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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Cloning *Rosa hybrid* phenylacetaldehyde synthase for the production of 2-phenylethanol in a whole cell *Escherichia coli* system

Yigal Achmon · Zohar Ben-Barak Zelas · Ayelet Fishman

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Abstract 2-Phenylethanol (2-PE) is a desirable compound in the food and perfumery industries with a characteristic rose fragrance. Until now, most of the studied biotechnological processes to produce 2-PE were conducted using natural 2-PE-producing yeasts. Only several researches were conducted in other genetically engineered microorganisms that simulated the Ehrlich pathway for the conversion of amino acids to fusel alcohols. Here, a novel metabolic pathway has been designed in *Escherichia coli* to produce 2-PE, using the *Rosa hybrid* phenylacetaldehyde synthase (PAAS), a pyridoxal 5′-phosphate (PLP)-dependent enzyme capable of transforming L-phenylalanine (L-phe) into phenylacetaldehyde by decarboxylation and oxidation. To overcome the enzyme insolubility in *E. coli*, several plasmids and host strains were tested for their expression ability. The desired results were obtained by using the pTYB21 plasmid containing the intein tag from the *Saccharomyces cerevisiae* VMA1. It was discovered that the intein PAAS activity is temperature-dependent, working well in the range of 25 to 30 °C but losing most of its activity at 37 °C. When external PLP cofactor was added, the cells produced 0.39 g l^{-1} 2-PE directly from L-phe. In addition, a biotransformation that was based only on internal de novo PLP synthesis produced 0.34 g l^{-1} 2-PE, thus creating for the first time an *E. coli* strain that can produce 2-PE from L-phe without the need for exterior cofactor additions.

Keywords 2-Phenylethanol · Phenylacetaldehyde synthase · Whole cell biotransformation · Pyridoxal 5′-phosphate · Ehrlich pathway

Introduction

The world's demand for using sustainable processes, in general, and in the food industry, in particular, creates the motivation to seek processes that are environmentally friendlier and involve materials that are neither dangerous to human health nor polluting the environment (Jenck et al. 2004; Notarnicola et al. 2012). 2-Phenylethanol (2-PE) is an aromatic substance with fragrance notes of a rose making it highly desired in the perfumery industry (Etschmann et al. 2002). In addition, 2-PE is used as an aromatic additive in the food industry as well as a preservative. Today, most of the world's 2-PE production (more than 10,000 tons per year; Hirata et al. 2012) is carried out by using chemical methods involving toxic reagents, such as benzene and styrene oxide, and also harsh production conditions (>300 °C; Etschmann et al. 2002). Replacing this process with a biotechnological alternative is therefore highly desired. It is also known that 2-PE is produced in nature by many organisms such as yeast and plants (Etschmann et al. 2002; Sakai et al. 2007). Yeast fermentations for producing 2-PE with or without an in situ product removal step are widely described in the art (Achmon et al. 2011; Eshkol et al. 2009; Etschmann et al. 2002; Sendovski et al. 2010). In contrast to the extensively studied yeast-based production methods, *Escherichia coli* is rarely used. *E. coli* is devoid of the Ehrlich pathway which enables the natural transformation of L-phenylalanine (L-phe) to 2-PE via three enzymatic steps: (i) transamination by aromatic amino acid aminotransferases, converting L-phe to phenylpyruvate; (ii) decarboxylation by phenylpyruvate decarboxylase, converting phenylpyruvate to phenylacetaldehyde; and (iii)

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reduction through dehydrogenation by phenylacetaldehyde dehydrogenase, converting phenylacetaldehyde to 2-PE (Fig. 1; Hazelwood et al. 2008). Yet there are several advantages in the use of *E. coli* as a host for whole cell biotransformation, among them, the rapid growth rate and ease of genetic manipulation (Yu et al. 2011). Two notable groups took this advantage and impressively managed to partially engineer the Ehrlich pathway into the central metabolism of *E. coli* by adding the two genes from the pathway that perform the decarboxylation and the reduction steps (Atsumi et al. 2008; Koma et al. 2012). An additional research described the cloning of the Ehrlich pathway into *E. coli* by the use of four different genes and two plasmids (Hwang et al. 2009). Until now, all studies on the production of 2-PE by *E. coli* used the Ehrlich pathway as a model. However, there are other metabolic pathways in nature for 2-PE production and among them is the phenylacetaldehyde synthase (PAAS) pathway (Fig. 1; Gonda et al. 2010; Kaminaga et al. 2006). This unique enzyme shows dual functionality and catalyzes L-phe directly into phenylacetaldehyde (Kaminaga et al. 2006), thus performing the first two reactions of the Ehrlich pathway in one step. PAAS is considered a cytosolic homotetrameric enzyme belonging to group II pyridoxal 5′-phosphate (PLP)-dependent amino-acid decarboxylases (Kaminaga et al. 2006). PAAS is found in several different types of plants such as roses and petunias (Kaminaga et al. 2006; Mohd-Hairul et al. 2010; Sakai et al. 2007). To date, PAAS has not been used in any biotechnological process for the production of 2-PE. The incorporation of PAAS instead of the three-enzyme Ehrlich pathway could lead to a simpler process that will be more convenient in terms of scaling up and with fewer variables (gene expression, exterior co-factors, etc.) that can influence the process in an unknown manner. Moreover, this one enzyme can be further subjected to protein engineering for improvement of its activity. Finally, since it is not part of the metabolic pathway of the cell, the growth phase and the production step can be disconnected unlike the yeast system.

