

Biocatalytic production of adipic acid from glucose using engineered *Saccharomyces cerevisiae*

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ABSTRACT

Adipic acid is an important industrial chemical used in the synthesis of nylon-6,6. The commercial synthesis of adipic acid uses petroleum-derived benzene and releases significant quantities of greenhouse gases. Biocatalytic production of adipic acid from renewable feedstocks could potentially reduce the environmental damage and eliminate the need for fossil fuel precursors. Recently, we have demonstrated the first enzymatic hydrogenation of muconic acid to adipic acid using microbial enoate reductases (ERs) - complex iron-sulfur and flavin containing enzymes. In this work, we successfully expressed the *Bacillus coagulans* ER in a *Saccharomyces cerevisiae* strain producing muconic acid and developed a three-stage fermentation process enabling the synthesis of adipic acid from glucose. The ability to express active ERs and significant acid tolerance of *S. cerevisiae* highlight the applicability of the developed yeast strain for the biocatalytic production of adipic acid from renewable feedstocks.

1. Introduction

Adipic acid (1,6 Hexanedioic acid) is a dicarboxylic acid that has immense importance from an industrial perspective. The primary use of adipic acid is in the manufacture of nylon 6,6 where it serves as one of the building blocks of the polymer. The global production of this bulk chemical was estimated to be 3.3 million tons/year in 2016 with a projected annual growth in demand of 3–3.5% per year (Bart and Cavallaro, 2015a; Polen et al., 2013). Conventional processes used for its production however, require the use of petroleum based feedstocks such as benzene, cyclohexanol and cyclohexanone and release copious amounts of nitrous oxide which is an extremely potent greenhouse gas into the atmosphere (Bart and Cavallaro, 2015a). With rising concerns about environmental sustainability and global climate change, it is imperative to develop a process that is more sustainable and causes limited or no damage to the environment. Over the past few years, significant progress has been made towards the use of renewable feedstocks such as glucose for adipic acid production (Bart and Cavallaro, 2015a, 2015b; Boussie et al., 2010; Draths and Frost, 1994; Raemakers-Franken et al., 2010; Vardon et al., 2015). The use of microorganisms for bioconversion of glucose into adipic acid is a promising alternative that addresses many of the concerns caused by the

current chemical processes. Recent advances in the fields of metabolic engineering and synthetic biology have allowed for the creation of industrial fermentation organisms that are capable of producing a wide variety of commercially important chemicals (Cheong et al., 2016; Curran et al., 2013; Kallscheuer et al., 2016; Lee et al., 2012; Yim et al., 2011; Yu et al., 2014). Adipic acid, being an important bulk chemical has also garnered significant interest in the research community as an important product for industrial fermentation (Cheong et al., 2016; Deng and Mao, 2015; Kallscheuer et al., 2016; Kruyer and Peralta-Yahya, 2017; Zhang et al., 2015).

Since adipic acid is not naturally produced by any known organism, attempts to produce it through fermentation require heterologous expression of pathways that can convert intracellular metabolites into adipic acid and several such pathways have been proposed. A comparison of these pathways based on their maximum theoretical yield has been provided in the supplementary information (shown in Figs. S1, S2 and Table S1). The cis, cis-muconic acid (ccMA) pathway (Fig. 1) begins with 3-dehydroshikimate, which is an intermediate metabolite in the aromatic amino acid synthesis pathway (Draths and Frost, 1994). Niu et al. implemented this pathway in *E. coli* and achieved a final ccMA titer of 36.8 g/L, which was then hydrogenated to adipic acid using a platinum catalyst (Niu et al., 2002). Recently, this ccMA pathway was

