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Production of aromatics in *Saccharomyces cerevisiae*—A feasibility study

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ABSTRACT

Aromatics are amongst the most important bulk feedstocks for the chemical industry, however, no viable bioprocess exists today and production is still dependent on petro-chemistry. In this article the production of aromatic precursors such as p-hydroxybenzoic acid (PHBA) and p-amino benzoic acid (PABA) in *Saccharomyces cerevisiae* was evaluated using metabolic network analysis. Theoretical mass yields for PHBA and for PABA obtained by metabolic network analysis were 0.58 and 0.53 g $g_{glucose}^{-1}$, respectively. A major setback for microbial production of aromatics is the high toxicity of the products. Therefore, PHBA and PABA toxicity was evaluated in *S. cerevisiae*. Minimal inhibitory concentrations of 38.3 g L^{-1} for PHBA and 0.62 g L^{-1} for PABA were observed. However, PABA toxicity could be alleviated in adaptation experiments. Finally, metabolic engineering was used to create proof of principle first generation strains of *S. cerevisiae*. Overall accumulation of 650 μM PHBA and 250 μM PABA could be achieved.

1. Introduction

Aromatic chemicals are essential feedstocks for the global chemical industry. The majority of the aromatic compounds are produced from BTX (benzene, toluene and xylene), which are obtained from both petroleum refining and natural gas. It is widely accepted that long term increase in demand and decreasing availability of these feedstocks as well as environmental compliance costs will drive prices up. Therefore there is a worldwide push in the chemical industry to move from fossil fuel derived feedstocks to renewable bio-derived feedstocks. It is estimated that the current 3–4% share of bio-feedstocks could increase to 17% of the global chemical business, equivalent to 425 billion $US, by 2025 (Reisch, 2009). Over the last years the first biological diamines for nylon production (Kind and Wittmann, 2011), dialcohols (Nakamura and Whited, 2003) and some dicarboxylic acids (Lee et al., 2008) for polyesters are being developed as bio-replacement chemicals, but to date only very few bio-replacement compounds are cost competitive. For many important compound classes there is either no bio-process available, or the discovered biological routes of manufacturing are not economically feasible. One class of compounds falling into this group are aromatics.

Aromatic compounds are among the most important building blocks in the chemical polymer industry. It is estimated that the aromatic molecule *terephthalic acid* will exceed a market volume of 50 million t by 2012. Based on the current price of US$ 1300/t this would equal a market of US$ 65 billion in 2012. The problem is that most commercial aromatics have no biological counterparts in biochemical pathways but are at best substrates in bio-remediation pathways of a few specialized microbes. The challenge is to find suitable precursor molecules that could be produced biotechnologically and then chemically converted to the desired compounds. One such candidate is *p-amino benzoic acid* (PABA), which is a potential precursor molecule for terephthalic acid. PABA could be chemically converted to *terephthalic acid mononitrile* using a Sandmeyer reaction and a subsequent reduction. PABA is an intermediate in folate biosynthesis in microbes but to date no over producing strains have been described. PABA is currently chemically synthesized and is used as a moiety in the pharmaceutical industry (Kluczyk et al., 2002) and as a

Abbreviations: PEP, phosphoenolpyruvate; PYR, pyruvate; aKG, α-ketoglutarate; AC-CoA, acetyl-coenzymeA; H-CoA, coenzymeA; CO_2, carbondioxide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; NADH/NAD, nicotinamide adenine dinucleotide (reduced/oxidized); NADPH/NADP, nicotinamide adenine dinucleotide phosphate (reduced/oxidized); PHBA, p-hydroxybenzoic acid; NH3, ammonia; TYR, tyrosine; GLU, glutamate; CHOR, chorismate.

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cross-linking agent for polyurethane resins, dyes, and feedstock additives.

Another interesting aromatic molecule is p-hydroxybenzoic acid (PHBA), which is used in liquid crystal polymers (LCPs) with a market volume of around 50,000 t per annum and an estimated price of around US$ 3000/t. LCPs are specialty chemicals that are used for electronics but also in the fiber industry for high strength applications. Moreover, PHBA is the base material for parabens, a key group of compounds used as preservatives in the cosmetic and pharmaceutical industries. PHBA is also an intermediate in microbial metabolism for the synthesis of ubiquinone. Both PABA and PHBA are derived from chorismate branching off the shikimate pathway (Fig. 1).

Unlike PABA, PHBA has been produced biotechnologically from glucose in recombinant strains of *Escherichia coli* (Barker and Frost, 2001), in *Klebsiella pneumoniae* (Müller et al., 1995) and in *Pseudomonas putida* (Verhoef et al., 2010; Meijnen et al., 2011). Green biotechnology has explored PHBA production outside the microbial world using sugar cane (McQualter et al., 2004), tobacco (Siebert et al., 1996) and potato (Köhle et al., 2003), but to date the highest titers achieved are 22.9 g L^{-1} in *E. coli* using in situ extraction of the toxic PHBA (Johnson and Amaratunga, 2000), which is probably still too low for a commercially viable process.

Apart from PABA and PHBA, the shikimate pathway also gives rise to shikimate (Krämer et al., 2003), anthranillic acid (Balderas-Hernandez et al., 2009), phenylethanol, p-hydroxycinnamic acid (Gosset, 2009), vanillin β-D-glucoside (Brochado et al., 2010) and phenylalanine and tryptophan (Hermann, 2003) to name a few.