In this work, we describe the cloning of the *Rosa hybrida* cv. PAAS using the intein tag from the *Saccharomyces cerevisiae* VMA1, in order to form the first *E. coli* strain capable of directly producing 2-PE from L-phe by the addition of only one heterologous gene and with endogenous alcohol dehydrogenases, without the need for external co-factors.

Materials and methods

Chemicals

2-PE, L-phe, PLP, sodium dodecyl sulfate (SDS), phenylacetaldehyde, kanamycin, and ampicillin were purchased from Sigma-Aldrich Chemical (Rehovot, Israel). All materials used were of the highest purity available and were used without further purification.

Enzyme

The *R. hybrida* cv. PAAS (Gen-Bank accession number DQ192639) was synthesized with an optimized codon usage for *E. coli* using OptimumGene™ algorithm (GenScript USA, Piscataway, NJ, USA) and deposited in Gen-Bank accession number KF500528.

Strains and plasmids

E. coli strains BL21(DE3), Rosetta (Novagen, Madison, WI, USA), ER2566, and SHuffle [New England Biolabs, Ipswich, MA, USA (NEB)] were used for expressing the PAAS enzyme. The plasmids that were used in this study were pETDuet-1,

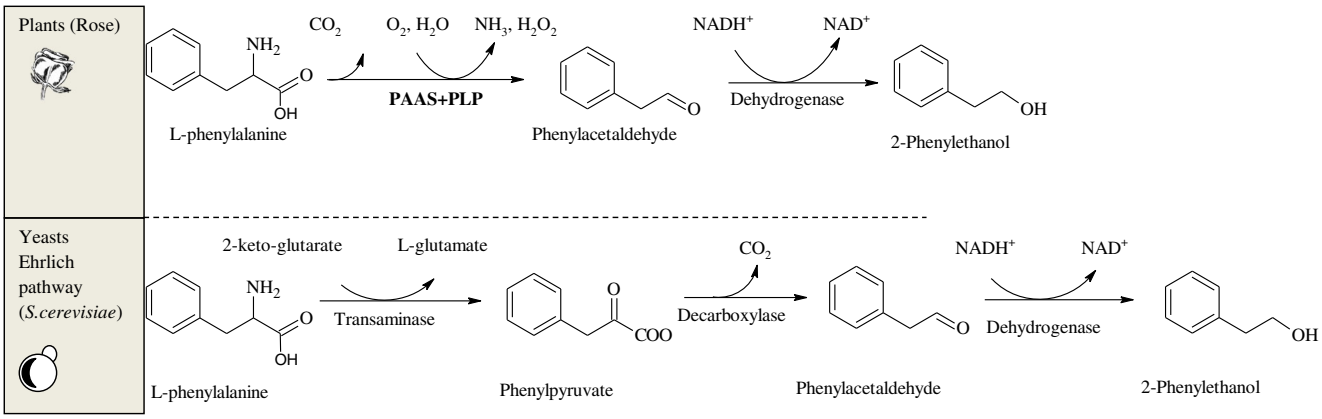

Fig. 1 2-PE metabolic pathways. On the top, the plant metabolic pathway with two enzymatic steps is presented: the first step is the conversion of L-phe into phenylacetaldehyde by PAAS with PLP as the cofactor. The second step is the conversion of phenylacetaldehyde to 2-PE by a dehydrogenase. On the bottom, the Ehrlich pathway abundant in yeast is shown including three well-described enzymatic steps (Hazelwood et al. 2008)

pET9a, and pET12a (Novagen), pTYB21 and pTXB1 from the IMPACT kit (NEB).

Construction of vectors

All the vectors were constructed using the following conditions: The PCR mix consisted of HF×5 buffer with 1.5 mM $MgCl_2$ (Thermo Scientific, Sunnyvale, CA, USA), 0.5 mM dNTPs (1:1:1:1 ratio), 2 ng μl^{-1} forward and reverse primers (see supplementary data Table S1), 0.5 ng μl^{-1} PAAS DNA template, 0.02 U μl^{-1} Phusion® high-fidelity DNA polymerase (Thermo Scientific), and sterile water to a final volume of 50 μl. PCR thermo cycles were as follows: 96 °C for 3 min followed by 30 cycles of amplification consisting of 10 s of denaturation at 98 °C, 30 s of annealing at 60 °C, extension at 72 °C for 1 min, and a final holding at 72 °C for 10 min. The PCR fragment was purified with a PCR purification kit (Qiagen, Hilden, Germany) and was cut by the appropriate restriction enzymes according to the primers (Table S1). The purified cut PCR fragment was ligated to one of the following vectors: pETDuet-1 to give pETDuet-1/PAAS and pETDuet-1/tPAAS [the latter containing the addition of an expression tag (Hansted et al. 2011a)], pET9a to give pET9a/PAAS, pET12a to give pET12a/ompTPAAS and pET12a/SHPAAS, pTYB21 to give pTYB21/PAAS, and pTXB1 to give pTXB1/PAAS. Plasmids were transformed into the desired *E. coli* strain by electrophoresis, except for pET12a/SHPAAS that was transformed into *E. coli* SHuffle® (NEB) by heat shock according to the SHuffle® protocol. The transformation was verified by colony PCR and the positive colonies were sequenced to validate that there were no sequence errors. A summary list of all the constructed strains that were used in this study is found in Table 1.