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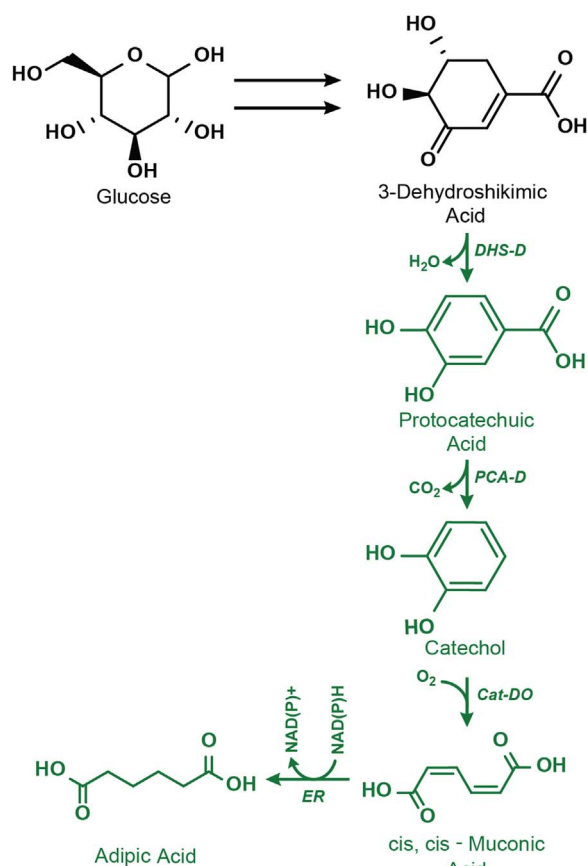


Fig. 1. The proposed pathway for adipic acid biosynthesis from glucose in *S. cerevisiae*. Metabolites and enzymes not native to yeast are shown in green. Abbreviations: DHS-D, 3-Dehydroshikimate Dehydratase; PCA-D, Protocatechuate Decarboxylase; Cat-DO, Catechol 1,2 – Dioxygenase; ER, Enoate Reductase.

introduced into a *S. cerevisiae* strain which enabled it to produce 140 mg/L of ccMA from glucose (Curran et al., 2013). More recently, this group implemented a biosensor based evolution strategy to increase ccMA titers of this strain to 0.5 g/L in a batch fermentation process (Leavitt et al., 2017). A recently published article examined the thermodynamic feasibility of several pathways for adipic acid biosynthesis and concluded that the muconic acid pathway is one among two pathways that remains thermodynamically feasible over a large range of pH and substrate concentrations (Averesch et al., 2017). The other feasible pathway described by the authors was also based on the shikimate pathway. In our work, we used this ccMA pathway due to the vast amount of prior work that has been done in both *E. coli* and *S. cerevisiae* which has resulted in several strains capable of producing high titers of ccMA. The non-availability of an enzymatic process for reducing the pi-bond of α -unsaturated carboxylic acids remained a significant bottleneck which prevented the realization of many proposed pathways for adipic acid biosynthesis. Though a chemo-catalytic hydrogenation could achieve this step as shown by Niu et al., a one-pot biosynthesis would be more feasible from an economic and environmental standpoint. Recently, we reported the biochemical characterization of enoate reductases (ER) from several bacteria that showed alkene hydrogenation activity on α -unsaturated carboxylic acids (Joo et al., 2017). In that work, we characterized a variety of ERs among which the ER from *Bacillus coagulans* was identified to be oxygen tolerant and thermostable. We demonstrated the in vitro (with purified enzymes) and in vivo (in *E. coli*) activity of these enzymes in the reduction of ccMA and 2-hexenedioic acid which are the penultimate metabolites in many adipic acid biosynthesis pathways.

The choice of microorganism for fermentation is also important in

determining economic feasibility, since it determines the composition of media and environmental conditions to be maintained during fermentation. The bacterium *E. coli* and yeast *S. cerevisiae* are particularly suited for fermentation to produce non-natural chemicals due to their fast growth and genetic tractability. Though *E. coli* has been previously used for adipic acid production, a complete conversion of glucose to adipic acid has not been achieved in *S. cerevisiae* or other yeasts. The use of yeast allows a lower pH to be maintained during fermentation. *S. cerevisiae* is a logical starting point for exploiting acidophilic yeasts and therefore, we sought to use it as our host organism.