Besides the prokaryotes and plants mentioned above, a potential candidate for the production of shikimate intermediates is the yeast *Saccharomyces cerevisiae*. This industrial microorganism has been extensively used in traditional biotechnological applications such as wine making, brewing and baking, and it has also been used for the production of therapeutic proteins (Gerngross, 2004), n-butanol (Steen et al., 2008), bioethanol (Bro et al., 2006), organic acids (Ishida et al., 2006), sugar alcohols (Toivari et al., 2007), steroids (Dumas et al., 2006), and isoprenoids (Maury et al., 2005; Westfall et al., 2012), among others.

Moreover, the available engineering tools and the ability of *S. cerevisiae* to survive under stress conditions such as high concentrations of solvents, pH, osmolarity, nutrient supply and temperature (Gibson et al., 2007), make it a very good candidate to be used as a host for aromatic hydrocarbon production. Indeed, yeast engineered for elevated production of aromatic amino acids by alleviating feedback inhibition in the shikimate pathway, readily accumulate aromatic compounds in the extracellular matrix (Luttik et al., 2008), indicating the potential of this organism for the production of these important aromatic chemicals.

In this work we first evaluated the theoretically possible product yields for PHBA and PABA in yeast using elementary mode analysis. Then, we evaluated PHBA and PABA toxicity in *S. cerevisiae*, which indicates the currently feasible product titers. Finally, we engineered yeast strains for the production of both PHBA and PABA.

2. Materials and methods

2.1. Culture media and chemicals

All chemicals used in this study were at least of analytical grade and were obtained from Sigma–Aldrich. High purity water (18.2 MΩ) was used for all preparations. Polypeptone and yeast extract were purchased from Oxoid (Adelaide, SA, Australia). Complex medium (YPD) contained 10 g L^{-1} yeast extract, 10 g L^{-1} polypeptone, and 20 g L^{-1} dextrose (glucose).

Chemically defined medium (CDM) contained $(NH_4)_2HPO_4$ 1 g L^{-1}, $(NH_4)_2SO_4$ 2 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.5 g L^{-1}, KCl 1 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 0.15 g L^{-1}, glucose 5 g L^{-1}, NaH_2PO_4 7.8 g L^{-1}, Na_2HPO_4 7.1 g L^{-1} and vitamins and trace metals. Trace metal concentration in 1 L of CDM consisted of EDTA 15 mg L^{-1}, $ZnSO_4{\cdot}7H_2O$ 4.5 mg L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.3 mg L^{-1}, $Na_2MoO_4{\cdot}2H_2O$ 0.4 mg L^{-1}, $CaCl_2{\cdot}2H_2O$ 0.45 mg L^{-1}, $MnCl_2{\cdot}2H_2O$ 0.84 mg L^{-1}, $CoCl_2{\cdot}6H_2O$ 4.5 mg L^{-1}, $FeSO_4{\cdot}7H_2O$ 3 mg L^{-1}, H_3BO_3 1 mg L^{-1}, KI 1 mg L^{-1}. Vitamin concentrations in 1 L of CDM were D-biotin 0.05 mg L^{-1}, calcium pantothenate 1 mg L^{-1}, thiamine hydrochloride 1 mg L^{-1}, pyridoxal hydrochloride 1 mg L^{-1}, nicotinic acid 1 mg L^{-1}, para-aminobenzoic acid 0.2 mg L^{-1} and myoinositol 25 mg L^{-1}. Glucose, vitamin and trace metal stock solutions were filter sterilized (0.22 μm) while the remaining solutions were autoclaved separately. The pH of CD medium was adjusted to 6. CDM and YPD solid media plates were prepared with 1.5% and 2% agar respectively.

For toxicity tests, saturated stock solutions of the respective substance in fermentation medium were prepared. The solubility of PABA and PHBA is pH dependent. At pH 6 the following concentrations were stable in CDM at 4 °C: PABA 10 g L^{-1}, PHBA 80 g L^{-1}.

2.2. Strains

The type strain *S. cerevisiae* S288C (Mortimer and Johnston, 1986) was used in the toxicity analysis (see below) and as a starting point for strain development (Table 1). All strains were obtained from The Australian Wine Research Institute culture collection (Adelaide, SA, Australia).

2.2.1. Gene deletions

Gene deletions were carried out using homologous recombination. The open reading frame (ORF) of the target gene was replaced with a polymerase chain reaction (PCR) product containing an antibiotic marker. The sequence encoding the antibiotic marker was flanked by sequences homologous to the upstream and downstream regions of the target ORF. In order to construct multiple deletions in the same strain two different antibiotic markers were used. Nourseothricin (Jena, Germany), encoded by the *nat1* gene from *Streptomyces noursei* and phleomycin (Sigma–Aldrich) encoded by the *ble* gene from the transposon Tn5. Markers were

Table 1
Yeast strains engineered for PHBA and PABA production.

Strains	Description
S. cerevisiae S288C	haploid, *MATα SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6*
S. cerevisiae S288C Δ*aro7*	Knockout strain in the *aro7* gene encoding chorismate mutase.
S. cerevisiae S288C Δ*aro7* pCV3-UBIC	Chorismate mutase knockout strain overexpressing *E. coli ubiC* gene encoding chorismate lyase from plasmid pCV3.
S. cerevisiae S288C Δ*aro7* Δ*trp3*	Double knockout strain in the aro7 and *trp3* genes encoding chorismate mutase and anthranilate synthase subunit, respectively.
S. cerevisiae S288C Δ*aro7* Δ*trp2*	Double knockout strain in the aro7 and *trp2* genes encoding chorismate mutase and anthranilate synthase subunit, respectively.
S. cerevisiae S288C Δ*aro7* Δ*trp3* pCV3-ABZ1	Double knock-out strain described above, overexpressing the wine yeast AWRI1631 *abz1* gene encoding para-aminobenzoate synthase from plasmid pCV3