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High-level biosynthesis of norleucine in *E. coli* for the economic labeling of proteins

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ABSTRACT

The residue-specific labeling of proteins with non-canonical amino acids (ncAA) is well established in shake flask cultures. A key aspect for the transfer of the methodology to larger scales for biotechnological applications is the cost of the supplemented ncAAs. Therefore, we established a scalable bioprocess using an engineered host strain for the biosynthesis of the methionine analog norleucine at titers appropriate for the efficient and economic labeling of proteins. To enhance the biosynthesis of norleucine, which is a side-product of the branched chain amino acid pathway, we deleted all three acetolactate synthase isoforms of the methionine auxotrophic *Escherichia coli* expression strain B834(DE3). Additionally, we overexpressed *leuABCD* to boost the biosynthesis of norleucine. We systematically analyzed the production of norleucine under the conditions for its residue-specific incorporation in bioreactor cultures that had a 30-fold higher cell density than shake flask cultures. Under optimized conditions, 5 g/L norleucine was biosynthesized. This titer is two times higher than the standard supplementation with norleucine of a culture with comparable cell density. We expect that our metabolically engineered strain for the improved biosynthesis of norleucine in combination with the proposed bioprocess will facilitate the efficient residue-specific labeling of proteins at a reasonable price in scales beyond the shake flask.
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1. Introduction

Non-canonical amino acids (ncAAs) show diverse and unusual side chain chemistries (Voloshchuk and Montclare, 2010; Wang et al., 2012). These make ncAAs attractive as building blocks for protein engineering (Zheng and Kwon, 2012). Many ncAAs are structural analogs of the canonical amino acids (cAA) prescribed by the genetic code. This is why the amino acid analogs can participate in ribosomal translation under tightly controlled conditions (Cohen and Munier, 1956) even though they are not encoded by the genetic code. Previously, it was shown that the incorporation of ncAAs into target proteins can improve enzyme activity (Acevedo-Rocha et al., 2013; Cirino et al., 2003; Hoesl et al., 2011). NcAAs can increase the stability of proteins under harsh conditions, such as the tolerance to organic solvents (Deepankumar et al., 2015; Deepankumar et al., 2014). Norleucine (Nle, Fig. 1A), a hydrophobic methionine (Met) analog prevented the aggregation of prion proteins while the hydrophilic analog methoxinine (O replaces S in Met) provoked it (Wolschner et al., 2009). Furthermore, Nle conferred resistance to chemical degradation (Anfinsen and Corley, 1969).

Many ncAAs with chemically favorable side chains are not commercially available, and those that are often come at a very high price. This can be neglected at small scale, but can seriously limit the application of ncAAs in large scale industrial bioprocesses. For instance, the supplementation of a one liter shake flask culture at a cell density of $D_{600} \sim 1$ with 0.5 mM Nle, a comparably inexpensive ncAA (~25–50 Euros/g), costs ~2 Euros. However, if this lab scale

Abbreviations: ALS, acetolactate synthase; cAA, canonical amino acid; FRT, Flp recombinase target; 2KB, 2-ketobutyrate; KO, knockout; M9 MM, M9 minimal medium; MetRS, methionyl-tRNA synthetase; ncAA, non-canonical amino acid; Nle, norleucine; Nva, norvaline; OYE, old yellow enzyme; RMD, relative mean deviation; SPI, supplementation incorporation; TTL, thermophilic lipase of *Thermoanaerobacter thermohydrosulfuricus*; YE, yeast extract.

