Improvement of yeast-based production of itaconic acid guided by in silico metabolic modelling

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Abstract

Itaconic acid (IA) is a dicarboxylic acid considered a promising building block that can be used in a wide range of industrial applications. In this work the yeast *Saccharomyces cerevisiae* BY4741 was used as a cell factory for the production of IA. Transformation of yeast cells with the pGAL1-AtCad1 plasmid, which drives expression the cis-acconitic acid decarboxylase cadA, enabled production of IA up to 65 mg/L. Together with itaconic acid it was observed a concomitant production of a given by product, this representing the main competitive pathway of IA biosynthesis. Based on this observation and on other evidences, it is proposed that the biosynthesis of IA in yeast cells does not require mitochondrial activity. The use of a strain devoid of pyruvate decarboxylase activity as a host for the production of IA resulted in a high production of IA. The improved performance of this strain was linked with a very reduced production of the above indicated by-product suggesting that this strain has an optimized metabolism towards IA biosynthesis. Using in silico metabolic modeling several candidate gene deletions were identified as promising strategies to improve IA biosynthesis in the BY4741 background. At least one of the deletion mutants identified exhibited a 4% increase in IA production and 14% increase in productivity, comparing with the levels attained in the wild-type strain.

Key-words: itaconic acid, S. cerevisiae, AtCad1, FBA, metabolic model
Introduction

Currently, world energy requirements are mainly fulfilled by fossil fuels. However, the limited deposits of fossil fuels, the increase in energy demand and the associated environmental problems, are forcing the society to find sustainable alternatives to the petrochemical industry. In particular, it is being searched new molecules that could be obtained based on renewable carbon resources and that could be used by the industry as precursors for chemical synthesis in alternative to molecules that are derived from oil or that are obtained from oil cracking [1]. Compared to chemical synthesis, microbial fermentation offers several advantages such as lower price, reduced impact in the environment and increased sustainability; these being factors that could, theoretically, lead to a progressive reduction in the utilization of chemicals derived from petrochemical industry [2]. Carboxylic organic acids are among the molecules considered to pose more important alternative as building block molecules due to their chemical versatility. The majority of the interesting organic acids are also part of microbial metabolic pathways and therefore can be obtained at relatively high purity by microbial fermentation. The present thesis is focused on the production of itaconic acid (IA), one of the most exciting and also more thoroughly studied (specially in the last years) organic acids. IA is a white crystalline unsaturated dicarboxylic acid having one carboxyl group conjugated to the methylene group. IA is used worldwide in the industrial synthesis of resins such as polysteres, plastics, and artificial glass and in the preparation of bioactive compounds in the agriculture, pharmacy, and medicine sectors [3]. The number of molecules that can be obtained from IA is very large and the fields where this organic acid can be used.

The work described in the present thesis follows previous studies undertaken at the BSRG group in which itaconic acid production has been successfully implemented in S. cerevisiae [4][5]. For this, the AtCAD1 gene from Aspergillus terreus was cloned under the control of a galactose-inducible promoter (GAL1) yielding plasmid pGal-AtCad1. Yeast cells transformed with pGAL-AtCad1 were found to produce titers of itaconic acid around 10 mg/L. In these previous works it was also characterized the influence exerted by some experimental parameters that could affect itaconic acid production by yeast cells including the size of the inoculum and the genetic background of the strain used [4]. Using in silico metabolic modeling several mutant strains were identified as candidates to produce higher titers of itaconic acid, preliminary results showing that at least 9 of these (Δacs1, Δadh6, Δadh7, Δifa38, Δpot1, Δhnf2, Δynl1) produced more itaconic acid than the parental strain, the improvements ranging between 17% and 145%. In the work undertaken by Vila Santa et al, (2015) it was also explored the S. cerevisiae A strain, a strain previously demonstrated to be a particularly good host for the production of carboxylic acids [6][7], as a potential host for the production of itaconic acid as it produced about 36-fold more itaconic acid than wild-type cells. Despite this, on the one hand the overall the yields and titers of itaconic acid produced by yeast cells remained low, especially when compared with those achieved by other hosts. It was thus clear that there was still a significant margin of improvement for this process and the main goal of the work described in this thesis aimed at that. In the first part of the work it was performed a thorough characterization of the production of itaconic acid undertaken by yeast cells leading to the identification of metabolic pathways involved in the production of this organic acid and also pinpointing competitive pathways that may be contributing for carbon diversion. The effect of various nutrients in the production of itaconic acid was also examined including the concentration of carbon, nitrogen, vitamins and trace elements; in an attempt to optimize the best culture medium to be used in larger-scale fermentations. Finally, a new approach exploring FBA and several optimization algorithms was undertaken aiming to accurately simulate the yeast metabolic network and thus identifying gene deletions enhancing itaconic acid production in yeast.

