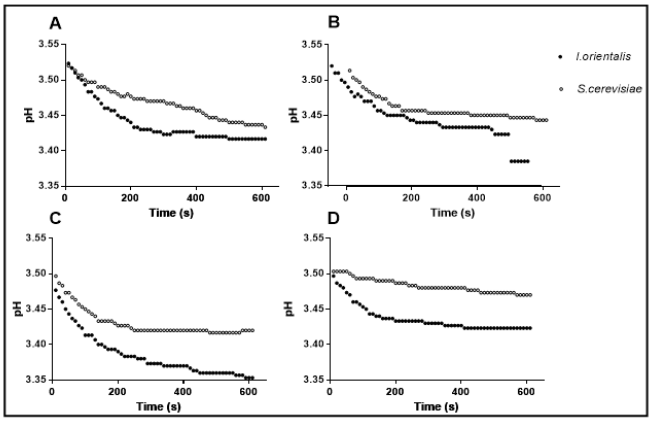

Figure 3. Graphic representation of pH variation over time for *I.orientalis* (closed symbols) and S.cerevisiae (open symbols) during cultivation in MM pH=3.5 under varying supplementations with itaconic acid **A**- 0 mM **B**- 2 mM **C**- 4 mM **D**-8 mM. Each data point displayed results from the mean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

Table 2. 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

Exploration of I.orientalis as a cell factory for the production of itaconic acid

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 a 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 chromatograms 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.5 the profile of citrate accumulation in the culture medium is displayed in comparison with the one of itaconic acid.

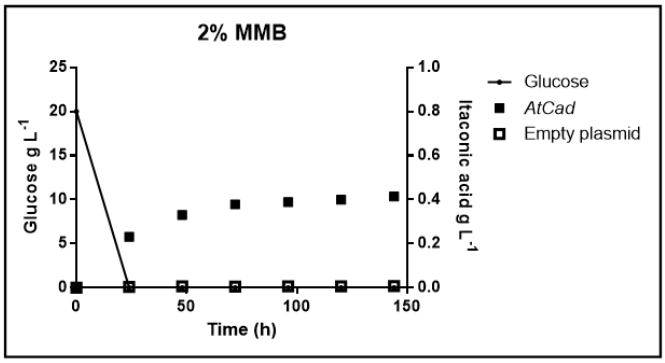

Figure 4- Concentration of glucose (circles) and of itaconic acid (squares) present in the culture supernatant of *I.orientalis* cells transformed with empty plasmid (closed symbols) or with the plasmid that drives *AtCad* expression (open symbols). *Each data point displayed results from the mean value of 3 biological replicates*

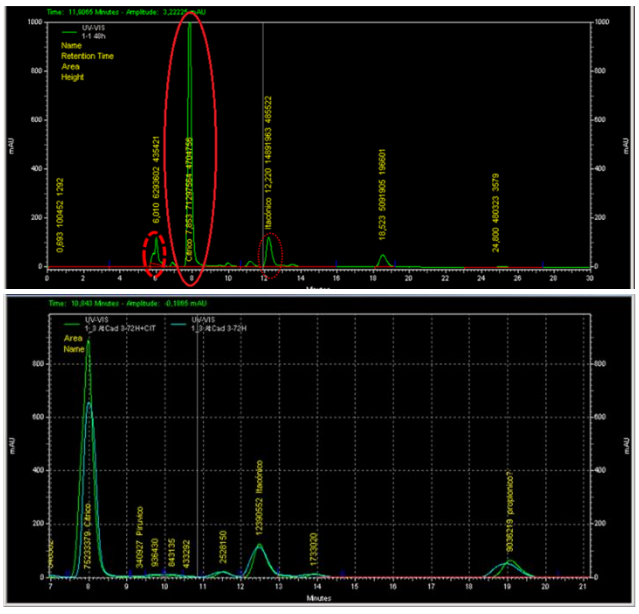

Figure 4. Chromatogram analysis of AtCad transformants fermentation médium supernatant. Upper: accumulation of two C-compounds, one identified as citric acid (line in full) and the other not being recognized by HPLC method calibration (dashed line). Itaconic acid corresponding peak is verified indicating 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*

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 [5,23]. 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 [24]. 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 compared to 0.42), 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).

