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Highly efficient Michael-type addition of acetaldehyde to β-nitrostyrenes by whole resting cells of *Escherichia coli* expressing 4-oxalocrotonate tautomerase

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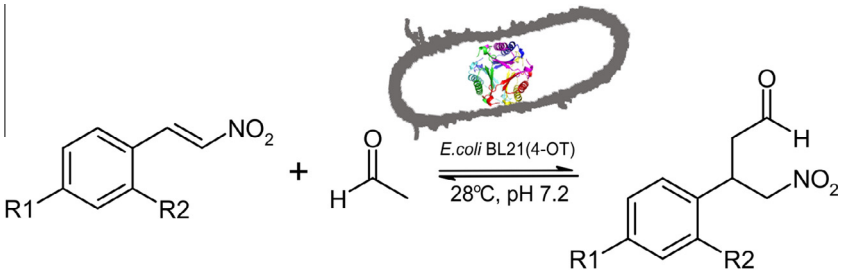
HIGHLIGHTS

- Biocatalyst based on whole cells and 4-oxalocrotonate tautomerase was developed.
- (*S*)-4-nitro-3-phenyl-butanal was obtained in high optical purity (>99% *ee*) and yield.
- Biotransformation products were amenable to further chemical derivatizations.
- Michael addition of acetaldehyde to halo substituted β-nitrostyrenes was achieved.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel whole cell system based on recombinantly expressed 4-oxalocrotonate tautomerase (4-OT) was developed and shown to be an effective biocatalyst for the asymmetric Michael addition of acetaldehyde to β-nitrostyrenes. Optimal ratio of substrates (2 mM β-nitrostyrenes and 20 mM acetaldehyde) and biocatalyst of 5 g of cell dry weight of biocatalyst per liter was determined. Through further bioprocess improvement by sequential addition of substrate 10 mM nitrostyrene biotransformation was achieved within 150 min. Excellent enantioselectivity (>99% *ee*) and product yields of up to 60% were obtained with β-nitrostyrene substrate. The biotransformation product, 4-nitro-3-phenyl-butanal, was isolated from aqueous media and further transformed into the corresponding amino alcohol. The biocatalyst exhibited lower reaction rates with *p*-Cl-, *o*-Cl- and *p*-F-β-nitrostyrenes with product yields of 38%, 51%, 31% and *ee* values of 84%, 88% and 94% respectively. The importance of the terminal proline of 4-OT was confirmed by two proline enriched variants and homology modeling.

1. Introduction

Processes based on enzymes either free, immobilized, or in whole cells, have been implemented in a broad range of industries because they are specific, fast and often save raw materials (i.e. water, energy) compared to synthetic processes (Jegannathan and Nielsen, 2013; Straathof et al., 2002). In the context of Green Chemistry, biocatalysis presents many appealing features such as gentle reaction conditions, use of water as the usual reaction medium and circumvention of the need for functional group activation (Nestl et al., 2011). These properties afford shorter processes which produce less waste and therefore are both environmentally and economically smarter than conventional routes.

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Direct asymmetric Michael-type addition is a fundamental reaction for carbon–carbon bond formation that provides many valuable compounds (Perlmutter, 1992). Achieving good chemical and stereochemical efficiencies in these reactions is of particular interest for the manufacture of numerous precursors used in the chemical and pharmaceutical industries (Munoz Solano et al., 2012). Nitroaromatic compounds such as β-nitrostyrenes are interesting and important groups of industrial chemicals as they have antibacterial properties (Milhazes et al., 2006) and the nitro group is a versatile precursor for many additional functionalities and a wide range of other possible biological activities (Ju and Parales, 2010).

Although a number of reports have appeared to describe efficient Michael-type additions of either aldehydes or ketones to β -nitrostyrenes using organocatalytic approaches usually employing proline-based catalysts (Kotrusz et al., 2004; List et al., 2001; Mase et al., 2006; Wang et al., 2008), biocatalytic routes capable of operating efficiently at high concentrations and high enantioselectivity have not been achieved. For example, promiscuous activity of acidic proteinase from *Aspergillus usamii* was employed in the Michael addition of ketones to nitroolefins in organic media in the presence of water, with reaction times in a range of 100 h (Xu et al., 2011).