Materials and Methods

Strains and Growth Media

Yeast strains used in this work were derived from the Euroscarf collection, as described in table 1. A strain was kindly provided by Prof. Antonius van Maris from Delft University of Technology. The yeast strains were cultivated in different growth media, depending on the application. YPD growth media, used to propagate the strains and for cultivation, contains 20 g/L of glucose (Merck), 20 mg/L yeast extract (Difco) and 10 mg/L peptone (Difco). The MM8 medium, used to select yeast transformants, contains 20 g/L glucose(Merck), 1.7 mg/L Yeast Nitrogen Base without aminoacids and ammonium sulphate (Difco) and ammonium sulphate 2,65 mg/ml (Merck). To supplement BY4741 auxotrophies MM8 media was supplemented with 20mg methionine 20 mg histidine and 60 mg leucine. MMFgal, growth medium, used for fermentation assays, contains: 20 g/L glucose (Merck), 1.7 mg/L Yeast Nitrogen Base without aminoacids and ammonium sulphate (Difco), 2,65 mg/L ammonium sulphate (Merck), 2 g/L potassium phosphate and a solution of aminoacids, trace elements and vitamins, having the composition that is described in table 2. When needed MMFgal medium was used, in which galactose was exchanged by glucose. Cultivation of Escherichia coli XL1-Blue was made in rich LB growth media. For plasmid selection 150 mg/L of ampicillin was added to the LB medium. Solid MMB, YPD, and LB medium were obtained by supplementing the corresponding liquid growth medium with 2% agar (iberagar).

Yeast transformation

The pGal1-AtCad1 plasmid was used to transform S. cerevisiae BY4741 cells using the Alkali-Cation TM Yeast Transformation kit (MP Biomedicals). For this, the cells were cultivated until mid-exponential fase (OD600 nm=0.4) 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 then centrifuged again using the same conditions described above. The
pellet was resuspended in 2.5 mL of Lithium/Cesium Acetate solution and the obtained suspension was then incubated for 30 minutes at 30°C with 100 rpm agitation. The cells were centrifuged as described above and finally resuspended in 500 μL of TE (pH 7.5) to obtain competent cells. Each transformation mixture used 100 μL of competent cells, 1.5 μL of purified plasmid, 5 μL of carrier DNA and 5 μL of histamine solution. The mixtures were 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 30°C. Cells were then heat shocked for 10 minutes at 42°C and subsequently cooled to 30°C. The reactions mixtures were centrifuged at 8000 rpms for 3 minutes and cells were resuspended in 100 μL of YPD and plated on MMB plates lacking uracil.

**Batch fermentations for the production of itaconic acid in yeast cell using strains derived from the BY4741 background**

To assess itaconic acid production the yeast strains (derived from BY4741 and 2344c backgrounds) harboring the Atcad1 plasmid were cultivated overnight in 250 mL shake flasks containing 50 mL MMF<sub>Glucose</sub> medium (containing 2% glucose), at 30° and 250 rpm. On the next day, an appropriate volume of the culture was re-inoculated in fresh MMF<sub>Glucose</sub> medium yielding a cell suspension with an OD<sub>600nm</sub> of 0.1. This culture was incubated at 30°C and 250 rpm until OD<sub>600nm</sub> reached 0.8 after which a new re-inoculation was performed in MMF<sub>Galactose</sub> and aiming to obtain an initial OD<sub>600nm</sub> of 0.1. The cultures were incubated at 30°C with orbital agitation (250 rpm) during 7 or 14 days. During this period 1mL-aliquots of the culture supernatant were collected and OD<sub>600nm</sub> measured every 24h. The accumulation of metabolites of interest in the culture broth was measured by HPLC separating 10 μL of the supernatant in an Aminex HPX- 87H column (Biorad) eluted with 0.05% sulfuric acid at a flow rate of 0.6 mL/min. 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. Under the experimental conditions used the retention times for the following compounds were obtained: citric acid- 8.5 min, pyruvic acid- 9.5 min, succinic acid-11.3 min, itaconic acid-12 min, glucose -8.7 min, galactose-9.9 min, glycerol-13.3 min and ethanol-20.5 min.

**Batch fermentations for yeast-based production of itaconic acid using the A strain**

Cells of A strain harboring the pAtCad plasmid were cultivated under the same conditions described above for BY4741 background with the difference that the initial precultures were performed for 3 days in MMF<sub>Glucose</sub> after which these were re-inoculated in MMF<sub>Galactose</sub> at an initial OD<sub>600</sub> of 0.1 for 5h before inoculation in MMF<sub>Galactose</sub>.

