Metabolic and genetic engineering strategies to explore *Saccharomyces cerevisiae* as a cell factory for the production of itaconic acid

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Microbe-based production of itaconic acid is essential for the implementation of biorefineries since this building block can in the future be used to replace oil derivatives. Microbial production of this acid is already a reality, however the processes for production have a low yield, partly due to the toxic effect exerted by the acid in the producing cells. In this work the experimental model yeast *Saccharomyces cerevisae* was used for the production of itaconic acid using an optimized version of the *A. terreus* Cad1 enzyme. Using this enzyme lead to the successful production of itaconic acid, even though in a very low amount (25 mg/L).

Introduction

Nowadays, products as polymers, acidulants, preservatives, flavor compounds and precursors for the synthesis of pharmaceuticals are mostly produced through catalytic processing of petroleum-based precursors [1]. The US and the EU have given firm political steps for the successful implementation of biorefineries [2] establishing ambitious goals to reduce liquid petrol and industrial organic chemicals in 25-35% by 2025 [2]. However, the successful implementation of biorefineries is dependent on the identification of building block molecules that will be used in replacement of oil-derivatives [3]. Several products of microbial metabolism are recognized to have the potential to be used as building block chemicals and for this reason the exploration of microbes as cell factories for the production of numerous interesting metabolites has gained a lot of attention in the recent years. Itaconic acid is one of those interesting building block molecules, being a C_5 saturated dicarboxylic that contains a conjugated double bond and a carboxyl group activated by an ethyl-methyl group which makes it an effective intermediate for the synthesis of complex organic compounds through several possible routes including salt formation with metals, esterification, addition reaction and polymerization. Trough those modification a variety of products that can be obtained ranging from paints, to deodorants, adhesives, plastics and coatings, water treatment to ion exchange resins[4]. Itaconic acid results from decarboxylation of cis-aconitic acid, a by-product of the Krebs cycle, in a rection that is mediated by the Cad1 enzyme (Figure 4). Until recently natural production of itaconic acid has only been reported in a very limited number of filamentous Fungi including *Aspergillus terreus* and *Ustilago medyis*.roduction of itaconic acid at an industrial scale is mostly performed by fermentation carried out by *A. terreus* TN-484-M1 strain (an isolate that was obtained in a survey for fungi exhibiting resistance to high itaconic acid concentrations [3, 5]) or by *A. niger* strains engineered to synthesize the Cad1 enzyme. Production of itaconic acid via chemical synthesis is also possible, however, it is a multi-step reaction which increases the cost of the process to prohibitive values. The yields obtained for production of itaconic acid mediated by *A. terreus* or *A. niger* is around 90 g/L (using glucose as a carbon source) [4], this value being significantly below the theoretical yield which is estimated in 240 g/L[6].This low yield is, in part, attributed to the fact that the metabolism is not optimized for the production of itaconic acid. *S. cerevisiae*

can be a valuable host system for the production of carboxylic acids, including itaconic acid since can grow at low pH, avoiding the problems related to the downstream processing to obtain the acid, it is not filamentous, which facilitates the fermentation process in reactors and is highly amenable for *in silico* metabolic engineering because of the availability of information gathered so far allowing alterations to improve productivity.

Results

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

To be expressed in yeast cells the optimized *A. terreus AtCad1* gene was cloned in the pGREG586 plasmid, one of the vectors of the "drag and drop" system[7]. A schematic representation of the cloning strategy used is shown in Fig.1. The engineered plasmid, named pAtCad1(opt)::His6, drives expression of *AtCAD1* N-terminally tagged with a six-histidine tail and under the transcriptional regulation of the galactose-inducible promoter *GAL1* [7] (Figure 1). The *OptAtCAD1* gene (having a predicted size of 1500 pb) was amplified by PCR using the pUC57_AtCad1 plasmid as a template (this plasmid was acquired from GeneScript and it has cloned the synthesized version of the *AtCAD1* gene).