4-Oxalocrotonate tautomerase (4-OT) (E.C. 5.3.2.6) belongs to the tautomerase superfamily whose members share catalytic amino-terminal proline and β-α-β fold (Whitman, 2002). 4-OT, encoded by *xylH* gene, as a part of aromatic compounds degradation pathway in *Pseudomonas putida* mt-2 naturally catalyzes the conversion of 2-hydroxyhexa-2,4-dienedioate into 2-oxo-3-hexendioate with terminal proline (Pro1) functioning as a catalytic base during proton transfer (Harayama et al., 1989). This particular enzyme has recently been suggested as an important bridging link between organo- and biocatalysis (Zandvoort et al., 2012a). Due to its promiscuous activity, 4-OT was described to catalyze the Michael-type addition of acetaldehyde to β-nitrostyrene (Zandvoort et al., 2012a), the isomerisation of *cis*-nitrostyrene to *trans*-nitrostyrene (Zandvoort et al., 2012b) as well as aldol condensation and dehydratation activities (Zandvoort et al., 2012c) as free enzyme in aqueous buffer with good enantioselectivity.

We have for the first time developed a biocatalytic route for the efficient synthesis of 4-nitro-3-phenyl-butanal (**3**) based on whole cells *E. coli* BL21 (DE3) expressing recombinant 4-OT (Fig. 1). The majority of biocatalytic studies report the production of bioproducts by enzymes and microorganisms but they do not investigate the recovery of these products from the biotransformation medium and their further transformations. Herein we also investigate the isolation and purification of **3** from aqueous media and the subsequent chemical reduction to biologically important γ-amino alcohol **10**, in this way demonstrating the whole process for the first time. Furthermore, halogen substituted β-nitrostyrenes were included in this study to widen the scope of the biocatalysis and improve product functionality. Finally two new variants of 4-OT with mutations in N-terminus were also generated to provide further insights into promiscous activity of this versatile biocatalyst.

2. Methods

2.1. Reagents

β-Nitrostyrene, 4-chloro-, 2-chloro, and 4-fluoro-β-nitrostyrene were of analytical grade and purchased from Sigma–Aldrich (Munich, Germany). Acetaldehyde and all other chemicals were of analytical grade and purchased from Sigma–Aldrich (Munich, Germany). Ethylacetate, ethanol and other solvents were of HPLC reagent grade and purchased from Fisher Scientific (Hampton, NH, USA). Silica Gel 60 (230–400 mesh ASTM) and TLC aluminum silica gel sheets 60 F254 pre-coated were purchased from Merck KGaA (Darmstadt, Germany).

Pfu Turbo DNA polymerase and T4 DNA ligase and QuikChange® Site-Directed Mutagenesis Kit were purchased from Stratagene (La Jolla, CA, USA). Restriction and DNA-modifying enzymes, *Nde*I and *Bam*HI were obtained from Promega (Madison, WI, USA). The QIAprep spin plasmid mini-prep kit, QIAEX II gel purification kit, and QIAquick PCR purification kit were purchased from QIAGEN (Hilden, Germany).

Ampicillin, isopropyl- β-D-1-thiogalactopyranoside (IPTG), dithiothreitol (DTT), other salts and reagents were purchased from Sigma–Aldrich (Munich, Germany). BugBuster solution was purchased from Merck (Darmstadt, Germany). Glucose, tryptone, yeast extract, casamino acids and other media components were purchased either from Oxoid (Cambridge, UK) or Becton Dickinson (Sparks, MD, USA).

2.2. Strains, plasmids, primers and growth conditions

Pseudomonas putida mt-2 (NCIMB10432), toluene and *m*- and *p*-xylene degrader was obtained from NCIMB culture collection (Aberdeen, Scotland, UK) and used as the source of *xylH* gene (Worsey and Williams, 1975). *Escherichia coli* BL21(DE3) strain and T7 polymerase based expression vector pRSET-B were purchased from Invitrogen (Paisley, UK). Oligonucleotide primers were also obtained from Invitrogen (Table 1).

Standard recombinant DNA techniques were performed as previously described (Sambrook and Russell, 2001). Tautomerase (*xylH* gene) was amplified by PCR using total genomic DNA from *P. putida* mt-2 as template and DNA Engine Thermal Cycler (Bio-Rad, Hercules, USA). The 192 bp fragment generated by PCR was digested with *Nde*I and *BamH*I, and ligated into pRSET-B to generate pRSET-TAUT (Table 1).

Site directed mutagenesis (SDM) was carried out using a QuikChange® Site-Directed Mutagenesis Kit according to the manufacturer's instructions using appropriate primers (Table 1) for the introduction of single mutations. New plasmids (Table 1) were also transformed into *E. coli* BL21(DE3). To verify recombinant plasmid