**Reactor fermentations for yeast-based production of itaconic acid**

To access production of itaconic acid production in reactor fermentations, BY4741 and A strains harbouring the pAtCad1 plasmid were cultivated overnight in a shake flask containing 125 mL of MMF<sub>Glucose</sub>. On the next day a necessary volume of this pre-inoculum was used to inoculate fresh MMF<sub>Glucose</sub> medium at an initial OD<sub>600mm</sub> of 0.1. Experiments were undertaken, during 14 days, in a 1 L-capacity reactor. During this period, 1mL-aliquots were taken and OD<sub>600mm</sub> measured every 24h. Reactor dissolved oxygen (DO) was controlled by the air flow (maintained between 0.1 and 0.75vvm) and the agitation was maintained between 150 and 750 rpm, in a cascade loop control. Temperature was controlled and maintained at 30°C using a heating jacket and continuously recirculating cold water. Conditions such as agitation, pH, DO and temperature were monitored using Labware software. The measurement of accumulation of metabolites in the fermentation broth was performed by HPLC, as described above for batch fermentations.

**Simulation of yeast metabolism in itaconic acid-producing cells**

In silico simulation of yeast metabolism was performed using COBRA toolbox and MATLAB R2016a software. The metabolic model used was Yeast 5.0, the SBML file being downloaded from http://yeast.sourceforge.net/. In order to enable production of itaconic acid by the stoichiometric model, the following reactions were added: cис-aconitate transport to cytosol, cis-aconitate decarboxylase and itaconic acid export. An additional sink reaction for itaconic acid was provided to preserve the maintenance of mass balance. Simulation experiments were carried out with a glucose maximum uptake rate of 10 mmol/gDCW/h. No additional constraints were applied to the stoichiometric model. Afterwards, OptKnock, OptGene and RobOKoD frameworks were used to suggest possible gene knockout strategies that could improve itaconic acid production in BY4741 and A strains.

**OptKnock**

OptKnock algorithm was available for COBRA toolbox and it was implemented in the MATLAB environment. The constrOpt structure was used to constraint the glucose and itaconic acid sink reactions which were set to 10 and 0 mmol/GDCW/h, respectively. OptKnock selectedRxnList function encompasses all possible genes allowed to be deleted by OptKnock algorithm. In the simulations performed, all reactions were allowed to be deleted with the exception of growth and itaconic acid production reactions. To acquire multiple single gene deletions, several runs of OptKnock were performed. Additionally, at each run, the solution given by the previous run (included in selectedRxnList) was simultaneously deleted, avoiding getting the same solution.

**OptGene**

OptGene algorithm was used in the OptFlux platform (freely available at http://www.optflux.org/). All the reactions associated with itaconic acid production, were provided to the Yeast 5 metabolic model. The objective function value was calculated using minimization of metabolic adjustment (MOMA) and the glucose maximum uptake rate was constrained to 10 mmol/GDCW/h.
RobOKoD associated scripts were downloaded from http://journal.frontiersin.org/article/10.3389/fcell.2015.00017/full and applied in the MATLAB environment. The maximum glucose uptake rate was set to 10 mmol/GDCW/h.

The itaconic acid production titer obtained upon the gene deletions suggested by OptKnock, OptGene and RobOKoD were further examined using a maxBm/flux objective function. All the scripts were obtained from https://github.com/rs-costa/ObjComparison. Afterwards, a flux-by-flux comparison was performed and the predicted mutants with increased itaconic acid flux were selected. Mutants that were unable to grow were not considered.

**Results**

To obtain insights into how the production of IA undertaken affected metabolism of *S. cerevisiae* cells, fermentations were undertaken along a period of 14 days in shake-flasks and using BY4741 cells transformed with the pGAL-AtCad1 plasmid. As said above, this plasmid yields expression of cis-aconitic decarboxylase enzyme which allows conversion of cis-aconitic acid in itaconic acid. In previous works the fermentations were stopped after 3 or 7 days and the increase in time could provide a clearer picture on the set of byproducts that could be formed together with IA along fermentation. The fermentations were performed in MMF medium supplemented with galactose following the same experimental setup used before [4]. The cells used as inoculum were cultivated in MMFgal, supplemented with glucose, so that AtCad1 expression could only be triggered upon inoculation in the MMFgal broth. Such strategy allowed a tight control over AtCad1 expression providing a more thorough understanding of how the production of itaconic acid affected the overall yeast physiology. The results obtained are schematically represented in figure 1. After 7 days of fermentation 32 mg/L of itaconic acid was measured in the broth, which is a value that is in agreement with those reported before [4]. Itaconic acid production increased constantly during 12 days until it finally reached a plateau of 65 mg/L of itaconic acid. The consumption of galactose decreased steeply with the increase in itaconic acid production sustaining this carbon source is being consumed to produce itaconic acid biosynthesis (Fig.1). Nevertheless, since the production of itaconic acid kept increasing after 5th day of fermentation, the time at which galactose was exhausted from the growth medium, other carbon sources are also used by the yeast cells to produce itaconic acid. The yield obtained was 3.51±0.71 mg of itaconic acid/gram of galactose. In the previous study that has also explored *S. cerevisiae* for the production of itaconic acid reported a titer of 60 mg/L of itaconic acid after 3 days, which is roughly the same that it was obtained in this work after 14 days. The different strengths of the promoters used to drive AtCad1 expression as well as the different compositions of the growth media used for the fermentations could underlie the differences observed.