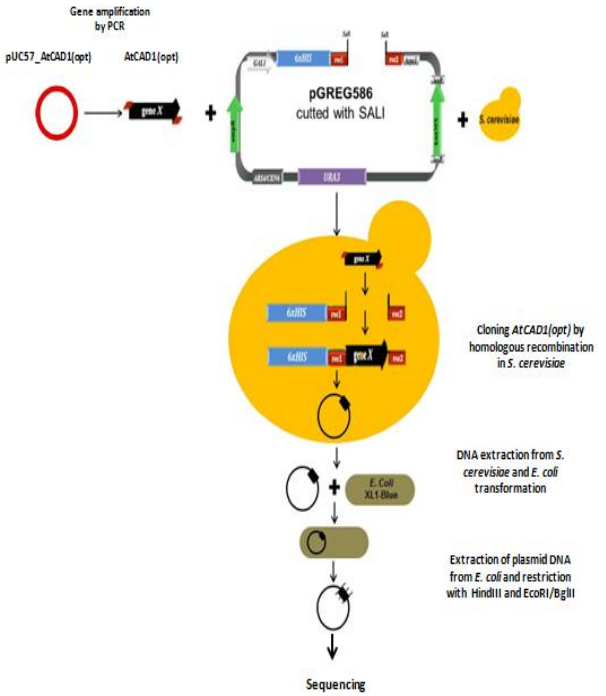

Figure 2- Schematic representation of the cloning engineering strategy used to clone the optimized *AtCAD1(opt)* gene in the pGREG586 plasmid.

After the PCR reaction the product of amplification was run in an agarose gel and the result obtained is shown in Figure 2. The size of the amplified product corresponds to the predicted size of the optimized *AtCAD1* gene confirming the success of the amplification reaction (Figure 2). The pGREG586 vector was digested with the *SalI* restriction enzyme and the result of that digestion was also confirmed by running the digested plasmid in an agarose gel (Figure 2 lane 6). The PCR-amplified *AtCAD1* gene was cloned in the *SalI*-digested pGREG586 vector by *in vivo* gap repair in *S. cerevisiae* cells.

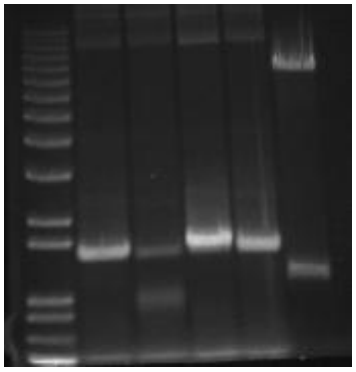

Figure 1 - Gel obtained after the digestion of plasmid DNA recovered from E. coli colonies considered to be candidates to have the pAtCad1(opt)::His6 recombinant plasmid. Lane 0: 1kb DNA ladder; Lane 1: pAtCAD1(opt)::His6 restricted with HindIII ; Lane 2: pAtCAD1(opt)::His6 restricted with EcoRI and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

After transformation of yeast cells with the *OptAtCAD1* gene and with the Sal*I*-digested pGREG586 vector around 100 transformants were obtained. To confirm that the transformants obtained harbored the pAtCAD1(opt)::His6 plasmid a colony PCR was carried out using 40 transformants. The same primers used for amplification of the optimized *AtCAD1* gene were used in this colony PCR. Only 4 of the PCR reactions performed produced a product of amplification with a size of 1.5 kb, these candidates being selected for further work. Total DNA was extracted from the positive yeast candidates and used to transform *E. coli* XL1-Blue cells. Plasmid DNA was recovered from 12 *E. coli* ampicillin-resistant transformants and then subjected to digestion with *HindII* and *EcoRI/BglII* enzymes (Figure 3).

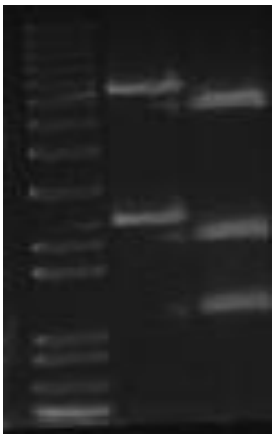

Figure 3 - Gel obtained after PCR amplification of the *OptAtCAD1* gene from the pUC57_AtCad1 vector and digestion of pGREG586 with *SalI*. Lane 1: 1 kb DNA ladder; Lanes 2-5: *AtCAD1(opt)* amplified fragment; Lane 6: pGREG586 digested with the *SalI* restriction enzyme.