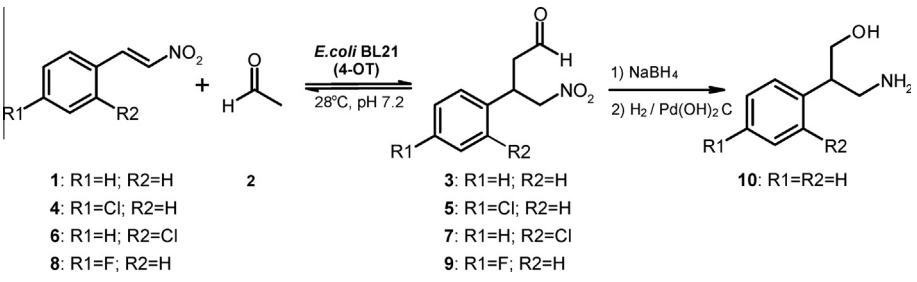

Fig. 1. Biotransformation of nitrostyrenes by *E. coli* BL21cells expressing 4-OT and subsequent chemical transformation to obtain amino alcohol. (1 = β-nitrostyrene; 2 = acetaldehyde; 3 = 4-nitro-3-phenylbutanal; 4 = 4-chloro- β-nitrostyrene; 5 = 3-(4-chlorophenyl)-4-nitrobutanal; 6 = 2-chloro- β-nitrostyrene; 7 = 3-(2-chlorophenyl)-4-nitrobutanal; 8 = 4-fluoro- β-nitrostyrene; 9 = 3-(4-fluorophenyl)-4-nitrobutanal).

Table 1
Oligonucleotide primers, plasmids and *Escherichia coli* strains used in this study.

Primer, plasmid or recombinant *E. coli* strain	Relevant characteristics	Source or reference
Primers (5′–3′)[a]		
TAUT_NdeI_F	ATACATATGCCTATTGCCCAGATC	This work
TAUT_BamHI_R	CATGGATCCTCAGCGTCTGACCTTGCT	This work
TAUT_ins_P_F	ATACATATGCCTCCTATTGCCCAGATC	This work
TAUT_ins_P_R	ATCTGGGCAATAGGAGGCATATGTAT	This work
TAUT_ins_2P_F	TATGCCTATTCCCCCGATCCACATTC	This work
TAUT_ins_2P_R	GAATGTGGATCGGGGGAATAGGCATA	This work
T7R_seq	GCTAGTTATTGCTCAGCGG	Invitrogen
Plasmids		
pRSET-B	expression under T7 promoter, amp^R	Invitrogen
pRSET-TAUT	pRSET-B containing 192 bp *xylH* fragment, amp^R	This work
pRSET-TAUT_P	pRSET-TAUT derivative, insertion of additional P at position 1 introduced by site directed mutagenesis, amp^R	This work
pRSET-TAUT_2P	pRSET-TAUT derivative, mutations A3P, G4P introduced by site directed mutagenesis, amp^R	This work
Recombinant *E.coli*		
BL21(DE3)	F$^-$, *omp*T, high level expression regulated by T7 promoter	Invitrogen
BL21(DE3_C)	control strain carrying pRSET-B	This work
BL21(4-OT)	expressing pRSET-TAUT	This work
BL21(4-OT_P)	expressing pRSET-TAUT_P	This work
BL21(4-OT_2P)	expressing pRSET-TAUT_2P	This work

[a] seq = primers used for sequencing, underlining indicates restriction nuclease recognition sites.

constructs DNA sequencing was conducted by GATC Biotech (Konstanz, Germany), using appropriate primers (Table 1). Sequence data were aligned and compared to GenBank database using BLAST programme (Altschul, 1997). Sequences of variants obtained and described were deposited in GenBank under GenBank Submission group 4044606.

For culture propagation, Luria–Bertani (LB) or M9 minimal medium was used (Sambrook and Russell, 2001). M9 medium was supplemented with casamino acids (0.5%, w/v). Ampicillin (50 μg mL^{-1}) was routinely used to select for ampicillin resistance (amp^R). Stock cultures of all strains were maintained at −80 °C in LB broth with glycerol (20%, v/v).

2.3. Whole cell biotransformations: determination of substrate depletion rates and by E. coli cells expressing 4-oxalocrotonate tautomerase and product formation analysis

Cultures of recombinant *E. coli* strains (Table 1) were firstly inoculated from frozen stock culture onto minimal M9 agar plates and incubated at 30 °C. Cultures were then grown in M9 medium (2 mL) at 30 °C for 16 h on rotary shaker (200 rpm). These cells (500 μl, 0.1% inoculum, v/v) were then used to inoculate 500 mL of M9 medium supplemented with glucose (1%, w/v), casamino acids (0.5%, w/v) and ampicillin (50 μg mL^{-1}), which was incubated at 30 °C with shaking at 200 rpm. Once the culture had reached an optical density of 0.5 (600 nm; Spectrophotometer Ultrospec 3300pro, Amersham Biosciences), the cells were induced with 0.1 mM IPTG at 28 °C. After 12 h expression, the cells were harvested by centrifugation 5,000×*g* for 10 min in Eppendorf 5804R bench top centrifuge. The wet cell pellets were then resuspended to a concentration of cell dry weight (CDW) of 5 g L^{-1} (OD_{600} = 20) in 20 mM phosphate buffer pH 7.2.