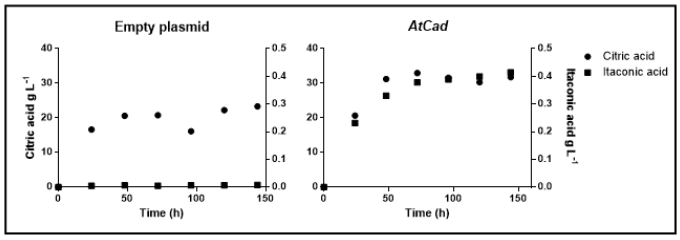

Figure 5. Concentration of citric acid (circles) and itaconic acid (squares) in *I.orientalis* transformants with the empty plasmid or with the plasmid that drives AtCad expression. Citric acid accumulation is verifiable in both transformants fermentation broth.

Table 3. Summary of *I.orientalis*' fermentations in tested MM medium variation growth parameters, concentration of itaconic 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_4SO_4	0.71	23.6	0.42	30.06	2.92	0.81	2
2% MM ¼ NH_4SO_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

Effect of the over-expression of AtmfsA, IoAcoA and AtMttA 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-acconitic acid. As such, it was decided to test the effect of over-expressing the aconitase enzyme as a mean to improve the pool of cis-aconitic acid. Since *A.terreus CadA* is a cytosolic enzyme [25], it was decided to truncate the aconitase by removing the predicted mitochondrial localization signal (comprised by 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-acconitic 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 [26]. A similar approach in *A.*terreus also yielded positive results [27]. 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* concerns with the decrease observed in citric acid accumulation in the fermentation broth.

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-

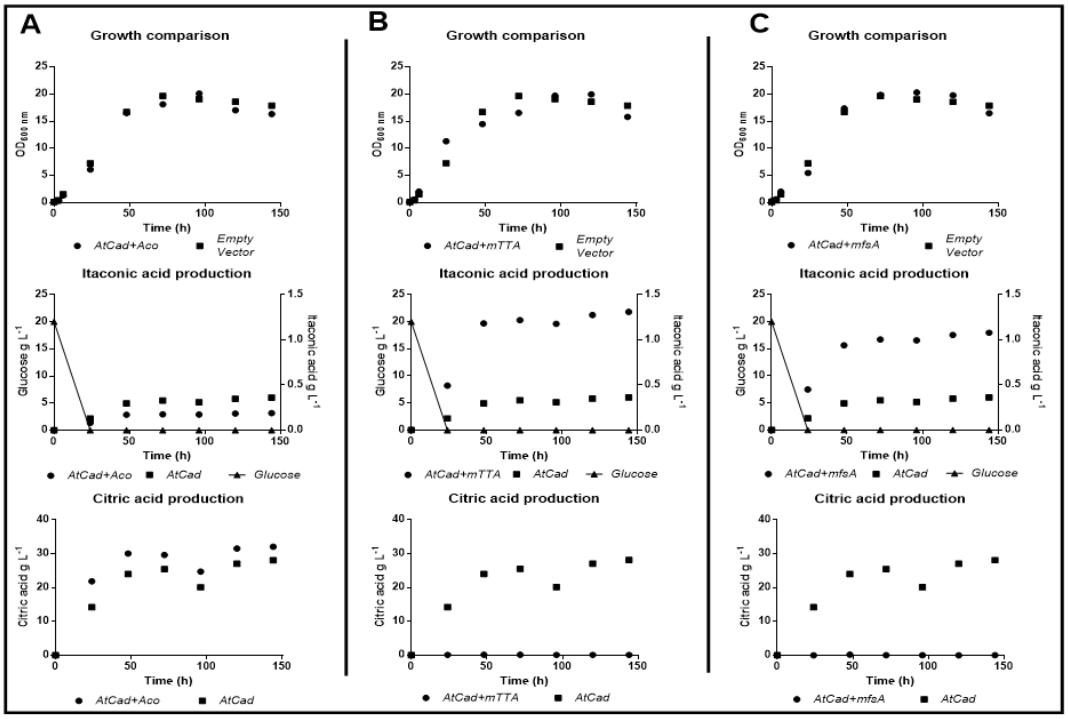

Figure 7. Panel. A- AtCad+Aco. Panel B- AtCad+*MttA* Panel C-AtCad+mfsA Transformant *I.orientalis* growth curves and fermentation profiles. For each panel, graphics depict OD600 of cell culture plotted as a function of time, concentration of itaconic acid (right Y axis) versus concentration of citric acid (left Y axis) 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.