The pattern of bands obtained after digestion of the plasmid DNA recovered from the *E. coli* candidates with the different restriction enzymes is consistent with these clones harboring the pAtCad1(opt)::His6 recombinant plasmid. The HindIII enzyme has two recognition sites in the pGREG586 vector located in the surroundings of the cloned gene (see restriction map in annex) and therefore digestion with this enzyme is expected to generate a fragment that has the approximate size of the insert, which in this case should be close to 1.5 kb. *EcoRI* has two cut sites in the pGREG586 vector, one located a few nucleotides upstream of the insert start codon, and one site in position 1124 of the AtCad1 gene. A band with approximately 1200 bp is obtained when the plasmid DNA of the clones was digested with the EcoRI/BglII mixture (Figure 17). The other bands obtained in the EcoRI/BglII double digestion also have the size that was expected for the pAtCad1(opt)::His6 vector. Correct insertion of the AtCad1 optimized gene in the pGREG586 vector was confirmed by DNA sequencing.

To confirm expression of AtCad1 in *S. cerevisiae* cells transformed with the pAtCad1(opt)::His6 vector it was assessed the ability of these cells to produce itaconic acid, a compound that is not naturally produced by this yeast species. For this *S. cerevisiae* BY4741 transformed with the pGREG586 cloning vector or with the pAtCad1(opt)::His6 plasmid were cultivated in MM Gal growth medium (containing 2% galactose) or in MMB (containing 2% glucose) supplemented with the CSM amino acid mixture. The decision to supplement the growth medium with CSM was based on previous indicatives obtained in a recently published paper that has also explored *S. cerevisiae* as a host system for the production of itaconic acid [8]. The supernatants collected from the different cultures after 24, 48 and 72 h of fermentation was tested for the presence of itaconic acid by HPLC. Under the experimental conditions used in the HPLC, itaconic acid had a retention time of approximately 13 minutes, as confirmed when a standard solution of the acid was used (Figure 6).

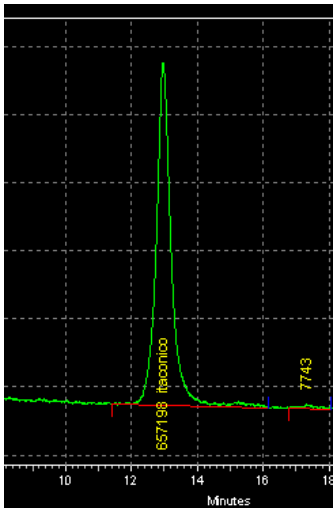

Figure 4 - UV-Vis spectrum of a standard solution (0.025mM) of itaconic acid.

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

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

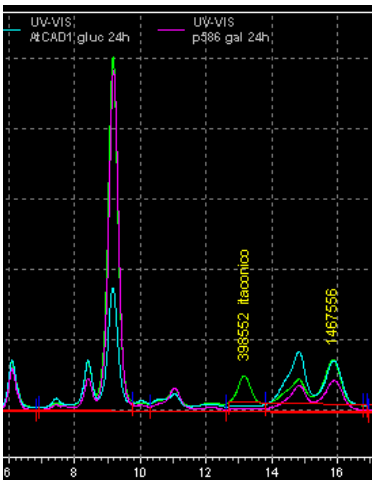

Figure 5 - UV-Vis spectrum analysis of the collected supernatants after 24h. Green: *BY4741* transformed with pAtCAD1(opt)::His6 plasmid cultured in MMB-U with 2% galactose supplemented with CSM, vitamins and trace elements; Blue: *BY4741* transformed with pAtCAD1(opt)::His6 plasmid cultured in MMB-U with 2% glucose supplemented with CSM, vitamins and trace elements; Pink: *BY4741* transformed with pGRER586 plasmid cultured in MMB-U with 2%galactose supplemented with CSM.

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

period of 72 hours.