2. Materials and methods

2.1. Plasmid construction

Lists of plasmids and DNA primers used in this study have been provided in the Supplementary information (Tables S2 and S4). Plasmid maps for pADP1 and pADP2 have also been provided in the Supplementary information (Figs. S3 and S4). The gene coding sequence for Enoate reductase from *Bacillus coagulans* (ERBC) was amplified from expression vectors described in our previous paper (Joo et al., 2017). The gene was then cloned into an empty yeast expression vector (pYES2) with a URA3 selection marker and the galactose inducible GAL1 promoter using the restriction enzymes *NotI* and *SacI*.

For the construction of the pADP2 plasmid, the gene ERBC – expressing Enoate reductase from *Bacillus coagulans* was amplified from the pADP1 plasmid and the gene ECL_01944_{opt} expressing Protocatechuate decarboxylase from *Enterobacter cloacae* was amplified from p426-GPD-ECL_01944_{opt}. These were then cloned onto the pSPGM1-KEX2 expression vector, with ERBC being controlled by a TEF1 promoter and ECL_01944_{opt} being controlled by a PGK1 promoter. This plasmid was constructed using a 4 part Gibson assembly.

The pYES expression vector used for the construction of pADP1 was purchased from ThermoFisher Scientific. The restriction enzymes *NotI* and *SacI* were obtained from New England Biolabs. All PCR reactions were conducted using Q5 DNA Polymerase obtained from New England Biolabs. The master mix for Gibson Assembly was prepared as described by Gibson et al., 2009. PCR product purification and plasmid purification from *E. coli* cells after cloning were done using GeneJET PCR purification and Plasmid Miniprep kits.

2.2. Strain construction and propagation

A complete list of strains used in this study has been provided in the Supplementary information (Table S3). The *S. cerevisiae* strain cenPK113-5d was used to construct the ADP1 strain. ADP2 was constructed using MuA12 as the parent which had a BY4741 background. Yeast strains were propagated at 30 °C in Yeast Synthetic Complete media. Yeast Synthetic Complete media was prepared by combining Yeast Nitrogen Base (with added ammonium sulfate) and Yeast synthetic dropout medium supplements without the appropriate auxotrophic markers. This media was supplemented with 20 g/L glucose. All shake flask cultures were grown in 250 mL baffled flasks with a 50 mL working volume and shaken at 250 rpm. The *E. coli* strain DH5 α was used for cloning and plasmid propagation. *E. coli* was grown at 37 °C in LB broth supplemented with 100 μ g/mL ampicillin.

The MuA12 strain consisted of three yeast expression vectors (listed in Table S3). We wished to express the ERBC gene and the ECL_01944_{opt} gene (expressing protocatechuate decarboxylase) using a divergent promoter system on a new expression vector. Therefore, we removed the plasmid p426-GPD-ECL_01944_{opt} by growth on 5-fluoroorotic acid to select against URA3 auxotrophy. We then transformed this strain with the newly constructed pADP2 plasmid to obtain the integrated adipic acid producing ADP2 strain. All plasmid transformations into *S. cerevisiae* were done by preparing chemically competent cells and applying a heat shock as described by Gietz and Schiestl, 2007.

2.3. Strain characterization

Precultures of the yeast strains were grown in baffled shake flasks at 30 °C under aerobic conditions in Yeast Synthetic Complete Media (with raffinose as the carbon source to allow for induction using galactose) lacking the appropriate auxotrophic markers. These cultures were then washed twice in fresh media and transferred into fermentation media. The fermentation media consisted of Yeast Synthetic Complete (YSC) medium lacking the appropriate auxotrophic markers, supplemented with 20 g/L of the carbon source (raffinose or glucose), 4 g/L oleic acid (in the form of Polysorbate 80), 40 mg/L ergosterol, 5 mg/L riboflavin and 1 mM glutathione. The cell density of the cultures was measured every 24 h by measuring the absorbance at 600 nm. The adipic acid concentration in the culture was measured through liquid chromatography – mass spectrometry as described in our previous paper (Joo et al., 2017). The setup for these fermentations used 250 mL media bottles which were modified to contain ports for sparging air or nitrogen gas to maintain aerobic or anaerobic conditions respectively in the media. The cultures were mixed using magnetic stirrers and maintained at 30 °C.