The whole cell biotransformation was carried out in 250 mL Duran Schott glass bottles with screw type plastic lids containing 60 mL cell suspension at 28 °C with shaking at 150 rpm. Unless otherwise stated the nitrostyrene substrate was added to a final concentration of 2 mM from a 200 mM stock solution in ethanol and acetaldehyde was added to 20 mM final concentration. Samples (800 μl) were withdrawn from the reaction over time, centrifuged at 13,000×*g* for 5 min and supernatants analyzed spectrophotometrically as previously described, following the depletion of β-nitrostyrene (**1**) by reduction of absorbance at 320 nm (ε = 14.4 mM^{-1} cm^{-1}) (Zandvoort et al., 2012a).

2.3.1. Optimization of bioconversion

In order to optimize the biocatalytic Michael-type addition of β-nitrostyrenes and acetaldehyde using whole cells expressing 4-OT, the increased initial amounts of **1** were used (Table 2, Experiments B, C and D) in comparison to reaction conditions described for the same type reaction catalyzed by purified 4-OT enzyme Table 2, Experiment A (Zandvoort et al., 2012a). Lower amounts of **2** were also included, using a fixed concentration of **1** (2 mM) and 5 g CDW L^{-1} of whole cell biocatalyst (Table 2, experiments E and F). Increased concentration of biocatalyst of 12.5 g CDW L^{-1} (OD_{600} = 50) was used during experimental setup (Table 2, experiment G). Halo substituted β-nitrostyrenes, namely *para*-Cl-, *ortho*-Cl- and *para*-F- β-nitrostyrene, were used at 2 mM concentration with acetaldehyde (20 mM) in experiments H, I and J respectively.

Biocatalyst recycling was assessed in the experimental setup where after the initial depletion of **1** (0.12 mmol, 2 mM) the removal of the product was achieved by centrifugation and resuspension of the catalyst in the fresh potassium phosphate buffer (pH 7.2; 60 mL) when another batch of **2** and **1** was added (2 and 50 mM final concentrations respectively), followed by another product removal and the new addition of **2** and **1** (1 and 25 mM). Sequential addition of **2** and **1** by addition of 2 and 50 mM final concentrations respectively was also applied to supply the overall 10 mM β-nitrostyrene and 200 mM acetaldehyde.

2.4. Protein expression analysis

Cleared cell lysates were obtained using 1 mL of cell suspension in phosphate buffer (OD_{600} = 20) before the addition of substrates using Novagen BugBuster reagent according to manufacturer's instructions. Proteins were resolved and analyzed by SDS–PAGE (Laemmli, 1970) under denaturing conditions with 15% and 4% acrylamide (w/v) in resolving and stacking gels, respectively.

Protein concentration was determined using the bicinchoninic acid method (Smith et al., 1985).

2.5. 4-Oxalocrotonate tautomerase structure analysis modeling

Mutated positions in the 4-OT protein were mapped onto the available crystal structure (pdb code 1BJP, (Taylor et al., 1998)) and the hexamer formed according to the biologically active form of 4-OT. The protein structure was refined and energy minimized using the minimize script in CNS (Brunger, 2007). Docking of substrates was performed in AutoDock Tools (Morris et al., 2009) and Vina (Trott and Olson, 2010) and figures prepared in PyMol (The PyMOL Molecular Graphics System, Version 1.4.1., Schrodinger, LLC).

2.6. Analytical procedures

2.6.1. Product extraction and purification

To recover the products from the biotransformation, the reaction mixture was extracted with ethyl acetate (2 × 100 mL). The combined organic extract was washed with brine (100 mL), and dried over anhydrous magnesium sulfate. After filtration and removal of the solvent under reduced pressure, the residue was purified by dry flash column chromatography (silica gel), eluting with petroleum ether/ethylacetate mixture in gradient (from 7: 3 to 9: 1) to afford pure nitroaldehydes.

2.6.2. Analyses of bioconversion products

Chemical structures of the products were confirmed by nuclear magnetic resonance (NMR) spectroscopy. ^{1}H and ^{13}C NMR spectra were obtained in deuterated chloroform with a Varian NMR system 200 MHz spectrometer and provided in the Supplementary Material.

The enantiomeric excess was determined by HPLC (Agilent Technologies HP1100) using CHIRALPAK IA 250 column (Chiral Technologies Europe, Cedex, France) under conditions described in Supplementary Material. $[\alpha]_D$ was recorded using Automatic Polarimeter AUTOPOL ® IV (Rudolph Research Analytical, Hackettstown, USA).

2.6.3. Synthesis of 4-amino3-phenyl-butanol from bioconversion product

Bioconversion product (4-nitro-3-phenylbutanal; **3**) was subjected to two subsequent reduction reactions to obtain **10** using standard procedures (Fig. 1; Supplementary Material S2(A–B)).