Expression culture conditions

All the strains were grown for protein expression in LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, and 10 g l^{-1} NaCl) or TB medium (12 g l^{-1} tryptone, 24 g l^{-1} yeast extract, 4 ml glycerol, 9.4 g l^{-1} K_2HPO_4, and 2.2 g l^{-1} KH_2PO_4) containing appropriate antibiotics. Fresh colonies were picked up from agar plats to 500 ml LB in baffled Erlenmeyer flasks. Cells harboring pETDuet-1/PAAS, PETDuet-1/tPAAS, pTYB21/PAAS, and pTXB1/PAAS were grown at 37 °C after induction at OD_{600} 0.5 with 0.2 mM isopropyl β-D-1-thio-galactopyranoside (IPTG), then incubated at 15 °C and 250 rpm (Orbital Shaker Incubator, MRC, Holon, Israel) for 18 h. The pET9a/PAAS, pET12a/ompTPAAS, and pET12a/SHPAAS were grown in TB at several temperatures (37, 30, and 25 °C) overnight. All the strains were harvested using centrifugation at 5,000×*g* for 15 min (Sigma, 4 K15 centrifuge with 12166 rotor, Osteroid, Germany) for biotransformation reactions.

Whole cell biotransformation

The pellets of the above strains were washed twice in distilled water in order to remove growth media residues. The pellets were then resuspended in M9 minimal medium (Sambrook and Russell 2001) consisting of 12.8 g l^{-1} $NaHPO_4$ $7H_2O$, 3 g l^{-1} KH_2PO_4, 2.5 g l^{-1} NaCl, 5 g l^{-1} NH_4Cl, 2 mM $MgSO_4$, 20 % dextrose, and 20 ml 0.1 mM $CaCl_2$ to give the desired OD_{600} value (5 or 10). The reaction started with the addition of 0.25–0.8 g l^{-1} L-phe and 0.2 mM PLP into 25 ml M9 minimal medium. The biotransformation was conducted by incubating the samples at three different temperatures [room temperature (RT), 30 and 37 °C] at 250 rpm for 48 h. Another set of reactions was conducted at RT and at 250 rpm for 48 h in

Table 1 Strains and plasmids used in this study

Vector	Strain	Plasmid	Description	Source
pETDuet-1/PAAS	*Escherichia coli* Rosetta™	pETDuet-1	Rosetta (DE3) used for overcoming codon bias. pETDuet-1 co-expression of two target genes, T7 promoter/lac operator.	Novagen®
pETDuet-1/tPAAS		pETDuet-1	*InfB* (1–21) sequence as an expressivity tag.	
pET9a/PAAS		pET9a	pET9a for constitutive protein expression.	Novagen®
pET12a/*ompT*PAAS	*E. coli* SHuffle®	pET12a	pET12a ompT sequence for potential periplasmic export of target proteins.	Novagen®
pET12a/SHPAAS		pET12a	SHuffle expresses constitutively a chromosomal copy of the disufide bond isomerase DsbC.	New England Biolabs®
pTYB21/PAAS	*E. coli* ER2566	pTYB21	ER2566 is provided as a host strain for the expression of a target gene cloned into the IMPACT vectors. pTYB21 vector utilizes an intein from the *Saccharomyces cerevisiae* VMA1 gene.	New Englend Biolabs®
pTXB1/PAAS		pTXB1	pTXB1 contains a mini-intein from the *Mycobacterium xenopi gyrA* gene	New England Biolabs®

the absence of the PAAS co-factor PLP. Samples of 500 μl were taken at various time points, centrifuged at 13,500 rpm for 1 min and then the supernatant was filtered through 0.22 μm PVDF filter (Millex HV, Millipore, Cork, Ireland) in order to stop the reaction and prepare the samples for high-performance liquid chromatography (HPLC) analysis.

Analytical methods

Cell density OD_{600} was measured with an Ultrospec 2100 pro-spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA). The conversion of L-phe to 2-PE was determined by a high-performance liquid chromatography (HPLC) Dionex UltiMate 3000 (Thermo Scientific) using a Gemini 5 μ C18 110A column (5 μm, 4.6×250 mm; Phenomenex, Torrance, CA, USA), and the results were analyzed with Thermo Scientific Dionex Chromeleon software version 7.1 SRI. The solvent composition changed from 99 % water and 1 % acetonitrile to 70 % water and 30 % acetonitrile within 10 min followed by a 2 min hold time and a successive gradual increase to the initial conditions within 10 min. A 1 ml min^{-1} constant flow rate was kept. Under these conditions, elution times of L-phe and 2-PE were 9.2 and 14.8 min, respectively (see supplementary data Fig. S1).