3. Results and discussion

3.1. Expression of enoate reductase in *S. cerevisiae*

The first goal in our path towards biosynthesis of adipic acid in yeast was to express functionally active enoate reductase from *B. coagulans* (ERBC) in *S. cerevisiae*. Though the purified ERBC enzyme was found to be relatively oxygen tolerant (Joo et al., 2017), we expected that maintaining the cells under anaerobic conditions would assist in the cytosolic assembly of its (4Fe-4S) iron-sulfur cluster by reducing oxidative stress. The commonly used lab strain of *S. cerevisiae* - CEN.PK113-5D was transformed with a plasmid containing the ERBC gene under the control of the galactose inducible promoter GAL1 to develop the ADP1 strain. To test for ER activity, the ADP1 precultures were transferred to anaerobic media supplemented with 2 mM ccMA and 10 g/L galactose for induction of the ERBC gene. We expected to observe ERBC activity through the detection of adipic acid in the fermentation broth. However, liquid chromatography-mass spectrometry (LC-MS) analysis of the fermentation broth revealed that no adipate was formed, therefore implying lack of ER activity (data not shown). Sipos et al. (2002) demonstrated in their work that while proteins with iron-sulfur clusters can be assembled under anaerobic conditions in the mitochondria of *S. cerevisiae*, maturation of iron-sulfur proteins in its cytosol requires the presence of glutathione even if the media is maintained anaerobic. It has been shown that glutathione protects iron-sulfur proteins from oxidative stress, thereby allowing for their successful maturation (Ozer et al., 2015). Therefore, ADP1 cells were induced for ER synthesis under anaerobic conditions in the presence of 1 mM glutathione for 12 h, followed by incubation under anaerobic or aerobic conditions. Both cultures showed similar cell densities during incubation (Fig. 2A). A completely aerobic control was also included in this experiment to examine whether active ER is synthesized under these conditions. LC-MS analysis of supernatant from the cultures revealed the formation of adipic acid after 48 h of incubation in the presence of glutathione, suggesting that glutathione facilitates the synthesis of active ER. In addition, it appears that after anaerobic expression of ER, the mature enzyme remains functional in yeast cells even under aerobic conditions as indicated by the production of adipic acid (Fig. 2B). This suggests that after synthesis of active ERBC, these yeast cells can be used for aerobic fermentation if required. The yeast cells grown under completely aerobic conditions failed to produce any adipic acid as expected. The final titers of adipic acid achieved in these experiments were in the range of 2.0–2.2 mg/L.