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procedure (Hoesl et al., 2011; Wiltschi, 2012) is extrapolated to an industrial scale, i.e. a 10 m^3 bioreactor culture at a cell density of $D_{600} \sim 300$ the cost would amount to at least five million euros. The biosynthesis of ncAAs from simple and cheap precursors, such as glucose, could remedy the lack of availability and lower the price. Thus, the metabolic engineering of host strains to biosynthesize ncAAs could increase the cost effectiveness of ncAAs for protein labeling in an industrial environment. Fortunately, ncAAs are very abundant in nature (Hunt, 1985) even though the biosynthesis pathways of only a handful of these compounds are known (Walsh et al., 2013). A few selected examples of the recombinant biosynthesis of ncAAs and their incorporation were previously reported (Ehrlich et al., 2015; Lepthien et al., 2008; Ma et al., 2014; Mehl et al., 2003; Zhang et al., 2010). However, the applicability in scales larger than shaker flasks was largely neglected so far.

Here, we aimed to biosynthesize an ncAA from sugar and salts at levels that would allow its incorporation into a target protein beyond lab applications. We chose Nle since its biosynthesis pathway is well understood and several possible ways to biosynthesize it have been reported (Bogosian et al., 1989; Brunner et al., 1997; Kisumi et al., 1976; Kisumi et al., 1977; Soini et al., 2008; Sycheva et al., 2007). Nle is a side-product of the branched chain amino acid pathway (Fig. 1A shows an overview of the biosynthesis pathway) and in *E. coli* it is readily inserted at Met sites in leucine (Leu) rich proteins (Bogosian et al., 1989). The translation of Leu-rich proteins taps the intracellular Leu pool, which results in the derepression of *leuABCD* (Fig. 1A). The isoleucine (Ile) precursor 2-ketobutyrate (2-KB) is a substrate for derepressed *leuABCD* and can be converted to norvaline (Nva) and Nle (Fig. 1A, orange and red branches). Brunner et al. (1997) reported the biosynthesis of Nle in the lower mM range by *E. coli* expressing bovine somatotropin (14% Leu) in medium containing low concentrations of yeast extract. Soini et al. (2008) showed that Nva is biosynthesized in the low mM range under oxygen limitation on pure glucose mineral salt medium. Under these conditions, pyruvate accumulated and the low Leu concentration in the medium caused a derepression of the *leuABCD* operon. Pyruvate might be directly elongated to 2-KB by *leuABCD*. Using an *E. coli* K-12 strain that lacked acetolactate synthase (ALS) activity and that overexpressed the *leuABCD* operon, Sycheva et al. (2007) produced 4 g/L Nle. ALS is a key player in the formation of the branched chain amino acids and *E. coli* has three ALS isoenzymes, encoded by *ilvBN*, *ilvIH*, and *ilvGM* (Fig. 1A).

Nle is an isosteric carba-analog of Met. The carbon atom of the methylene group of Nle is slightly less electronegative than the corresponding sulfur atom of Met (2.48 *vs* 2.56, (Xie et al., 1995)). In comparison to the sulfur atom the methylene group is less polar and more hydrophobic (Thomson et al., 1994). It has a larger covalent radius than the sulfur atom (1.84 Å *vs* 1.02 Å (Richmond, 1962)) and the C—C bond is slightly shorter than the C-S bond (1.54 Å vs 1.81 Å (Richmond, 1962)). The thioether group of Met is readily oxidized to two diastereomeric sulfoxides and the sulfone (Shechter et al., 1975). Since Nle lacks the sulfur atom, it cannot form a sulfoxide and the global replacement of Met by Nle protects proteins from oxidative stress (Gilles et al., 1988). However, global proteome-wide replacement of Met by Nle deprives the cell of its antioxidative defense (Luo and Levine, 2009). Nle has a long history in bacteria. Very early, norleucine-resistant strains provided insight in the control of Met biosynthesis in *E. coli* (Rowbury, 1965). Pine, (1967) demonstrated that substitution of Met by Nle potentiated the catabolism of the microbial proteome. Nle is attached to $tRNA^{Met}$ by the methionyl-tRNA synthetase (MetRS) and Nle on the initiator $tRNA^{Met}$ is formylated (Trupin et al., 1966). Thus, it can replace Met at the protein start (Kerwar and Weissbach, 1970) as well as within proteins (Barker and Bruton, 1979; Gilles et al., 1988).

