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From zero to hero – Production of bio-based nylon from renewable resources using engineered *Corynebacterium glutamicum*

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ABSTRACT

Polyamides are important industrial polymers. Currently, they are produced exclusively from petrochemical monomers. Herein, we report the production of a novel bio-nylon, PA5.10 through an integration of biological and chemical approaches. First, systems metabolic engineering of *Corynebacterium glutamicum* was used to create an effective microbial cell factory for the production of diaminopentane as the polymer building block. In this way, a hyper-producer, with a high diaminopentane yield of 41% in shake flask culture, was generated. Subsequent fed-batch production of *C. glutamicum* DAP-16 allowed a molar yield of 50%, a productivity of 2.2 g L^{-1} h^{-1}, and a final titer of 88 g L^{-1}. The streamlined producer accumulated diaminopentane without generating any by-products. Solvent extraction from alkalized broth and two-step distillation provided highly pure diaminopentane (99.8%), which was then directly accessible for poly-condensation. Chemical polymerization with sebacic acid, a ten-carbon dicarboxylic acid derived from castor plant oil, yielded the bio-nylon, PA5.10. In pure form and reinforced with glass fibers, the novel 100% bio-polyamide achieved an excellent melting temperature and the mechanical strength of the well-established petrochemical polymers, PA6 and PA6.6. It even outperformed the oil-based products in terms of having a 6% lower density. It thus holds high promise for applications in energy-friendly transportation. The demonstration of a novel route for generation of bio-based nylon from renewable sources opens the way to production of sustainable bio-polymers with enhanced material properties and represents a milestone in industrial production.

1. Introduction

Polyamide (PA), commonly known as nylon, is a polymer with a myriad of pharmaceutical and industrial applications. Chemically, the polymer backbone is composed of repetitive units of diamines and dicarboxylic acids that contain different numbers of carbon atoms, imparting a variety of material properties. Commercialized in the 1940s, polyamides have entered the market place on a large scale for the manufacturing of fibers for clothing or thermoplastics for carpets, cogs, car parts, tire reinforcements, and other products. Currently, the global market requires 6.6 million tons per annum, making polyamides one of the most important industrial polymers. Its good biocompatibility has further led to its implementation in medical applications, such as providing a scaffold for tissue cultures (Yoo et al., 2011), foil for orbital implants (Park et al., 2008), and bone support in arthroplasty (Edwards et al., 2011). Novel developments extend the application range of polyamides to diagnostics (Cox, 2001) and cellular control devices in molecular medicine (Schmitz and Schepers, 2004). Interest in a "green" route for polyamides has arisen due to the inevitable stoichiometric wastes of classical petrochemical production routes, commonly thought to cause global warming and ozone depletion, as well as acid rain and smog (Sato et al., 1998). Compared to other industrial polymers, the production of petrochemical nylon has a markedly severe impact on global climate change, expressed as carbon dioxide equivalents released (Vink et al., 2003). Moreover, it exhibits an exceptionally high requirement for fossil energy, which is particularly unfavorable in the light of the increasing shortage and rising price of fossil resources. Alternative chemical routes, such as the direct oxidation of cyclohexene to the nylon precursor adipic acid (Sato et al., 1998), promise attractive solutions for the reduction of the carbon footprint of polyamides, but these methods still rely on fossil fuels.

Without doubt, a highly promising bio-block for polyamides is 1,5-diaminopentane (cadaverine, diaminopentane), a five-carbon diamine (Qian et al., 2010). Polyamides based on diaminopentane exhibit excellent and well-known material properties (Carothers,

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Table 1
Lysine and diaminopentane-producing *Corynebacterium glutamicum* strains used and constructed in the present study.

Strain	Modification	Reference
LYS-12	Wild-type ATCC 13032 + nucleotide exchange resulting in the amino acid exchange T311I in *lysC*, encoding aspartokinase ($lysC^{T311I}$); over expression of $lysC^{T311I}$ by replacement of the native promoter with the promoter of *sod*, encoding superoxide dismutase ($P_{sod}lysC^{T311I}$); overexpression of *dapB*, encoding dihydropicolinate reductase, under the *sod* promoter ($P_{sod}dapB$); duplication of *ddh*, encoding diaminopimelate dehydrogenase (2x*ddh*); duplication of *lysA*, encoding diaminopimelate decarboxylase (2x*lysA*); deletion of *pepck*, encoding PEP-carboxykinase (Δ*pepck*); nucleotide exchange resulting in a V59A change in *hom*, encoding homoserine dehydrogenase (hom^{V59A}); overexpression of *pycA*, encoding pyruvate carboxylase, by replacement of the native promoter with the *sod* promoter ($P_{sod}pycA$); nucleotide exchange resulting in the P458S change in *pycA* ($P_{sod}pycA^{P458S}$); replacement of the ATG start codon with the rare GTG in *icd*, encoding isocitrate dehydrogenase (icd^{gtg}); replacement of the natural promoter of *fbp* by the *tuf* promoter, encoding elongation factor tu ($P_{tuf}fbp$); replacement of the natural promoter of the *tkt*-operon, comprising the genes *zwf* and *tal*, encoding transaldolase, *tkt*, encoding transketolase, *opcA*, encoding a putative subunit of glucose 6-phosphate dehydrogenase, and *pgl*, encoding 6-phosphogluconolactonase, by replacement of the native promoter with the *sod* promoter ($P_{sod}tkt$)	Becker et al. (2011)
DAP-13	LYS-12 + heterologous, genome-based expression of a codon-optimized variant of *E. coli ldcC*, encoding lysine decarboxylase, with replacement of the native promoter by the *tuf* promoter ($P_{tuf}ldcC^{opt}$), insertion at the *bioD* locus, encoding dithiobiotin synthetase	This study
DAP-14	DAP-13 + deletion of *NCgl1469*, encoding a *N*-acetyltransferase (Δ*NCgl1469*)	This study
DAP-15	DAP-14 + deletion of *lysE*, encoding the lysine exporter (Δ*lysE*)	This study
DAP-16	DAP-15 + overexpression of *cg2893*, encoding a major facilitator permease, by replacement of the native promoter by the *sod* promoter (P_{sod} *cg2893*)	This study