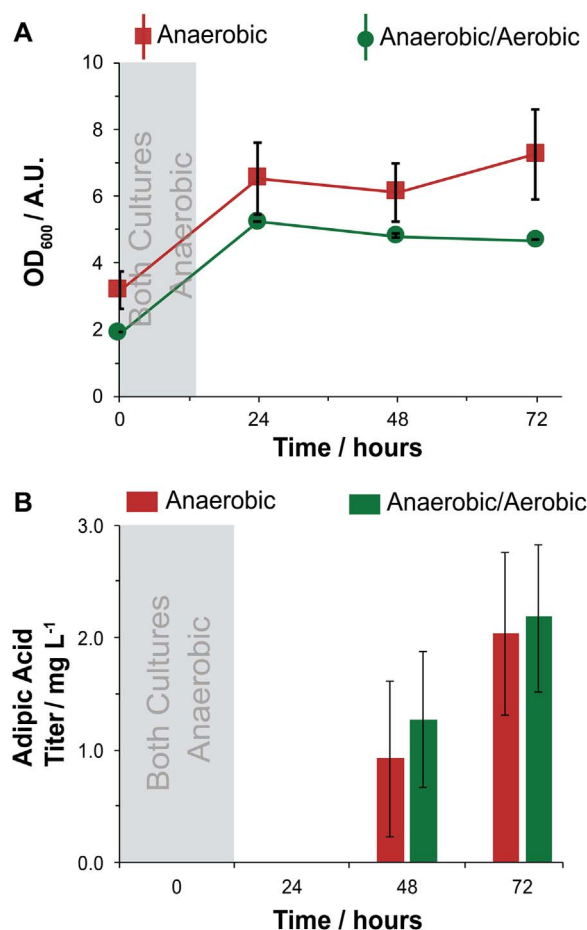


Fig. 2. Induction of ER and adipic acid production by the *S. cerevisiae* strain ADP1 during incubation with glutathione and ccMA. A: Cell densities (Optical Density at 600 nm) after induction with galactose at time 0. B: Adipic acid titers obtained during incubation. In both plots, “Anaerobic” refers to the cultures that were maintained anaerobic throughout, while “Anaerobic/Aerobic” refers to cultures that were maintained anaerobic for the first 12 h, followed by incubation under aerobic conditions.

3.2. Biosynthesis of adipic acid in *S. cerevisiae* from glucose

After establishing the experimental conditions for expression of active ERBC in yeast, we sought to develop an integrated strain of *S. cerevisiae* expressing ERBC and producing adipic acid from glucose as the sole carbon source. Our goal was to examine the efficacy of ERBC in the conversion of endogenously produced ccMA to adipic acid in a *S. cerevisiae* strain. We used the *S. cerevisiae* strain MuA12 which had been shown to produce the highest titer of ccMA from glucose at the time (Curran et al., 2013). To allow ERBC expression, we constructed the pADP2 plasmid that co-expressed ERBC and a protocatechuate (PCA) decarboxylase from *Enterobacter cloacae*. This plasmid was used to construct the integrated adipic acid producing ADP2 strain (Fig. 3A).

Finally, we used the ADP2 strain to perform a one pot conversion of glucose to adipic acid. This is not a straightforward task due to varying oxygen sensitivity and requirement by enzymes of the engineered adipic acid pathway. Specifically, catechol dioxygenase uses molecular oxygen as a substrate for converting catechol to ccMA, whereas ERBC requires anaerobic conditions for active enzyme maturation (iron-sulfur cluster synthesis and integration). Therefore, our strategy was to modulate oxygen levels in the fermentation vessels during a three-stage fermentation to allow both anaerobic ERBC maturation and aerobic adipic acid production (Fig. 3B). During the first fermentation stage (48 h), the *S. cerevisiae* cells were grown to a reasonably high cell density using aerobic conditions. Then, to allow for the production of functionally active ERBC enzymes, the culture was supplemented with