3. Results and discussion

Purified 4-OT enzyme was recently shown to be able to catalyze the formation of 4-nitro-3-phenyl-butanal (**3**) from nitrostyrene (**1**) and acetaldehyde (**2**) using either both recombinantly produced and purified protein or a commercially synthesized version (Zandvoort et al., 2012). Having in mind that the whole cell approach is usually one of the strategies for the bioprocess improvement (Illanes et al., 2012) we have designed and assessed a novel biocatalyst based on resting *E. coli* BL21 whole cells expressing 4-OT.

3.1. Whole cells biotransformation

The Michael-type addition of **2** to **1** was investigated as the model reaction as a recently reported study, employing pure 4-OT enzyme, had demonstrated the conversion of **1** (2 mM) and **2** (50 mM) in 3 h with a product yield of 46% (Zandvoort et al., 2012a).

By UV–Vis spectroscopy monitoring **1** (λmax = 320 nm) the 2 mM initial concentration of β -nitrostyrene was utilized within 30 min using whole resting cells of recombinant *E. coli* BL21(4-OT) (Table 2, experiment A). This represented a 6-fold improvement in the reaction time in comparison to that of the previously reported for the pure protein (Zandvoort et al., 2012a). Reaction product 4-nitro-3-phenyl-butanal (**3**) was subsequently purified and analyzed by NMR spectroscopy (Supplementary Material S1-A) and the comparable yield of 42% was obtained. Interestingly, enantioselectivity of the whole-cell catalyst was > 99% while reported *ee* values for the pure enzyme by Zandvoort and co-authors was 89% (Supplementary Material, S3-A). It is worth noting that the obtained aldehydes (**3**, **5**, **7** and **9**) were not stable upon prolonged storage at −20 °C, so purified aldehydes were subjected to reduction by $NaBH_4$ to yield corresponding nitro-alcohols that were more stable and more amenable to chiral HPLC analysis (Supplementary Material S3 (A–D)). Appropriate controls using whole cells of BL21(DE3_C) bearing plasmid without tautomerase gene, as well as reactions without biocatalyst exhibited no β-nitrostyrene depletion (data not shown).

After successfully establishing that *E. coli* BL21 whole cells expressing 4-OT, under described conditions were better biocatalysts in terms of the time required for the reaction, as well as in stereoselectivity we have optimized bioprocess conditions by altering substrate and biocatalyst load (Table 2). Higher initial concentrations of **1** of 3 and 5 mM caused 1.9- and 2.4-fold decreased overall β-nitrostyrene depletion rates in comparison to 2 mM (Table 2, experiments B and C), while product **3** yields were comparable

Table 2
Biotransformation of nitrostyrenes and acetaldehyde by recombinant *E. coli* BL21(4-OT) using 5 g CDW L^{-1} at 28 °C, pH 7.2 in 60 mL volume.

Experiment	Substrates	Product	Reaction time (h)	Nitrostyrene depletion rate (μmol/min/g CDW)[d]	Yield (%)	*ee* (%)
A[a]	**1**[c] (2 mM) and **2** (50 mM)	**3**	0.5	13.3[e]	42	>99
B	**1** (3 mM) and **2** (50 mM)	**3**	1.5	6.7	40	>99
C	**1** (5 mM) and **2** (50 mM)	**3**	3	5.6	45	>99
D	**1** (10 mM) and **2** (50 mM)	/	nr[d]	0	0	/
E	**1** (2 mM) and **2** (20 mM)	**3**	0.5	13.3	60	>99
F	**1** (2 mM) and **2** (10 mM)	**3**	1.3	5	32	>99
G[b]	**1** (2 mM) and **2** (20 mM)	**3**	0.3	8	58	>99
J	**4** (2 mM) and **2** (20 mM)	**5**	1.5	4.4	38	84
K	**6** (2 mM) and **2** (20 mM)	**7**	1	6.7	51	88
L	**8** (2 mM) and **2** (20 mM)	**9**	1.5	4.4	31	94

[a] Reaction setup is explained in detail in Methods Section 2.3.1.
[b] Cells were harvested and brought to the same biomass of 5 g CDW l^{-1} except in experiment G, when the biomass was 12.5 g CDW L^{-1}.
[c] 1 = β -nitrostyrene; 2 = acetaldehyde; 3 = 4-nitro-3-phenylbutanal; 4 = 4-chloro- β -nitrostyrene; 5 = 3-(4-chlorophenyl)-4-nitrobutanal; 6 = 2-chloro- β -nitrostyrene; 7 = 3-(2-chlorophenyl)-4-nitrobutanal; 8 = 4-fluoro- β -nitrostyrene; 9 = 3-(4-fluorophenyl)-4-nitrobutanal.
[d] nr = no reaction occurred under described conditions.
[e] Standard errors were between 2% and 6%.

and stereoselectivity unchanged. However, the initial concentration of **1** of 10 mM apparently had inhibitory effect on the biocatalyst resulting in no observable β-nitrostyrene depletion by UV–Vis spectra monitoring, while no product was detected under used experimental conditions (Table 2, experiment D). A common limitation of biocatalytic systems is the inhibition or toxicity posed by the starting substrate as well as limited aqueous solubility (Kim et al., 2007). Researchers usually resort to the reaction engineering to optimize substrate (Schmölzer et al., 2012).