The production of other fermentation products was followed by HPLC including X, Y and Z. The results obtained showed a very high concentration of X in the broth, reaching a maximum of 120 mM in the 5th day and decreasing to 80 mM after 14 days. Y and Z were also produced achieving a maximum titer (of 1.6 mM and 0.7%, respectively) by the 5th day of fermentation and being almost fully exhausted from the media after 14 days. The decrease in the concentration of Y, Z and, less significantly, of X was followed by the depletion of galactose (occurring at the 5th day). This observation indicates after galactose is exhausted, itaconic acid production is sustained at the expense of X, Y and Z catabolism. The production of X followed the same pattern as the production of itaconic acid suggesting that it is competing with itaconic acid production and therefore representing an important diversion of the carbon source provided, especially because the concentrations present in the broth were relatively high compared with the Y concentrations. A similar elevated production of X was also observed in fermentations carried out using a plasmid that drives AtCad1 expression from the constitutive promoter TEF1 (results not shown), this indicating that the phenomenon was independent of the carbon source used. Since A cells were previously shown to produce itaconic acid titer at levels above those obtained for the wild-type strain, we have monitored fermentations undertaken by these cells for 14 days. In the end of the fermentation, ~400 mg/L of itaconic acid were detected in the broth of A cultures (Fig.2), which corresponds to an increase of about 7 fold the levels achieved by the wild-type strain. No Z was detected in the broth as expected since the strain lacks the pyruvate decarboxylase complex (results not shown). Also in line with previous observations, Y accumulated in the medium of the A cultures up to 4.2 mM (369 mg/L, well above the titer obtained for the wild-type strain) as well as X although the titers obtained were much smaller than those obtained for the wild-type strain (20 compared to ~140 mM), and it was followed by an abrupt decline after that period. Previously was demonstrated [9] that fermentations undertaken by the A strain produced 130 g/L, comparing to ~400 mg/L produced herein. Apparently under the conditions that we have used there was some consumption of Y by the A strain since the concentration decreased after achieving a peak at the second day of fermentation.

The consumption of galactose in the A strain was very slow only being detectable a significant decrease in the initial concentration at the third day of the fermentation and leaving about half of galactose initially provided in the broth after the 14 days of fermentation. The overall yield of the production undertaken by the A strain was of 35.23±1.88 mg/g of itaconic acid, comparing with 3.51±0.71 mg/g previously obtained for the BY4741 strain, representing a 10-fold increase.
Yeast-based production of itaconic acid is improved in pH-controlled fermentations

The accumulation of very high concentrations of X in the acidic fermentation broth, similar to those present in the culture broth of the BY4741 strain, has been described to be very toxic for the yeast cells compromising their viability [9][10]. Taking this into account, it was tested whether the production of itaconic acid by yeast cells could be improved by having the cells cultivated in fermentations with a pH set at 4.5. This pH value is near the pKa (4.75) of X thereby decreasing the concentration of the toxic undissociated acid form. To do this the fermentations were performed in 1L-capacity reactors with pH control, in collaboration with the group of Professor Frederico Ferreira from the Institute of Bioengineering and Biosciences. All the other experimental conditions used were similar to those used in shake flask fermentations. As a control, non-pH controlled fermentations in the 1L-bioreactor were also performed. The results obtained show that maintenance of the fermentation broth at the pH of 4.5 increased the titers of itaconic acid produced by BY4741 cells by about 2 fold (Fig.3), comparing with the titers obtained in non-pH controlled fermentations. This improvement was linked with a higher viability of the fermenting cells in the pH-controlled fermentations (results not shown), consistent with the idea that the accumulation of X in the broth is indeed exerting a toxic effect. In comparison with the results obtained in shake flask fermentations, the utilization of the bioreactor improved the yield from 3.51 mg/g (obtained at the 7th day of fermentation undertaken in the shake flask) to 4.9 mg/g. It was also tested the performance of the A strain in producing itaconic acid in the bioreactor (Fig.3), although in this case the concentration of X present in the broth was smaller and therefore a lower toxic effect of this acid was anticipated. Buffering of the pH in the case of the A culture improved the titer of acid produced, although not was as much as in the BY4741 strain. This increase in the titer produced could be attributable to a faster consumption rate by the cells in the pH-buffered fermentation. In fact, in the pH-buffered fermentation galactose was fully exhausted from the medium while in the non-pH buffered fermentation a significant amount was still left (Fig.3), these observations suggesting that the acidic conditions could perturb sugar uptake by these cells. The cultivation in the bioreactor did not improved the yield of itaconic acid production 28 mg itaconic acid/g of sugar was obtained comparing with 35 mg/g obtained in shake-flask fermentations.