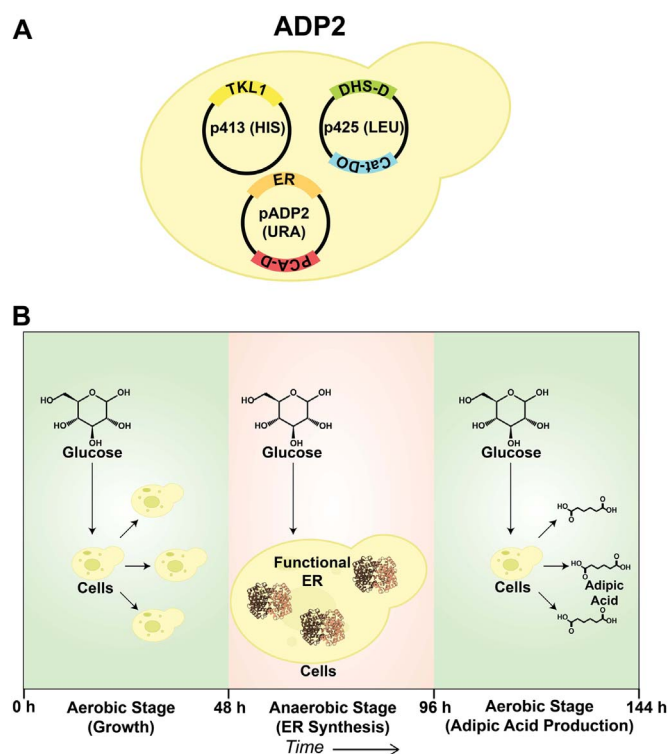


Fig. 3. The integrated adipic acid producing ADP2 strain and a fermentation strategy for adipic acid production. **A:** The plasmids present in the adipic acid producing ADP2 strain have been depicted. The names of the plasmids have been shown within them, followed by their auxotrophic markers (given in parenthesis). The genes encoding for various enzymes required for the adipic acid pathway have also been shown on each of the plasmids in colored boxes. Abbreviations - TKL1: Transketolase, DHS-D: 3-Dehydroshikimate Dehydratase, Cat-DO: Catechol Dioxygenase, PCA-D: Protocatechuate Decarboxylase, ER: Enoate Reductase, URA: Uracil, HIS: Histidine, LEU: Leucine. **B:** The proposed three-stage process for adipic acid production involves alternating between sparging the fermentation media with air and nitrogen to maintain aerobic and anaerobic conditions respectively.

Table 1

Final titers of adipic acid and muconic acid from three stage fermentations for adipic acid synthesis from glucose using the *S. cerevisiae* strain – ADP2.

Metabolite	Titer (mg L ⁻¹)
cis, cis - Muconic acid (ccMA)	> 284
Adipic acid	2.59 ± 0.5

1 mM glutathione and sparged with nitrogen to create anaerobic conditions in the second stage (48 h). After this, the culture was switched back to aerobic conditions by continuous sparging with air so that freshly added glucose (20 g/L) can be taken through the entire pathway to make adipic acid in the third stage (48 h). Our discovery that ERBC retains its functional state in *S. cerevisiae* cells after the culture is switched from anaerobic to aerobic conditions allows this strategy to work. The extracellular concentration of adipic acid in the fermentation broth at the end of the batch was analyzed using LC-MS. The growth profile of the strain was also monitored by measuring the optical density of the fermentation broth (Fig. S5). The final titers of adipic acid and ccMA are shown in Table 1. ADP2 produces nearly 2.6 mg/L of adipic acid using glucose as the sole carbon source (after 144 h), which is within the range of adipic acid titers produced by the recently reported *E. coli* strains expressing the reversed β -oxidation pathway (0.6–36 mg/L) (Kallscheuer et al., 2016; Yu et al., 2014). ADP2 also accumulated large quantities of ccMA in the process (> 284 mg/L) indicating that there is a significant potential for increasing adipic acid production by improving ERBC expression and activity. Muconic acid

and adipic acid titers obtained during the course of the fermentation are presented in the Supplementary information (Fig. S6 and Table S5). Though ERBC was successfully expressed in yeast cells and ADP2 is capable of adipic acid biosynthesis, there needs to be further investigation into achieving higher expression and activity of ERBC to allow complete conversion of ccMA, whose titers in yeast cells can reach 2.1 g/L in a bioreactor based fermentation process (Leavitt et al., 2017). Significant improvements in titer, yield and productivity through metabolic engineering and optimization of process conditions are necessary before a commercial process for adipic acid biosynthesis using *S. cerevisiae* can be conceived.

4. Conclusion

In summary, we have established the experimental conditions for the biosynthesis of an active microbial enoate reductase, an iron-sulfur enzyme, in yeasts and demonstrated the biosynthesis of adipic acid from glucose in *S. cerevisiae*. A three-stage fermentation strategy was developed to ensure optimal conditions for the biosynthesis of adipic acid and activity of all enzymes involved in the pathway. The successful expression of enoate reductase in *S. cerevisiae* also allows for engineering novel pathways for the biosynthesis of adipic acid and other chemicals in yeast. Thus, we have designed the first yeast-based biocatalytic system (ADP2) capable of adipic acid biosynthesis from glucose. Although this yeast strain cannot yet match the efficiency of more established ccMA producing strains (Leavitt et al., 2017), our work expands the biocatalytic toolkit and opens several avenues for engineering organisms that are capable of producing adipic acid from glucose at low pH. Future efforts will be directed towards further improvements in expression and activity of enoate reductases in yeast cells and also, the metabolic optimization of the bioconversion of glucose to adipic acid.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.meteno.2018.02.001>.

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