Michael-type addition of aldehydes to nitroalkenes in general requires the use of a large excess of aldehyde substrate which poses a problem in the case of commercially unavailable substrates (Palomo et al., 2006). Thus, the decreased amount of acetaldehyde (20 and 10 mM) was supplied to biotransformation reaction using 2 mM β-nitrostyrene (Table 2, experiments E and F). Using a 2.5-fold lower amount of **2** in comparison to initial experiment A resulted in unchanged reaction rate and enantioselectivity, while the product **3** yield was 1.4-fold improved (Table 2, experiment E). Nevertheless, further lowering the amount of **2** (10 mM) resulted in a 2.7-fold decrease of **1** overall depletion rate and reduced product yield by almost 2-fold (Table 2, experiment F). This may be due to technical difficulties of low amounts of highly volatile acetaldehyde addition, when some of the aldehyde possibly had evaporated prior to be able to react.

Once we have established that whole cells *E. coli* BL21(4-OT) exhibited improved activity of carbon–carbon bond formation with **2** present in 20 mM concentration, we have an increased amount of biocatalyst by 2.5-fold which resulted in a slightly decreased reaction time, while the overall reaction rate was 1.6-fold lower (Table 2, experiment G) in comparison to optimized experimental conditions (Table 2, experiment E). Therefore, these conditions have been applied in all subsequent bioconversions.

In order to explore the scopes of the developed biocatalyst, the reactions of halogen substituted β-nitrostyrenes, namely 4-Cl- β-nitrostyrene (4), 2-Cl- β-nitrostyrene (6), and 4-F- β-nitrostyrene (8) with acetaldehyde were carried out (Table 2). In all cases appropriate products of Michael-type additions of acetaldehyde to these substrates were observed and confirmed by NMR analysis (Supplementary Material S1(B–D)). The intrinsic chemical reactivity of a substrate is also an important consideration, in determining the rate of reaction catalyzed by an enzyme. The electron-withdrawing substituents on the aromatic ring of the β-nitostyrene negatively influenced the biotransformation rate (Table 2, experiment H–J). Substrate depletion rates of both *para*-substituted β-nitrostyrenes were comparable and 3-fold lower in comparison to unsubstituted β -nitrostyrene, with Cl-derivative having a slightly better product yield and lower *ee* value in comparison to *p*-F- β-nitrostyrene (Table 2, experiments H and J). When *o*-Cl- β-nitrostyrene was used as the substrate in biocatalysis, 1.3-fold improved product yield was achieved in comparison to *p*-Cl- β-nitrostyrene and slightly higher enantioselectivity (Table 2, experiment I). The effect of electron-withdrawing groups on aromatic nitroalkenes was also reported when acidic protease from *Aspergillus usamii* was used as the catalyst for Michael-type additions of cyclohexanone to β-nitrostyrene (Xu et al., 2011). On the contrary, in this study, 4-chloro- and 4-bromo substituted nitrostyrene gave improved reaction times and product yields in comparison to unsubstituted nitrostyrene (Xu et al., 2011). Similarly, *p*-hydroxy-β-nitrostyrene used as substrate in the 4-OT catalyzed reaction with acetaldehyde resulted in improved product yield, but reduced enantioselectivity (51% *ee*) in comparison to β-nitrostyrene (89% *ee*) (Zandvoort et al., 2012a).

High stereoselectivity of the reaction was observed in the case of all substrates used (Table 2). Comparison of $[\alpha]_D$ of the obtained products with literature data revealed *S* configuration for 4-nitro-3-phenyl-butanol and other products, while *o*-Cl-nitroaldehyde, based on $[\alpha]_D$, was of *R* configuration. This could be due to a different orientation of the substrate in the active site consistent with different steric requirements.