Effect of nutrient availability in yeast production of itaconic acid

The simplification of the feed is a way by which the cost of biotechnological production processes could be reduced, specially of those in which the cost of the raw materials has an important contribution in the overall profit that can be obtained, as it is the case of itaconic acid [11]. In that sense, in this part of the work it was examined the effect of manipulating the concentration of several nutrients in the fermentation medium including the carbon source, the nitrogen source and the presence of vitamins and trace elements. For this fermentations were performed using BY4741 cells transformed with the pAtCad1 plasmid and cultivated in MMFgal or in this same growth medium having: i) a duplicated concentration of NH4SO4 (5.3 g/L); ii) a duplicated concentration of CSM (500 ml/L), used as the source for amino acids; iii) a duplicated concentration of NH4SO4 (5.3 g/L) and of CSM (500 ml/L); iv) without ammonium sulphate; v) without CSM (but providing the amino acids for which the BY4741 strain is auxotrophic to (250 ml/L each); vii) without vitamins and trace elements. The results obtained are summarized in figure 4. The results obtained show that doubling the concentration of CSM alone or together with ammonium sulphate improves the final titer of itaconic acid to 59.8 mg/L and 58.5 mg/L, respectively; comparing with 35.1 mg/L that are produced when only MMFgal medium is used. Removal of CSM or of trace elements and vitamins from the culture medium impaired production of itaconic acid by hampering yeast growth. The duplication of ammonium sulphate concentration did not resulted in improved production of itaconic acid but its removal from the growth medium slightly improved the production of the acid. On the overall these results suggest that during fermentations for the production of itaconic acid yeast cells are mainly using the amino acids present in CSM as nitrogen sources for which the manipulation of this nutrient concentration is the only one affecting the production of
the acid. The doubling of CSM concentration considerably enhanced galactose consumption, this being attributable to the faster growth rate that is observed. Manipulation of ammonium sulphate, vitamins and trace elements concentration did not result in significant modifications of itaconic acid production. The second nutrient of the MMF medium that was manipulated was the carbon source. In specific it was examined the ability of yeast cells to produce itaconic acid in growth media containing solely galactose (2%), solely glucose (2%) or an equimolar mixture of the two sugars (1% each). For this, both the BY4741 and A strains were used. The production of itaconic acid based on crystalline sugars has a very high cost, which increases even more in case of galactose. Nevertheless, galactose and glucose are often found present in several organic raw materials such as lignocellulosic or beet molasses and therefore the production of itaconic acid using the pAtCad1 plasmid could be an interesting strategy. As expected, cultivation of the two strains in the glucose-supplemented growth medium led to residual levels of itaconic acid, this being attributable to the repression of the cadA gene from the pAtCad1 plasmid. In the case of the A strain this observation was unexpected since this strain had been described to have an alleviated glucose repressive effect and therefore it was expected that the AtCad1 enzyme could still be expressed in this genetic background, albeit at low levels. When the cells were cultured in a medium containing the two sugars, both strains were able to produce itaconic acid, although at lesser extent than it was demonstrated when cells were cultured in MMF containing 1% glucose and 1% galactose, the BY4741 strain produced a maximum titer of 26 mg/L whereas the A strain achieved 208 mg/L.