1938). However, due to the lack of efficient petrochemical routes for production of this monomer, the corresponding polymers are not considered industrially valuable. In particular, bio-based diaminopentane would open up green routes to production of novel bio-nylons, such as PA5.10 and PA5.4, copolymerized with sebacic acid from natural castor oil (Ogunniyi, 2006) and succinic acid from microbial fermentation (Hong et al., 2004), respectively (Kind and Wittmann, 2011). Moreover, systems metabolic engineering is currently available to facilitate design and improvement of the performance of microorganisms for fermentative production of an increasing number of chemicals, materials, and fuels from low-cost renewable resources (Becker and Wittmann, 2012a, 2012b; Jang et al., 2012). This opens new possibilities for the creation of bio-based polymers at the required titers, yields, and productivities. Recently, pioneering studies have enabled diaminopentane production from sugar in engineered cells of the soil bacterium *Corynebacterium glutamicum* (Mimitsuka et al., 2007). These synthetize the desired chemical from the natural amino acid lysine through heterologous expression of the *E. coli* lysine decarboxylase CadA (Mimitsuka et al., 2007) or LdcC (Kind et al., 2010a). Further rounds of systems metabolic engineering have allowed improvement of the biosynthetic capacity and supporting reactions (Kind et al., 2010a) and elimination of competing pathways generating undesired by-products (Kind et al., 2010b), and have increased product secretion (Kind et al., 2011) and extended the substrate spectrum to starch (Tateno et al., 2009) and xylose (Buschke et al., 2011). Currently, the reported product yields of the best strains are still far from economic applicability, but at least demonstrate promising proof-of-concepts (Becker and Wittmann, 2012a, 2012b).

In this work, we describe a sustainable value chain, extending from renewable resources to a novel, bio-based polyamide PA5.10. The development integrated engineering of *C. glutamicum* at the cellular and the process level to ensure fermentative supply of the polyamide building block, diaminopentane. Thereafter, we further developed processes for the fermentation and down-stream purification of the monomer, poly-condensation into a novel bio-nylon, and further conditioning by generating enforced industrial polymers.

2. Materials and methods

2.1. Strains and plasmids

The lysine producer *C. glutamicum* LYS-12 (Table 1) was used as parent strain (Becker et al., 2011). For genetic engineering work, the *Escherichia coli* strains DH5α and NM522 (Invitrogen, Karlsruhe, Germany) and the plasmids pClik int *sacB* and pTc were applied as described previously (Kind et al., 2010a).

2.2. Genetic engineering

All modifications were introduced into the genome of *C. glutamicum* using homologous recombination and a two-step selection, using kanamycin resistance and the *sacB* system (Becker et al., 2005; Jäger et al., 1992). Construction, purification, and analysis of plasmid DNA, as well as transformation of *E. coli* and *C. glutamicum* were performed as described previously (Becker et al., 2011; Kind et al., 2010a). Targeted gene deletion was carried out by replacement of the coding region of the gene of interest by a shortened gene fragment. For overexpression, the strong *sod* and *tuf* promoter was inserted in front of the candidate gene. The primers used for construction and verification of the introduced genetic changes have been described previously (Becker et al., 2011; Kind et al., 2010a; Kind et al., 2010b).

2.3. Chemicals

Tryptone, beef extract, yeast extract, brain heart infusion (BHI), and agar were obtained from Difco Laboratories (Detroit, MI, USA). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), or Fluka (Buchs, Switzerland). Ultramid B27 and Ultramid A27 were supplied by BASF SE (Ludwigshafen, Germany).

2.4. Media

For shake flask studies, a complex medium was applied for the first pre-culture, whereas the second pre-culture and the main culture were carried out in glucose-based minimal medium (Kind et al., 2010a). For fed-batch production in the bioreactor, the pre-culture was grown in shake flasks in a complex medium containing 37 g L^{-1} BHI and 20 g L^{-1} glucose. The production process started with a batch medium that contained the following amounts of substances per liter: 90 g glucose, 15 g yeast extract, 2 g citric acid, 25 g $(NH_4)_2SO_4$, 1.25 g KH_2PO_4, 1.25 g Na_2HPO_4, 1.25 g $MgSO_4 \cdot 7H_2O$, 70 mg $FeSO_4 \cdot 7H_2O$, 30 mg $ZnSO_4 \cdot 7H_2O$, 14 mg $MnSO_4 \cdot 6H_2O$, 168 mg $CaSO_4 \cdot 2H_2O$, 0.43 mg boric acid, 0.34 mg $CoSO_4$, 0.42 mg $CuSO_4$, 0.07 mg Na_2MoO_4, 4.5 mg biotin, 7.5 mg thiamin, 9 mg nicotinamide, 30 mg pantothenic acid, 10 mg pyridoxal hydrochloride, and 1 mL antifoam (polyoxyethylene

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