To replace all Met residues by Nle in a target protein, a Met auxotrophic strain is used that facilitates the control of the intracellular levels of both amino acids. This is necessary because the MetRS accepts a variety of Met analogs but strongly prefers Met as the substrate (Kiick and Tirrell, 2000). In a typical shake flask experiment, the Met auxotrophic host cells are first grown on minimal medium with limiting amounts of Met (growth phase). As soon as the Met in the medium is consumed, growth stalls (Met depletion). At this point, the cells are supplemented with Nle and the expression of the target protein is turned on (protein production phase; supplementation based incorporation, SPI) (Budisa et al., 1995; Wiltschi, 2012). Shake flask cultures are usually supplemented with 0.3–4 mM or approximately 20–300 mg/L ncAA (Cirino et al., 2003; Hoesl et al., 2011; Kiick and Tirrell, 2000; Wiltschi, 2012).

For the cost-effective labeling of proteins with Nle at larger scales, the supplementation of Nle in the SPI bioprocess should be replaced by its biosynthesis. In the present study, we engineered an ALS-deficient Met auxotrophic *E. coli* strain to provide significant levels of Nle to prevent its cost-intensive supplementation. Here, we focused on the high-level biosynthesis of Nle under SPI conditions while the incorporation into a target protein is beyond the scope of this study.

2. Materials and methods

2.1. Chemicals and enzymes

All standard chemicals used in this work were purchased from Sigma (St. Louis, MO), Merck KGaA (Darmstadt, Germany) or Roth (Karlsruhe, Germany), if not stated differently. Norleucine and norvaline were obtained from IRIS Biotech GmbH (Marktredwitz, Germany). Enzymes for cloning and PCR were from Thermo Fisher Scientific (Waltham, MA). PCRs were performed using TaKaRa Ex Taq® High-Fidelity DNA Polymerase (Clontech Laboratories, Inc., Mountain View, CA) or Dream Taq® DNA polymerase (Thermo Fisher Scientific). PCR primers were ordered from IDT Inc. (Coralville, IA) in standard desalted quality. Aqueous stock solutions were sterilized by filtration through 0.20 μm CA syringe filters (Lab Logistic Group GmbH, Meckenheim, Germany).

2.2. Strains and plasmids

E. coli B834(DE3) (*E. coli* B F^- *ompT hsdS*(r_B^- m_B^-) dcm^+ *gal* λ(DE3) *endA met*; Merck KGaA) was the host for ALS deletion and DH5α (*E. coli* K–12 F^- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-; Thermo Fisher Scientific) was used for cloning experiments and plasmid propagation. Transformation of the *E. coli* strains was carried out by electroporation as described by Seidman et al. (2001). To generate $pLEU^{fbr}$, pREP4 (Qiagen, Hilden, Germany) containing a synthetic constitutive HCE promoter (Poo et al., 2002) was PCR-amplified with primers BPp251 and BPp252 (Supporting information, Table S1). The PCR fragment was circularized by *in vivo* assembly (Sawitzke et al., 2007) with *leuA*^{fbr}*BCD* from plasmid pIAA16 (Connor and Liao, 2008) (kindly provided by Prof. James C. Liao). Plasmid $pLEU^{fbr}$ was verified by sequencing (Supporting information, Sequence S4). Plasmid pOYE (Schittmayer et al., 2011) was obtained from an in-house strain collection and pTTL was reconstructed according to Hoesl et al. (2011). Briefly, the coding sequence of the lipase from *Thermoanaerobacter thermohydrosulfuricus* was PCR-amplified from synthetic DNA (IDT Inc.) using primers BPp244 and BPp245 (Supporting information, Table S1). The PCR fragment was inserted into pQE80L (Qiagen) cut with EcoRI and HindIII by Gibson isothermal assembly (Gibson et al., 2009).

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