Metabolic engineering strategies to boost production of itaconic acid in yeast cells

In this point of the work it was attempted to use metabolic engineering strategies to improve the production of itaconic acid in yeast, either using the data described above (or gathered from previous studies) or using in silico metabolic modeling. The first attempt that was made was to use a strain devoid of the B gene. B encodes a transcriptional repressor which controls the overall glucose repressive effect in S. cerevisiae [13]. In the presence of glucose B is translocated to the nucleus where it binds to the promoter region of genes required for metabolism of alternative carbon sources including genes involved in glycolysis, in tricarboxylic acids cycle, in fermentative and in the glyoxylate shunt and in gluconeogenesis, among others [14]. There were three main reasons to test the ability of BY4741 ΔB strain to produce itaconic acid: i) on one hand it could be a way to implement production of itaconic acid using the pAtCad1 plasmid in media containing glucose as the sole carbon source; ii) the alleviation of glucose repression could contribute to improve the expression of enzymes involved in the biosynthesis of precursors of itaconic acid, namely of citrate synthase and aconitase which are enzymes of the Krebs cycle that are required for synthesis of cis-aconitic acid; iii) the better performance of the A strain in producing organic acids was suggested to result from this strain having an alleviated glucose repression [8] and the deletion of B could mimic this. Deletion of B gene in BY4741 background impaired production of itaconic acid when glucose was used as the sole carbon source, presumably because the deletion of this transcription factor was not enough to alleviate the repression of the pAtCad1 plasmid (not shown). Cultivation in the presence of galactose enabled production of itaconic acid but this was less favorable in the ΔB strain than in the parent strain BY4741. Comparing with the titers obtained in 2% galactose (48 mg/L, as shown in Fig.5), cultivation in growth medium having 1% galactose and 1% glucose improved production of itaconic acid by ΔB cells (58 mg/L, as shown in Fig.6) and this effect was even more prominent when cultivation was undertaken in a growth medium having 0.5% of each sugar (85 mg/L, as shown in Fig.6). In the case of the BY4741 strain the production of itaconic acid decreased with the decrease of galactose availability in the medium. Altogether, these results suggest that using ΔB cells in a medium having a limiting concentration of galactose and glucose could be a strategy to boost itaconic acid production in yeast.

![Figure 4](image-url)  
**Figure 4:** (Left) Itaconic acid concentration in supernatant of cultures of By4741 cells harboring the pGAL1-AtCad1 plasmid along 7 days of fermentation in the different variants of MMF medium: without CSM (●), twice the ammonia (▲), without ammonia (■), twice the ammonia and CSM (◆), twice the CSM (□) and without trace elements and vitamins (○); (Right) Galactose concentration in supernatant of cultures of BY4741 cells harboring the PGAL-AtCad1 plasmid along 7 days of fermentation in MMF medium variants: without CSM (●), twice the ammonia (▲), without ammonia (■), twice the ammonia and CSM (◆), twice the CSM (□) and without trace elements and vitamins (○).

![Figure 5](image-url)  
**Figure 5:** (Left) Growth curve of the the wild-type (●) and By4741ΔB (○) cells harboring the pGAL1-AtCad1 plasmid in different MMF variants; (Right) Concentration of itaconic acid in supernatant of cultures of BY4741 (●) and BY4741ΔB (○) cells harboring the pGAL1-AtCad1 plasmid along 14 days of fermentation in MMF fed with 2% galactose.
Metabolic modelling

A second approach that was used for the metabolic modeling was the in silico simulation of the yeast metabolic network during production of itaconic acid. For this, FBA analysis was undertaken as described (in materials and methods) using the Yeast 5.0 model which was modified to include reactions yielding production of materials and methods) using the Yeast 5.0 model. The approach that was followed in this work differed from the one used previously in Vila-Santa et al., (2015) because a new optimization algorithm was tested (RobOKoD, while in Vila-Santa et al., (2015) only OptGene and OptKnock were used) and because an iterative strategy of deletions was employed to analyze the results given by the three optimization tools (OptGene, OptKnock and RobOKoD). The computational utilization of the 3 different frameworks resulted in a total of 67 (data not shown) genes whose deletion is suggested to augment the production of itaconic acid. This list of genes was ranked accordingly to their respective CAD flux, using a maxBmi/Flux objective function (maximization of biomass per flux unit, yielding a total of eight strains (listed in table 6) that were considered to be more interesting to be further tested. Most of these solutions correspond to genes related with mitochondrial function. It is important to stress that the herein followed approach obtained solutions differed considerably from those obtained in the study of Vila-Santa et al., (2015) since in this work the flux attributed to the CAD reaction (the one that yields production of itaconic acid from cis-acconitic acid) could not be detected because the FBA was being maximized for biomass and therefore there was no carbon left available for reactions not involved in biomass production such as it is the case of CAD reaction. Differently, in this work growth and CAD fluxes were estimated using an alternative objective function (maximization of biomass per flux unit), which better describes the metabolism of cells enabling flux quantification of biomass and itaconic acid related reactions.