Proteomic analysis

pTYB21/PAAS overexpressing cells were lysed by sonication with Vibra Cell sonicator (Sonics, Newtown, CT, USA, pulser 9.9 s on, 5.5 s off, for 3 min) and then were centrifuged at 15,000×*g* for 30 min at 4 °C. The supernatant was loaded onto the IMPACT chitin column and was washed according to the IMPACT kit protocol. After on-column cleavage for 40 h with 50 mM dithiothreitol (DTT), the eluted fraction was run on SDS-PAGE gel. The suspected band was digested by trypsin and chymotrypsin and analyzed by LC-MS/MS on LTQ-Orbitrap (Thermo Scientific). The identification was done by the Discoverer software version 1.3 and the mass spectrometry data was analyzed using the SEQUEST® 3.31 software searching against the UniProt database and the PAAS-specific amino acids sequence. The proteomic analysis was performed at the Smoler Proteomics Center (Faculty of Biology, Technion, Israeli Institute of Technology, Israel).

Results

Expression and solubility of PAAS in different vectors

The *paas* synthetic gene was first cloned into the pETDuet-1 vector that contains two multiple cloning sites. The assumption was that by cloning the *paas* gene with the *adhI* gene originating from *S. cerviciae*, it would be possible to convert L-phe directly to 2-PE (Fig. 1) similarly to the Ehrlich pathway. Unfortunately, protein overexpression or biological activity was not observed for PAAS (although ADH was both expressed and active). Several experiments with different expression temperatures and different IPTG concentrations were conducted but without any success. The first step to overcome the expression problem was to add to the *paas* gene the expression tag *infB* derived from *E. coli*, that was shown to be a highly efficient solubility tag sequence (aug aca gau gua acg auu aaa; Hansted et al. 2011b; Table 1, PETDuet-1/tPAAS). With the expression tag, overexpression was observed randomly, but only in the bacterial pellet and in an insoluble form (see supplementary data Fig. S2).

Examination of the PAAS amino acid sequence revealed the presence of 13 cysteine residues, suggesting the probability of disulfide bonds in the PAAS tertiary structure. The possibility that disulfide bonds were involved in stabilizing the tertiary structure of PAAS led to the use of pET12a and *E. coli* SHuffle (Table 1, pET12a/ompTPAAS and pET12a/SHPAAS). The SHuffle strain was designed to facilitate correct expression of disulfide bonds in active proteins and lead to high yields within its cytoplasm. This strain is based on the *trxB gor* suppressor strain SMG96, in which cytoplasmic reductive pathways have been diminished, allowing for the formation of disulfide bonds in the cytoplasm. The SHuffle strain was further engineered with a major improvement by integration of disulfide bond isomerase, *DsbC*, into its chromosome, and by that assisting the formation of correctly folded multidisulfide bonded proteins (Lobstein et al. 2012). Likewise, the *ompT* tag was used for potential periplasmic export of target proteins. The periplasm mimics the oxidative environment of eukaryotic cells in the ER, thus helping correct folding of proteins with disulfide bonds (de Marco 2009). Unfortunately, the use of these vectors did not yield overexpression or enzymatic activity.

Another approach was aimed at making the PAAS expression system simpler and therefore the gene was introduced into the "leaky" pET9a vector to avoid the use of IPTG as an inducer (Table 1, pET9a/PAAS). Biological activity was then observed for the first time with the use of pET9a/PAAS in a whole cell biotransformation as described in the next section. Nonetheless, the lack of clear overexpression of the protein led to the use of the Intein Mediated Purification with an Affinity Chitin-binding Tag (IMPACT) system. The IMPACT system is designed for protein expression and purification by utilizing the inducible self-cleavage activity of protein splicing elements (termed inteins) to separate the target protein from the affinity tag (Chong et al. 1997). IMPACT was successfully used to overexpress several enzymes with similar activity to PAAS such as *Arabidopsis thaliana* aromatic acetaldehyde synthase, *Papaver somniferum* tyrosine decarboxylase, and *Catharanthus roseus* tryptophan decarboxylase (Torrens-Spence et al. 2013). The *paas* gene was cloned into a plasmid

with a C-terminus intein tag (pTXB1) or to the plasmid with an N-terminus intein tag (pTYB21). Both vectors enabled overexpression of the enzyme (Fig. 2), however, the expression from pTXB1/PAAS was in an insoluble form, while the pTYB21/PAAS showed soluble protein on the SDS gel and was used for activity tests and protein purification.