3.2. Bioprocess improvement for the 4-nitro-3-phenyl-butanal production and further transformation to 4-amino-3-phenyl-1-butanol

In order to increase 4-nitro-3-phenyl-butanal production yields and to further improve the bioprocess, two strategies of sequential addition of substrate were attempted (Fig. 2). Firstly, due to evident inhibitory effect of higher initial nitrostyrene concentrations (Table 2, experiments C and D), biocatalyst recycling by product removal was attempted (Fig. 2A). After initial 2 mM load of **1** and 20 mM of **2** was transformed to **3** within 30 min, cells were centrifuged and resuspended in a fresh buffer containing another 2 mM load of **1** following the addition of 20 mM of **2**. In the following 30 min of the reaction, only half of the substrate **1** was consumed and evident was disruption of cells (suspension became more viscous). Another centrifugation and addition of 2 mM final concentration of **1** was attempted, when only 40% of the **1** was consumed within 30 min, suggesting that the biocatalyst was not efficiently recycled using this strategy (Fig. 2A). Overall, 6 mM final concentration of **1** and 60 mM of **2** was supplied during this biotransformation obtaining only 18% product yield indicating that product loss could have occurred during centrifugation steps.

When the similar sequential addition of substrates was applied without product removal by centrifugation, highly efficient bioprocess was achieved (Fig. 2B). In this experiment slightly higher biocatalyst concentration of 5.5 g CDW L^{-1} (OD_{600} = 22) was used, and final concentrations of 1 (2 mM) and 2 (20 mM) were added at the start of the reaction, as well as after each depletion of **1**. Using this strategy, within 2.5 h 10 mM β-nitrosty-

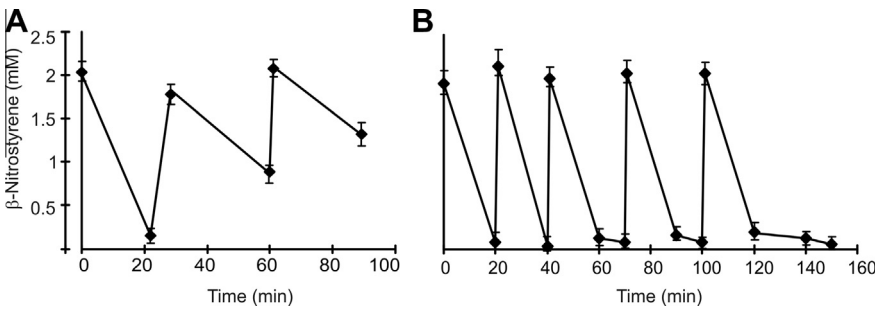

Fig. 2. Bioprocess improvement of nitrostyrene transformation to 4-nitro-3-phenyl-butanal through: (A) biocatalyst recycling by product removal and (B) sequential addition of substrate.

rene was biotransformed to 4-nitro-3-phenyl-butanal with product yield of 56% and enantioselectivity of > 99% *ee*. Through the simple substrate supply strategy we have achieved much higher overall biotransformation rates (up to 12 times improved in comparison to free enzyme system reported by Zandvoort and coauthors (Zandvoort et al., 2012a)). In addition, compared with isolated enzymes, whole cell catalysts can be much more readily and inexpensively prepared and are much easier to handle (Ishige et al., 2005).

3.2.1. Production of aminoalcohol

Biotransformation product 3 was successfully purified in good quantities and subjected to two subsequent reduction reactions (Fig. 1) according to standard procedures in yields of > 95%. Thus, we have demonstrated that the biotransformation product could subsequently be used as a substrate for further chemical synthesis. Indeed, *p*-Cl-substituted γ -amino-alcohol has previously been used in the synthesis of baclofen which is an important $GABA_B$ receptor agonist (Wang et al., 2008).

3.3. Structural implications of proline enriched 4-OT variants on bioprocess

Terminal proline has been postulated to be the general base in the active center of 4-OT while Arg11, Arg39 and Arg61 play important roles in orienting the substrate in the active site (Czerwinski et al., 1997; Harris et al., 1999; Stivers et al., 1996). In addition, Arg39 might also act as a general acid delivering a proton to the Cα carbon of the substrate nitrostyrene (Zandvoort et al., 2012a). Two mutants were generated using site directed mutagenesis to have additional N-terminal proline (4-OT_P), as well as variant with two substituted amino acids to proline, namely Ala3Pro and Gln4Pro (4-OT_2P) and their activity assessed (Fig. 3A).

Biotransformations were carried out using optimal conditions determined in the optimization experiment of **1** (2 mM) and **2** (20 mM) and 5 g CDW L^{-1} of each biocatalyst. Protein analysis revealed comparable amounts of overexpressed 4-OT and both variants as determined by SDS–PAGE analysis (Fig. 3B). A complete amount of β -nitrostyrene (2 mM) was biotransformed by 4-OT and 4-OT_P within the first 30 min of the reaction, while 35% of substrate was still left in the reaction using 4-OT_2P variant after 60 min (Fig. 3A). The highest initial rate of the nitrostyrene depletion of 47.2 μmol min^{-1} g CDW^{-1} was observed in the reaction catalyzed by whole cells expressing 4-OT_P variant, and it was 1.4- and 2.9-fold higher in comparison to wild type 4-OT and 4-OT_2P respectively. However, overall nitrostyrene depletion rate of 13.3 μmol min^{-1} $g^{-1}$was achieved by wild type 4-OT and 4-OT_P variant, that was 3-fold higher in comparison to the overall nitrostyrene depletion rate of 4-OT_2P variant (Fig. 3A).