The 5 mutant strains were cultivated in MMF medium together with the wild-type strain BY4741 for 14 days, as described above. The results obtained showed that only the deletion of G led to an improvement in the titer and production rate of itaconic acid, comparing with the wild-type strain (Fig.7).

Discussion

In the first part of this thesis a characterization of itaconic acid production undertaken by S. cerevisiae BY4741 cells was performed. The results demonstrated that transformation of the yeast cells with the galactose-inducible plasmid pGal1-AtCad1 enabled production of ~65 mg/L of itaconic acid after 14 days of fermentation. This final titer is slightly above the one previously obtained in the study of Blazcek et al., (2014) in which the maximal production ranged between 20 mg/L and 60 mg/L, depending on the strength of the promoter used to drive expression of the CadA enzyme. Nevertheless, it has to be stressed that the productivity herein obtained was considerably smaller than the one reported by Blazcek et al., (2014) since their maximal titers were obtained after 3 days of fermentation, while in this case the maximum titers were achieved after 12 days. There are some differences in the experimental setup used in the two studies which could contribute to explain these differences including the different composition of the fermentation media and the different promoters that were used to drive expression of the CadA enzyme: while in this study the strong inducible GAL1 promoter was used, in Blazcek et al., (2014) it was explored an enhanced constitutive promoter based on GPD. The strength of GAL1 promoter is known to be proportional to the amount of galactose present in the growth medium for which it can be anticipated that in the later stages of the fermentation the production of itaconic acid could be limited by CadA availability. Duplication of the concentration of CSM in the fermentation medium led to an improvement of about 2-fold in the titers of itaconic acid that were obtained, this correlating with an improved growth rate of cells cultivated in these conditions, comparing with cells cultivated in MMF medium. It is important to stress that this improvement in the titer was obtained after 7 days of fermentation and thus as future work it will have to be determined how the duplication of CSM concentration impacts itaconic acid titer after 14 days of fermentation to have a more thorough comparison with the final titer of ~65mg/L.

The results obtained show that the production of itaconic acid is accomplished at the expense of galactose, Y, Z and X utilization, although in the latter case the contribution is less prominent. A very high accumulation of X was found to be present in the fermentation broth after the 14 days of fermentation, the production of this organic acid being observed to accompany the production of itaconic acid.
The accumulation of X in the medium (and also of Y and of Z) is a strong indication that the Y produced during glycolysis is not being fuelled up to the mitochondria at a significant rate, being rather decarboxylated into acetaldehyde and afterwards converted into X. The fact that the accumulation of X accompanies the production of itaconic acid (and this was observed for all the strains tested in the BY4741 background and under different conditions) suggests that these two metabolites are being produced through the same metabolic pathway. A closer inspection of the yeast metabolism shows that X can be converted into acetyl-CoA, which can then enter the glyoxylate cycle and produce cis-aconitic acid, the precursor of itaconic acid. This way of producing itaconic acid through cytosolic cis-aconitic acid is likely to be more advantageous for the biosynthesis of itaconic acid since the cadA enzyme is cytosolic [15] and therefore its substrate would be readily available for conversion. This hypothesis of itaconic acid being produced using cytosolic cis-aconitic acid is also consistent with the previous observation that targeting of CadA into the mitochondria does not result in improved production of itaconic acid [15], which would be expectable if only mitochondrial cis-aconitic acid was being utilized. An additional support for this hypothesis is the observation that deletion of E, encoding a mitochondrial tricarboxylate transporter homologous presumed to transport cis-aconitic acid from the mitochondria to the cytosol, did not reduced production of itaconic acid (see below). The accumulation of X in the medium results from the fact that its conversion to acetyl-CoA (catalyzed by acetyl-CoA synthetases Acsl or Acs2) is energetically unfavorable because it consumes 2 ATPs [16]. Thus the enzymes are not able to convert all X into acetyl-CoA resulting in its excretion. These observations point to new intervention strategies for strain engineering to boost itaconic acid production in yeast which could include, for example, the use of bacterial pathways to promote X activation, a strategy that was used before with success to improve cytosolic acetyl-CoA biosynthesis [16]. This accumulation of X in the fermentation broth not only results in massive carbon diversion but it is also highly toxic for the yeast cells reducing their ability to produce itaconic acid. This was in fact herein shown since buffering of the fermentation broth at 4.5 improved the titers of itaconic acid produced in a bioreactor.