Whole cell biotransformation for the conversion of L-phe to 2-PE with pET9a/PAAS

The pET9a/PAAS system was tested in parallel for expression of the enzyme and for activity. As no IPTG induction was needed, the bacteria were grown for 16 h to reach a high cell concentration in TB media. In order to examine the influence of temperature on expression, cells were grown at 37, 30 and 16 °C. The biotransformation was conducted at RT and cell density was normalized to OD_{600}=10 with M9 medium containing 0.25 g l^{-1} L-phe and 0.2 mM PLP. The negative control (*E. coli* harboring pET9a without an insert) showed no significant amount of 2-PE, indicating that the activity was associated with the PAAS (Fig. 3). As shown in Fig. 3, pET9a/PAAS showed activity in all three growth temperatures. Interestingly, the metabolic pathway intermediate, phenylacetaldehyde, could not be detected throughout the biotransformation. The highest total 2-PE production level of pET9a/PAAS, 0.15 g l^{-1}, was at 37 °C (Fig. 3a), the lowest was at 30 °C [0.05 g l^{-1} (Fig. 3c)], while at 16 °C, 0.1 g l^{-1}(Fig. 3b) was produced. Thus, no direct correlation between PAAS activity and the growth/expression temperature using pET9a/PAAS vector was observed. In all of the systems tested, L-phe was consumed within the first 30 h. Accumulation of 2-PE was clearly correlated with L-phe consumption as described in Fig. 3d. As no overexpression of the protein was detected in the SDS-PAGE analysis of pET9a/PAAS (soluble or insoluble fraction), it is assumed that the activity was associated with low or instable expression levels (see "Discussion" section).

Optimization of the pTYB21/PAAS expression system

The IMPACT system is commercially available with two different inteins on two different plasmids: the pTXB1 for C-terminus expression with the desired target protein, and the pTYB21 for N-terminus expression (Table 1). The *paas* gene was cloned in both systems. Only the combination of N-terminus cloning (pTYB21) to the intein tag from the *S. cerevisiae* VMA1 gene (Sce VMA1 intein; 454 amino acids) enabled the expression of soluble and active PAAS enzyme (pTYB21/PAAS; Lane 3A in Fig. 2). The PAAS protein was purified according to the suppliers' protocol and the band (lane A5, Fig. 2) was verified as PAAS by proteomic analysis using trypsin and chymotrypsin digestion and analysis by LC-MS/MS (see "Materials and methods" section). Subsequently, the eluted fraction from the chitin column was also tested for PAAS activity (Fig. S3) and showed conversion of L-phe to phenylacetaldehyde. It is important to note that the whole cell biotransformation was carried out with the intein-PAAS (iPAAS) fusion construct. The expression conditions optimized for pTYB21/PAAS were induction with 0.2 mM IPTG after the culture has reached an OD_{600} of approximately 0.5. The culture was then transferred to 15 °C for 16–20 h for overexpression of the enzyme. For optimizing the biotransformation conditions, the cells were harvested and diluted to OD_{600} 5 or 10 to show true dependence of transformation rate on enzyme concentration and were subjected to three different

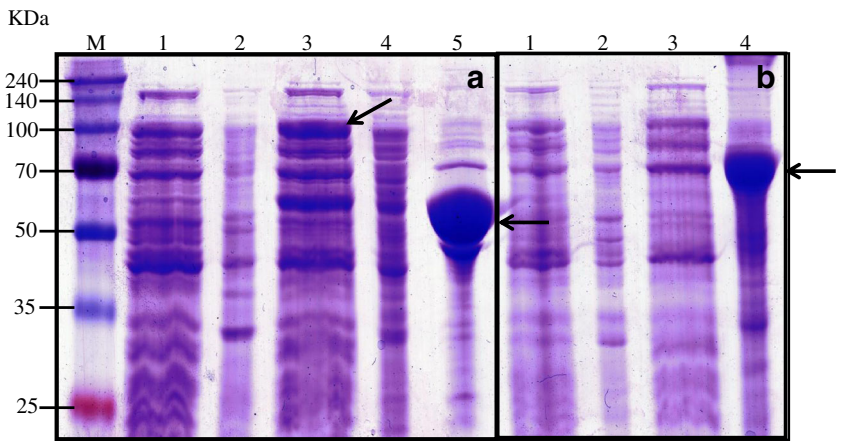

Fig. 2 PAAS expression in various *Escherichia coli* systems is presented in a 12 % SDS-PAGE gel. **a** *Lane M* molecular weight marker. The pTYB21/PAAS system: *Lanes 1* pTYB21 with no insert, cytosolic fraction, *2* pTYB21 with no insert, pellet fraction, *3* pTYB21/PAAS cytosolic fraction (the *arrow* indicates the fused VMA1 intein-PAAS with calculated MW of 107 kDa), *4* pTYB21/PAAS pellet fraction, and *5* the eluted fraction of the PAAS protein following the chitin column [the *arrow* indicates the PAAS enzyme with calculated MW of 57 KDa (without the intein tag)]. **b** The pTXB1/PAAS overexpression system: *Lanes 1* pTXB1 plasmid with no insert, cytosolic fraction, *2* pTXB1 with no insert, pellet fraction, *3* pTXB1/PAAS cytosolic fraction, and *4* pTXB1/PAAS pellet fraction (the arrow indicates the fused Mxe GyrA intein-PAAS with a calculated MW of 72 KDa). Pellet fractions were suspended in 8 M urea prior to loading. 300 μg of protein was loaded in all lanes except lane 5 (**a**), the purified PAAS (500 μg)