The effect of introduced mutations was examined by homology modeling (Supplementary Material S4). In the case of N-terminal addition of proline to the wild type sequence (4-OT_P), the effect on the protein fold was negligible since there was sufficient space for its placement in the structure and no hydrogen bonds were disrupted and no new ones were formed. In the docking experiment the nitro group of nitrostyrene is positioned so it can form hydrogen bonds with Arg11 while the distance to the Pro1 bound acetaldehyde is in the wild type 5.3 and in the 4-OT_P variant 3.8 A, bringing the active residues closer together (Supplementary Material S4). As predicted by docking experiments, binding of acetaldehyde to the terminal proline of 4-OT_P variant proceeds in the same manner as in the wild type variant, while slightly better positioning of the nitrostyrene substrate has been allowed between the proline bound acetaldehyde and Arg11, thus probably enabling higher reaction rates for β-nitrostyrene in the 4-OT_P variant. The double mutation in 4-OT_2P variant introduces twists into

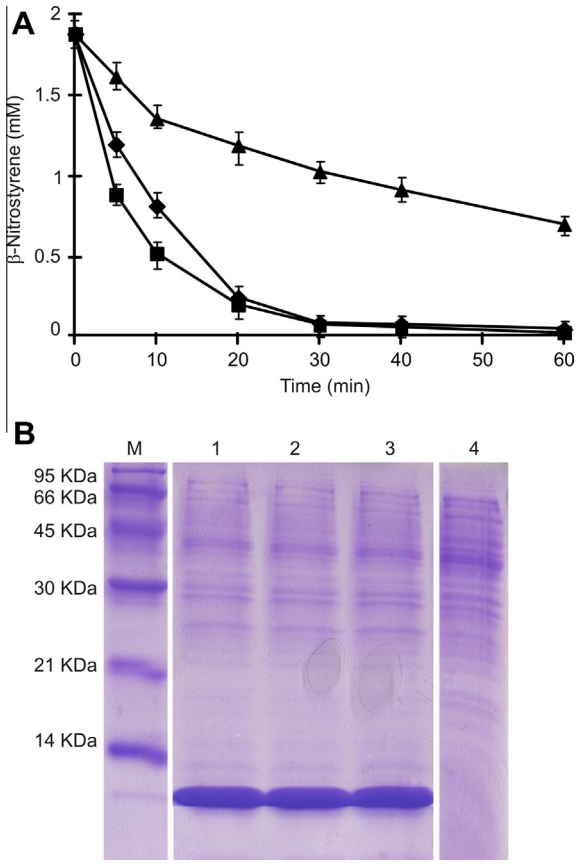

Fig. 3. Recombinant *E. coli* BL21 expressing 4-OT and variants. (A) Substrate depletion kinetics of three versions (♦, 4-OT; ■, 4-OT_P; , 4-OT_2P); (B) SDS–PAGE analysis of cell-free lysates of (1) 4-OT, (2) 4-OT_P, (3), (4) 4-OT_2P, 4-control strain carrying vector without insert, M- protein molecular weight standards (Low molecular weight protein mixture, GE Healthcare, UK).

the first beta strand while the backbone nitrogen atoms of the mutated residues are being prevented to form characteristic β-plate hydrogen bonds (Supplementary Material S4). Having in mind that this enzyme is one of the shortest enzymes (with only 62 amino acids) with six identical chains arranged into three dimers (Taylor et al., 1998), it is evident that the distortion could easily be carried through the chain resulting in the disruption of the overall conformation of the active site neighborhood. Described distortion would likely lead to reduced stability of the protein itself and of the biologically active hexamer, thus resulting in the reduced ability to catalyze Michael-type additions of **1** and **2** (Fig. 3A).

4. Conclusions

Whole resting cells of recombinant *E. coli* expressing 4-OT catalyze Michael-type addition of acetaldehyde to β -nitrostyrenes with high activity and high excellent enantioselectivity. Furthermore, the successful application of the whole-cell catalyst in repetitive batch conversions was demonstrated. Highly optically pure 4-nitro-3-phenyl-butanal (>99% *ee*) was produced in concentrations of 1.8 g l^{-1}. This bioprocess may be an efficient alternative for the practical synthesis of 4-nitro-3-phenyl-butanal that are demonstrated synthons for desirable amino alcohols.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.05.074.

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