The use of the A strain for the fermentations improved the titers of itaconic acid up to 413 mg/L, with a corresponding yield of 35.23 mg of itaconic acid/g of galactose. Up to now these are the highest titers described for the production of itaconic acid in a S. cerevisiae strain. The amount of X that was accumulated in the fermentation broth of A cultures was reduced by about ~4-fold the value obtained in BY4741 cultures. This result is consistent with the previously proposed model for itaconic acid biosynthesis since the A strain is not able to convert Y into X and thus the excretion of this latter acid should be smaller than the one registered in the parental strain. The reduced excretion of X could explain the increased performance of the A strain in producing itaconic acid since more carbon can be used to produce itaconic acid and also the deleterious effect of X accumulation is also less significant. It is known that the A strain (as well as other Pdc strains) are able to synthesize cytosolic acetyl-CoA, although the pathways leading to this had not been fully clarified. One hypothesis that was raised was the possibility of the cells having an enhanced flux of production of mitochondrial acetyl-CoA through a higher activity of the acetyl-CoA hydrolase Achi [17] which would result in production of X that could then be translocated to the cytosol and finally converted into acetyl-CoA by the Acs enzymes. It remains to be established if this is the case during fermentations for production of itaconic acid. The identification of these alternative pathways by which A strain produces acetyl-CoA should also provide new possibilities to engineer yeast strains optimized for the production of itaconic acid. On the overall, the results obtained in this work couple, for the first time, the production of itaconic acid to the availability of cytosolic acetyl-CoA.

Besides the higher titers of itaconic acid produced, the fermentations undertaken with the A strain also had a much higher specific yield (0.35 compared to 0.035) because the consumption of galactose in this strain was much smaller than the one observed in the parental strain. This lower consumption of sugars in the A strain was previously observed and attributed to a lower expression of sugar transporters [18]. Despite this, in the later days of the fermentation the consumption of galactose and the production of itaconic acid are significantly decreasing their rates. Notably, when the fermentations were undertaken in a bioreactor having a buffered pH at 4.5 galactose was fully exhausted. These observations suggest that the acidification of the broth could result in hampered ability of A cells to import galactose thereby resulting in stop of itaconic acid production.

To increase the versatility of the pAtCad1 plasmid and to see whether this could be used on a glucose-containing media, cells devoid of the B transcription factor, the key player involved in glucose repression in yeast, were used for the fermentations. This approach was pursued having in mind that the production of itaconic acid is a process limited by the cost of the raw material and the use of crystalline glucose or galactose would turn the process economically unviable. In that sense, the identification of a strain that could produce itaconic acid from cheap substrates containing glucose and galactose (such as molasses) could be an interesting way to value the developed pAtCad1 plasmid. The results obtained confirmed that the ∆B strain can be utilized for itaconic acid production with the pAtCad1 plasmid in media containing glucose and galactose and this was found to be particularly beneficial if the concentration of these carbon sources is reduced to 0.5%. In this case the ∆B mutant strain produced ~80 mg/L which is even above the titers produced by wild-type cells using 2% galactose. Necessarily, a significant increase in specific yield is obtained when the fermentations are undertaken by the ∆B mutant strain, 0.08 compared to 0.029. It is not clear the reason why the ∆B strain was able to produce such a high amount of itaconic acid using so little sugar, although this would be interesting to further characterize because it could provide hints for further strain optimization.

In the last part of this work three metabolic engineering algorithms (OptKnock, OptGene and RobOkoD) were used with the aim of identifying putative knockout mutants that could improve itaconic acid production in yeast cells. In silico prediction of itaconic acid production by these strains was performed using maximization of biomass per flux unit, an approach that had not been used before. The results obtained yielded 5 putative knockout mutants that were suggested to augment itaconic acid production and that were selected for experimental validation. Among the
predicted deletions was \(G\), encoding a mitochondrial dehydrogenase that converts isocitrate to alpha-ketoglutarate. This mutation would be an intuitive approach to improve the pool of cis-acconitic acid and therefore its identification by the in silico metabolic modeling tools was not surprising. Differently, other gene deletions (e.g. \(C\), \(J\), \(F\)) predicted were non-intuitive since it was not easy to understand how they could be connected with the itaconic acid biosynthetic pathway. Unfortunately, none of these non-intuitive deletions resulted in improved itaconic acid production and only the deletion of \(G\) gene improved the final titer of itaconic acid produced and also the productivity. Since mitochondrial isocitrate is the product of cis-acconitic acid degradation, it is possible that blockage of isocitrate degradation could result in a lower degradation of mitochondrial cis-acconitic acid which could then be channeled for itaconic acid production in the cytosol. It is also possible that the isocitrate produced in the mitochondria could be channeled to the glyoxylate cycle resulting in increased itaconic acid production. For the future, it should be tested other deletion mutants out of the candidate list identified. The inclusion of multiple deletions may also be considered as a possible option for future work, particularly starting from the \(\Delta G\) or \(Pdc\) mutant background.

References