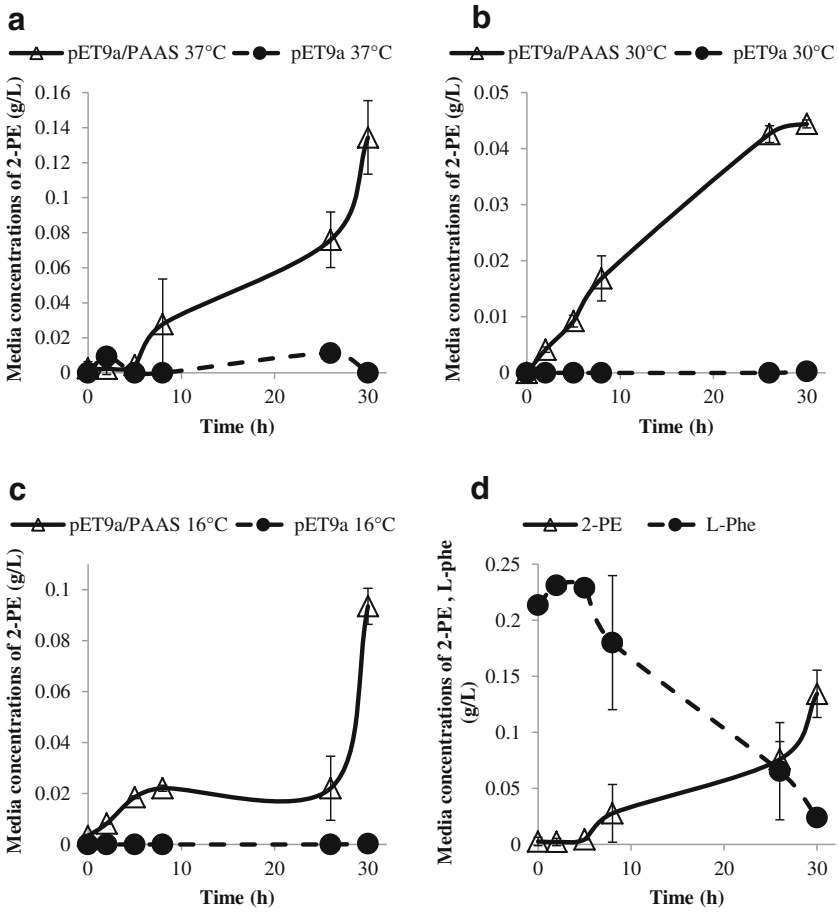

Fig. 3 Conversion of L-phe to 2-PE using *E. coli* cells harboring pET9a/PAAS grown at different expression temperatures. The pET9a was the negative control without a PAAS gene in the plasmid. **a** 37 °C, **b** 30 °C, **c** 16 °C, and **d** 2-PE production vs. L-phe consumption in the 37 °C expression system. The biotransformation reaction was performed at 25 °C, and the progress was monitored by measuring the substrate and the product using an HPLC. The biotransformation contained cells at OD=10, 0.25 g l^{-1} L-phe, 0.2 mM PLP. The results are an average of three independent measurements

temperatures of RT (~25°C), 37, and 30 °C. A maximal 2-PE concentration of 0.2 g l^{-1} was obtained at 30 °C for either of the cell concentrations (Fig. 4). The RT systems showed lower product concentrations than at 30 °C, but had a better initial production rate. The highest initial conversion rate was 0.0155 g l^{-1} h^{-1} at RT with OD_{600} 10. Interestingly, at 37 °C, the diluted cell system (5 OD_{600}) showed no activity at all (Fig. 4a), and the more concentrated system (10 OD_{600}) was only slightly active [0.04 g l^{-1} (Fig. 4b)]. It appears that the whole cell system comprising of the fused iPAAS possesses an optimal temperature range of 25–30 °C. The RT system and the 30 °C system, at the two cell densities, consumed the entire amount of L-phe (0.5 g l^{-1}) within 30 h of biotransformation (data not shown). Further experiments are needed to determine the optimal conditions for 2-PE production using this system.

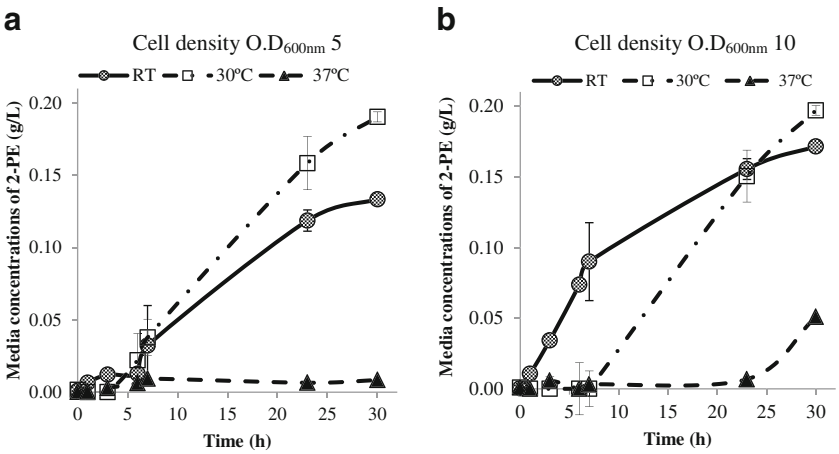

Fig. 4 Conversion of L-phe to 2-PE by a whole cell biotransformation exploiting *E. coli* pTYB21/PAAS at three different temperatures (RT, 30 and 37 °C) and at two different cell density matrices. The expression temperature was 15 °C for all systems. The reaction progress was monitored by measuring the product accumulation using an HPLC. **a** Cell density of OD 5. **b** Cell density of OD 10. The results are an average of three independent measurements