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

Discussion

In the first part of this thesis the work presented concerns the heterologous expression of an optimized version of the *AtCAD1* in *S. cerevisiae*. The approach utilized has allowed the expression of AtCad1 protein from a galactose-inducible promoter which was confirmed by the detection of itaconic acid in the supernatant of cultures harboring the pAtCad1(opt)::His6 plasmid. 25 mg/L of itaconic acid were obtained after 72h of the plasmid induction with galactose. This production was close to the one reported by Blazeck *et.al* [8](approximately 24 mg/L) using the BY4741 strain and glucose as the carbon source. Glucose and galactose are metabolized through similar metabolic pathways (only the initial steps of glycolysis diverge) and therefore a similar yield in the acid produced from the two sugars was expected. The work described in this thesis set the foundations for the development of metabolic engineering strategies to be undertaken in our laboratory aiming to optimize productivity of itaconic acid. Such improvement is absolutely required to setup yeast as a valuable cell factory for the production of itaconic acid since the yields produced by *A. terreus* reach up to 90 g/L [4]. The HLPC analysis performed showed that yeast cells produced a significant amount of ethanol together with itaconic acid, meaning that the cells mainly fermented galactose through alcoholic fermentation instead of channeling it to the Krebs Cycle where the *cis*-aconitate is transformed in itaconate. In order to bypass this issue it could be used a pyruvate-decarboxylase negative (Pdc^-) strain since this strain not perform the alcoholic fermentation process. In this work the only carbon source explored was galactose, which is not a very interesting substrate for large-scale production because of its price. The ideal scenario would be to use compounds that are considered waste in biorefineries such acetate, glicerol or xylose, as these compounds are highly abundant in lignocellulosic feedstocks. In this context besides the metabolic engineering it will also be required to assess the effect of

having AtCad1 protein being expressed from other promoters more active when yeast cells are growing in the above-referred carbon sources.

Materials and Methods

Strains and growth media

The *S. cerevisiae* strain BY4741 (MATa, *his3*Δ1, *leu*2C0, *met15*Δ0, *ura3*Δ0) was used as wild-type along this work. The deletion mutants used in this work, listed in Table1, were all derived from the BY4741 background and were acquired from the Euroscarf collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/).

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

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

In vitro DNA manipulations

The pGREG586 plasmid (2 µg) was digested with 20 U of SalI restriction enzyme (Takara) and the mixture was incubated at 37˚C for 3 hours. The AtCAD1(opt) region to be cloned in the pGREG586 plasmid was obtained by PCR amplification using the pUC57_AtCad1 plasmid as a template. The pUC57_AtCad1, obtained from GeneSpring, was the plasmid in which it was cloned the optimized version of the *CAD1* gene (*AtCAD1(opt)*). PCR reactions were performed using Phusion® high-fidelity polymerase (Thermo Scientific) and a set of primers that were specifically designed for the effect being the forward primer **5' - GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC A**AT GAC AAA ACA ATC TGC CG - **3'** and the reverse primer **5' - GCG TGA CAT AAC TAA TTA CAT GAC TCG AGG TCG AC**T CAG ACC ATT GGG GAT TTT AC **- 3'** (The underlined nucleotides correspond to the homologous regions of the pGREG586 vector). The mixture used for the PCR reaction was composed by 10 µl of HF buffer, 1 µl of sNTPs, 1 µl of forward primer, 1 µl of reverse primer, 2 µl of DNA template, 2 µl of MgCl, 1.5 µl of DMSO, 0.5 µl of Taq phusion enzyme and 31 µl of water. As for the experimental setup it was used athe following program: 1) 98°C for 3 seconds, 2) 98°C for 10 seconds, 3) 56°C for 20 seconds,4) 72°C for 1 minute and 5) 7°C for 7 minutes. The steps 2, 3 and 4 were repeated for 30 cycles. Confirmation of the amplification of *AtCAD1(opt)* region was performed by running the product of amplification in an agarose gel (0.8%). The DNA band corresponding to the *AtCAD1(opt)* was excised from the gel and further purified using the JETQUICK Gel Extraction Spin kit (Genomed).