Table 4. Summary of *I.orientalis* diferent transformants fermentation assays. Depicts growth parameters, final itaconic acid production titer, itaconic acid productivity, glucose consumption rate and carbon % yield (g itaconic acid / g glucose).

	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

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 [96]. 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 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 [81,97]. 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} [98]). 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 [99–101]. 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 [102,103]. 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[31,95]. 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.

MATERIALS & METHODS

Strains and growth media

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 who kindly provided the *I.orientalis* strain used in this study.

. **Table 5.** Scerevisiae 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 vitaconic acidmins 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 Vitaconic acidmin medium supplementation solution composition

Vitamins 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 the 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 mfsA gene under the 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 mTTA gene under the 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 Aco gene under the control of the PGK promoter

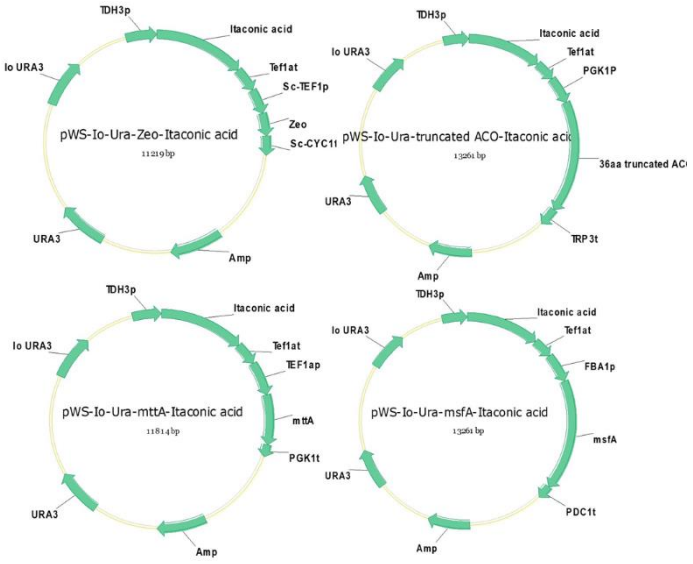

Figure 8. Plasmid maps for the plasmids used in this study based on pWS backbone.Each plasmid possesses selection markers for yeast and bacteria.

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 (OD600 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 MMB plates without uracil to allow for selection by mark complementation. Transformants were selected in solid MMB plates without uracil.

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 OD600 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 OD600 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 [13] 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_4SO_4)	Yeast Nitrogen Base	Aminoacid solution SC Ura$^-$	Vitamin 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 ½NH_4SO_4	20	1.34	1.7	50	-	-
2% MM ¼NH_4SO_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

Itaconic acid susceptibility assays

To assess growth of *I.orientalis* SD108 to inhibitory concentrations of itaconic acid, the cells were cultivated overnight at 30ºC in controlled pH=3.5 MMB 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 MMB 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

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 agitaconic acidtion, 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 1,2 or 4 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.

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 *Saccharomyces 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 MMB 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 orbitaconic acidl agitaconic acidtion (150 rev/min). Culture samples were taken after 1, 5, 10, 15, 20, 25 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 mL/mg dry biomass [39]. 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-Axiopian) in connection with CoolSnap fx (Photometrics) with 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 organelle and *a* to its width. This aproach has been previously used by Arango et al., to determine the celular volume of Candida tropicalis cells [40]. 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 30 measurements this procedure resulted in an estimated medium volume for *I.orientalis* cells corresponding to 120.93 µm3. It is worthy to note that the obtained volume is correspondent to the diploid growth stage and is higher than that of S.cerevisae whith a corresponding 83 µm3 [41]. Having obtained the individual celular volume we then proceeded to calculate the total internal volume (V_i) for the whole cell culture. This was performed using each individual assay culture OD_{600} and converting this to number of cells under the general assumption applied to most yeasts that is OD_{600}=1 corresponds to 10^7 cells.

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