Influence of the PLP co-factor

PAAS is a PLP-dependent enzyme and therefore addition of the co-factor to the whole cell system was investigated. The biotransformation was conducted in 25 ml M9 medium supplemented with 0.8 g l^{-1} L-phe and 0.2 mM PLP at RT and with a cell density of 10 OD_{600} for 48 h. The results (Fig. 5) proved unequivocally that the pTYB21/PAAS whole cell system can convert L-phe to 2-PE without the need for cofactor addition. A slightly higher product concentration was reached with the addition of PLP to the media (0.39 g l^{-1} vs. 0.34 g l^{-1}), but the overall performance of the systems were very similar. Both systems showed high initial production rates of 0.044 g l^{-1} h^{-1}, but then production stopped possibly due to product inhibition (Lucchini et al. 1993). Even though the production rate had decreased, the pTYB21/PAAS strain succeeded in consuming most of the L-phe (95 %, data not shown) with approximately 60 % conversion to 2-PE within 48 h.

Discussion

This study aimed to create a novel platform for an *E. coli* whole cell biotransformation system for the production of 2-PE from L-phe with the use of PAAS. There is a demand for creating a highly effective system for the production of 2-PE (Rabinovitch-Deere et al. 2013), and the fact that the rose metabolic pathway for the synthesis of 2-PE (Fig. 1 top) was never exploited in *E. coli* has made this an attractive route. Although an *E. coli* based biotransformation is an appealing system (Ishige et al. 2005), the expression of soluble heterologous eukaryotic genes remains a great challenge (Baneyx and Mujacic 2004). To overcome the insolubility problem, we initially cloned the *paas* gene into the IPTG-inducible vector pETDuet-1, aiming to overexpress the two enzymes, PAAS and alcohol dehydrogenase (ADH1), and thus design a short metabolic pathway for converting L-phe to 2-PE (Fig. 1). Although expression optimization was conducted using classical approaches (such as low temperature and various IPTG concentrations), the constructs pETDuet-1/PAAS and PETDuet-1/tPAAS were not active and did not express the PAAS protein.

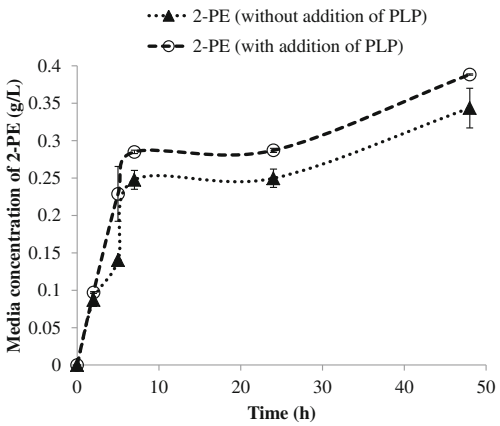

Fig. 5 Conversion of L-phe to 2-PE in a whole cell biotransformation by *E. coli* pTYB21/PAAS. Both systems were induced with 0.2 mM IPTG and were grown at 15 °C for PAAS expression. Biotransformation was conducted at room temperature in M9 medium with 0.8 g l^{-1} L-phe. PLP (0.2 mM) was added to one of the systems (○) and an equal amount of water to the second system (▲). The reaction progress was monitored by measuring the product accumulation using an HPLC. The results are an average of three independent measurements

The presence of 13 cysteine residues in the PAAS sequence indicates that there may be a number of disulfide bonds in the protein. In nonoptimal conditions for disulfide bonds, formation of the tertiary structure can cause damage and misfolding of the protein in *E. coli*. Such problems were described for example for bovine β-lactoglobulin containing five cysteine residues (Ponniah et al. 2010) and also for Nogo-A-specific exon 3 that has eight cysteine residues (Fiedler et al. 2002). An expression system such as the SHuffle strain and the *ompT* tag may resolve the problem by creating an oxidizing environment as was reported by several groups (Baneyx and Mujacic 2004; Berkmen 2012; de Marco 2009). This approach was attempted by creating two plasmids with those supporting systems, the pET12a/ompTPAAS (with *ompT* tag) and the pET12a/SHPAAS (with the *E. coli* SHuffle strain). None of these systems enabled the soluble expression of PAAS leading to the conclusion that disulfide bonds were not the obstacle for correct protein folding.

