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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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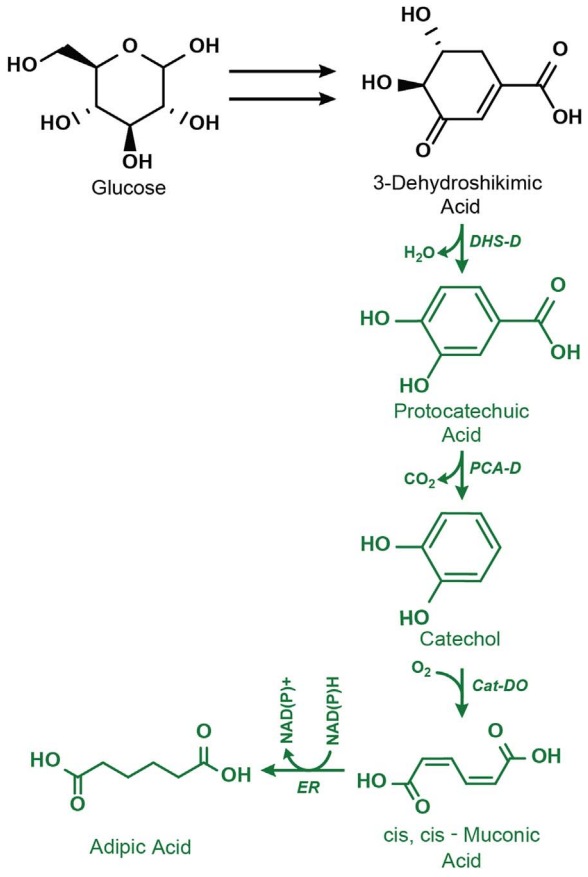

Fig. 1. The proposed pathway for adipic acid biosynthesis from glucose *in S. cerevisiae*. Metabolites and enzymes not native to yeast are shown in green. Abbreviations: *DHS-D*, 3-Dehydroshikimate Dehydratase; *PCA-D*, Protocatechuate Decarboxylase; *Cat-DO*, Catechol 1,2 – Dioxygenase; *ER*, Enoate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

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 *Not*I and *Sac*I 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 DH5α was used for cloning and plasmid propagation. *E. coli* was grown at 37 °C in LB broth supplemented with 100 μg/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.