2.1.2 Yeast transformation

The PCR-amplified *AtCAD1(opt)* fragment and the SalI-digested pGREG586 vector were used to transform *S. cerevisiae* BY4741 cells using the Alkali-Cation™ Yeast Transformation kit (MP Biomedicals). *S. cerevisiae* BY4741 cells (50 mL) cultivated until mid-exponential phase (OD_{600nm}=0.4) in YPD growth were harvested by centrifugation for 5 minutes at 6500 rpm, at 4°C, and the supernatant was discarded. The cell pellet was resuspended in 9 ml of TE (at pH 7.5) and centrifuged in the same conditions. The supernatant was discarded and the cells were resuspended in 2.5 mL of a Lithium/Cesium Acetate Solution. The cell suspension was incubated at 30°C for 25 minutes with 100 rpm agitation. After that time cells were centrifuged again in the same previous conditions and gently resuspended in 500µL of TE at pH 7.5. 100µL of competent yeast cells were taken for each transformation. Each transformation mixture contained: 10µL the *AtCAD1(opt)* fragment, 5µL of carrier DNA, 5µL of histamine solution and 1.5µL of the SalI-digested pGREG586 plasmid. The mixture was incubated at room temperature for 15 minutes and after that 0.2 ml of TE/Cation solution and 0.8 ml of PEG were added. This step was followed by two incubations: the first incubation lasted 10 minutes and was performed at 30°C and the second lasted 10 minutes and was performed at 42°C. After these two incubation steps the reaction was cooled to 30°C, centrifuged for 5 seconds and the supernatant was discarded. Cells were resuspended in 100µL of YPD medium and plated on top of agar selective MMB media without uracil (MMB-U) and incubated at 30°C until visible cell growth. The transformants obtained were streaked in MMB medium without uracil.

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

Confirmation of the integration of the *AtCAD1(opt)* gene in the pGREG586 plasmid was performed, in a first stage, by colony PCR. A loop of cells from each colony recovered after the transformation step was resuspended in 10 µl of 20 mM of NAOH solution in an 1.5 ml eppendorf and then incubated at 100°C for 10 minutes. After this, the samples centrifuged at 13000*g* for 1 minute to separate cell debris from soluble material which is expected to include the plasmid DNA. The same primers used for amplification of *AtCAD1(opt)* gene were used for the PCR reaction. As for the mixture the composition was the following: 2 µl of Taq buffer, 0.4 µl of dNTPs, 0.4 µl of forward primer, 0.4 µl of reverse primer, 2 µl of DNA template, 1.2 µl of MgCl, 0.2 µl of Taq Med enzyme and 13.4 µl of water. The programe used for the PCR reaction was the following: 1) 94°C for 1 minute, 2) 94°C for 30 seconds, 3) 56°C for 1 minute, 4) 72°C for 2 minutes and 5) 7°C for 10 minutes. The steps 2, 3 and 4 were repeated for 30 cycles. Plasmid DNA was recovered from colonies confirmed to harbor the *AtCAD1(opt)* in the pGREG586 vector (that is, those colonies that had produced a band of amplification after the colony PCR reaction) using the QIAprep® Spin Miniprep Kit (Quiagen). The DNA recovered was used to transform *E. coli* XL1-Blue cells by classical transformation. For this purpose a mixture of 15 µL of the total plasmidic DNA (obtained from each positive yeast candidate), 150 µL of *E. coli* competent cells and 50 µL of a TCM solution (10mM CaCl2, 10mM MgCl2 and 10 mM Tris-HCl pH 7.5). The mixture was incubated on ice for 15 minutes, followed by a heat shock at 42 °C for 3 minutes and another incubation on ice for 5 minutes. 800 µL of liquid LB medium was added to the mixture and the cells were incubated at 37°C with orbital agitation at 250 rpm. Cells were centrifuged (8000 rpm for 3 minutes), plated in agar LB medium with ampicillin (150 µL/mL) and incubated at 37°C overnight. Plasmid DNA was recovered from the *E. coli* transformants obtained using the QIAprep® Spin Miniprep Kit (Quiagen). The concentration of DNA obtained at the end of the extraction protocol was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Confirmation of the correct

integration of the fragment in the pGREG586 vector, herein designated pAtCAD1(opt)::His6 (Figure 14), was confirmed by performing the restriction map of the recovered plasmids with BglII, HIndII and EcoRI enzymes and, later on, by DNA sequencing.

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

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

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