Using a simple protein expression system, such as pET9a, is often the first choice when a nontoxic protein is produced (Yin et al. 2007). This vector allowed for the first time to get bioactivity in a whole cell system. However, the product detected by the HPLC was 2-PE rather than phenylacetaldehyde, suggesting that the aldehyde was further transformed by an ADH present in *E. coli* cells. Indeed, it was reported that the *E. coli* genome contains at least seven alcohol dehydrogenases (Torrens-Spence et al. 2013). This phenomenon was also observed by Koma et al. (2012) who introduced the phenylpyruvate decarboxylase gene (T7p-*ipdC*) and reported an automatic reduction of phenylacetaldehyde in vivo, apparently by endogenous aldehyde reductases present in *E. coli*, that lead to 2-PE accumulation. Despite the encouraging biological activity of the pET9a/PAAS strain, the PAAS expression was still insufficient and optimization could not bridge that gap. The biological activity may result from low expression levels of the PAAS under the T7 promoter. We ruled out the additional option that the pET9a/PAAS strain exhibited natural activity since cells harboring the plasmid devoid of the *paas* gene could not produce 2-PE. The possibility that insoluble enzyme in the form of inclusion bodies can be active in the whole

cell (García-Fruitós et al. 2012) was not feasible as well since strains with insoluble enzyme as pETDuet-1/tPAAS and pTXB1/PAAS (Fig. 2) were inactive.

A biotransformation system dependent on low expression levels of enzymes is not attractive for commercial purposes and therefore a more efficient vector was sought. The IMPACT system comprising a chaperone tag enabled the production of soluble intein-PAAS accompanied with bioactivity. The pTYB21/PAAS whole cell biotransformation system showed high sensitivity to temperature, and at 37 °C, almost no activity was observed. This can be attributed to the fact that the PAAS originated from roses (Bar-Even et al. 2011). *E. coli* harboring the pTYB21/PAAS is the first strain to date that have shown an active PAAS enzyme with a fused tag (intein). In a previous research (Farhi et al. 2010; Kaminaga et al. 2006) on PAAS from Petunia, the enzyme was purified from *E. coli* using a pET-28a vector without a tag and the activity of the purified enzyme was described. In our hands, only inclusion bodies were obtained using a similar pET system, and attempts to dissolve or refold the enzyme failed to produce active protein.

To further evaluate the system as a biotechnological process, we examined whether the addition of the cofactor PLP can be avoided. *E. coli* can synthesize PLP de novo and has free PLP inside the cell cytoplasm at concentrations of 120 μM (Fu et al. 2001) which can be sufficient for PAAS activity. Therefore, it was not surprising that the system showed activity in the absence of added PLP (Fig. 5). Another advantage that may lie in the use of pTYB21/PAAS whole cell system is the utilization of the cofactor PLP, thus avoiding feedback inhibition on its de novo synthesis by the endogenous pyridoxal kinase (pyridoxal kinase forms a complex with the product PLP to form an inactive enzyme complex; Ghatge et al. 2012). The whole cell biotransformation system expressing pTYB21/PAAS has several advantages over other systems. Koma et al. (2012) mimicked the Ehrlich pathway by incorporating the phenylpyruvate decarboxylase gene (*ipdC*) from *Azospirillum brasilense* and the phenylacetaldehyde dehydrogenase gene (*feaB*) from *E. coli* into the chromosomes of phenylalanine and tyrosine overproducing *E. coli* cells (2012). Atsumi and co-workers cloned α-ketoisovalerate decarboxylase (*Kivd*) from *Lactococcus lactis* and alcohol dehydrogenase 2 (ADH2) of *S. cerevisiae* using two different plasmids into *E. coli* cells (Atsumi et al. 2008). Our system avoids the use of several heterologous genes, such as phenylpyruvate decarboxylase (*ipdC*) and *Kivd* decarboxylase from *L. lactis* (Koma et al. and Atsumi et al., respectively), utilizing phenylpyruvate as the source for 2-PE rather than L-phe. Using phenylpyruvate may cause the creation of nonspecific products (Koma et al. 2012) or the need for an expensive substrate in order to produce higher titers (Atsumi et al. 2008). Similarly, the system that was created by Hwang et al. (2009), despite showing a high efficiency, is a complex system containing two different plasmids pET24ma and pETDuet-1 and thus evoking the need for additional antibiotics. Additionally, this system involves four heterologous enzymes (transaminase, carbonyl reductase, phenylpyruvate decarboxylase, and glucose dehydrogenase), and the system activity depends on all four genes and is therefore more problematic for future biotechnology processes. The pTYB21/PAAS exploits the bifunctionality characteristic of the PAAS (Kaminaga et al. 2006) and with the use of a single specific enzyme, 2-PE can be produced from L-phe.

In summary, we have created a novel system which may be a good starting point for a future biotechnological process to produce 2-PE. Our system showed for the first time the possibility of using the IMPACT system intein tag as a protein solubility supporter, not just as a purification aid. Expanding our knowledge about the structure of the PAAS will be needed for the sake of applying protein engineering for further development of the system and for the production of higher amounts of 2-PE. Parallel with protein engineering, in situ product removal will be needed to avoid product toxicity in a future commercial production system.

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