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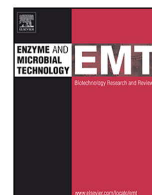

Chemoselective biocatalytic reduction of conjugated nitroalkenes: New application for an *Escherichia coli* BL21(DE3) expression strain

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ABSTRACT

Chemoselective reduction of activated carbon–carbon double bond in conjugated nitroalkenes was achieved using *Escherichia coli* BL21(DE3) whole cells. Nine different substrates have been used furnishing the reduced products in moderate to good yields. 1-Nitro-4-phenyl-1,3-butadiene and (2-nitro-1-propenyl)benzene were successfully biotransformed with corresponding product yields of 54% and 45% respectively. Using this simple and environmentally friendly system 2-(2-nitropropyl)pyridine and 2-(2-nitropropyl)naphthalene were synthesized and characterized for the first time. High substrate conversion efficiency was coupled with low enantioselectivity, however 29% enantiomeric excess was detected in the case of 2-(2-nitropropyl)pyridine. It was shown that electronic properties of the aromatic ring, which affected polarity of the double bond, were not highly influential factors in the reduction process, but the presence of the nitro functionality was essential for the reaction to proceed. 1-Phenyl-4-nitro-1,3-butadiene could not be biotransformed by whole cells of *Pseudomonas putida* KT2440 or *Bacillus subtilis* 168 while it was successfully reduced by *E. coli* DH5α but with lower efficiency in comparison to *E. coli* BL21(DE3). Knockout mutant affected in *nemA* gene coding for *N*-ethylmaleimide reductase (BL21Δ*nemA*) could still catalyze bioreductions suggesting multiple active reductases within *E. coli* BL21(DE3) biocatalyst. The described biocatalytic reduction of substituted nitroalkenes provides an efficient route for the preparation of the corresponding nitroalkanes and introduces the new application of the strain traditionally utilized for recombinant protein expression.

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1. Introduction

Chemical modification of compounds by biological systems is a powerful method to synthesize valuable multifunctional molecules from small and simple precursors. Biocatalysts are usually more efficient than chemical catalysts, require mild reaction conditions and usually provide high chemo- and stereoselectivity [1]. Hundreds of biotransformations have been described in the literature until now but there is a great need for discovery of new enzymes and reactions that could be used in the pharmaceutical, chemical and other industries [1–3]. There were about 150 bioconversion processes at industrial scale in 2006 while their implementation on a scale of over 100 kg of product per annum is steadily growing [4,5].

Biocatalytic reductions of unsaturated compounds based on reductases have been described as very versatile reactions including asymmetric reduction of aldehydes and ketones resulting in primary and secondary alcohols, reduction of C=N double bonds and reduction of activated C=C double bonds in α,β-unsaturated carboxylic acids, esters, lactones, aldehydes, ketones and nitro compounds [6,7]. Flavoproteins from the old yellow enzyme (OYE) family that are commonly associated with baker's yeast are usually used for bioreductions [8–10]. However, more recently recombinantly expressed enoate reductases from microbial sources have been employed for the same purpose [11]. Many bacterial enzymes with amino acid sequence homology to OYE have been reported and characterized. N-ethylmaleimide reductase (NemA) from *Escherichia coli* [12] was recently described to be involved in reductions of α-methylated nitroalkenes [13].

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Conjugated nitroalkanes have been proven valuable building blocks and intermediates in organic synthesis as their poly functionality offers a whole range of synthetic manipulations. The conjugated, electron deficient double bond is an excellent partner in Michael reaction [14,15] and related processes while the diene moiety involving nitro group finds application in hetero Diels–Alder cycloadditions [16,17]. The fact that the nitro functionality, due to strong electron withdrawing effect, activates α- or γ-CH (in conjugated substrates) enriches their chemistry further. In addition, they can be easily converted into versatile functionalities such as amines, carbonyl compounds or even corresponding alkanes [18,19].

E. coli apart for being the model microorganism for molecular genetic studies is the most used microorganism in research laboratories and biotech industry because of its fast growth and the achievement of high cell densities in inexpensive media [20,21]. *E. coli* BL21(DE3) in combination with T7 based vector expression is applied for recombinant protein production for almost twenty years now [22]. Recently this strain has been investigated in detail using DNA microarray, transcriptomic and proteomic analysis with a view of improved future process design and operations based on this host [21]. On the other side, this strain has often been used as whole-cell biocatalyst expressing different enzymes for useful biotransformations and generation of important molecules including pinostilbenes or nucleotide sugars [23,24]. In this study, new application of *E. coli* BL21(DE3) as whole-cell biocatalyst for chemoselective reductions of conjugated nitroalkenes was identified and investigated. The involvement of NemA protein in bioreductions of selected nitroalkenes was assessed through generation of *nemA* gene inactivation mutant. The presence of similar reduction activity was examined in *E. coli* DH5α and members of *Bacillus* and *Pseudomonas* genus as well. Efficient and versatile biocatalytic synthesis of nitroalkanes has been demonstrated in a system that utilizes *E. coli* BL21(DE3) strain, circumvents recombinant protein expression, protein purification, and cofactor addition, therefore offering greener alternative to chemical synthesis of these valuable compounds.

2. Materials and methods

2.1. Reagents

Bacteriological media components, ampicillin, IPTG and compounds **9** (*trans*-β-nitrostyrene), **10** (*trans*-1-phenyl-1,3-butadiene), **11** (3-phenyl-2-propenoic acid ethyl ester) and **13** (*trans, trans*-1,4-diphenyl-1,3-butadiene) were 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 pre-coated TLC aluminium silica gel sheets 60 F254 were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Synthesis of substrates and analytical standards

Compounds **1** [25], **2**–**8** [26] and **12** [27] were synthesized according to the literature procedures. For chiral analysis of biotransformation product **4a**, derivatization procedure yielding **4b** and **4c** was followed (Scheme 1; Fig. 4).

1-(4-Methoxyphenyl)propan-2-amine (**4b**). A solution of the precursor nitro compound **4a** (30 mg, 0.15 mmol) in anhidrous Et_2O (2 mL) was slowly added with continuous stirring to a solution of $LiAlH_4$ (24 mg, 0.60 mmol) in anh. Et_2O (2 mL) under N_2. The solution was refluxed for 3 h. The reaction was then allowed to cool to 0 °C and it was quenched by slow addition of H_2O (0.05 mL), NaOH (0.05 mL of 15% aq. solution). The mixture was dried ($MgSO_4$), filtered off and concentrated under reduced pressure to afford amine **4b**. The crude **4b** was used in the next step without further purification [26].

N-(1-(4-Methoxyphenyl)propan-2-yl)acetamide (**4c**). Compound **4b** (25 mg, 0.15 mmol) was dissolved in DCM (3 mL) and TEA (0.04 mL, 0.30 mmol). Acetic anhydride (0.01 mL, 0.20 mmol) was then added at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and at 25 °C for 23 h. Water (10 mL) was added carefully and the mixture extracted with DCM (3× 10 mL). The combined organics were washed with brine (10 mL), then dried ($MgSO_4$), filtered and concentrated under reduced pressure. The residue was purified by column chromatography (silica gel, Et_2O, Rf = 0.20) to afford the product in 71% yield [28].

2.3. Bacterial growth and biocatalysis

Bacterial strains *Bacillus subtilis* 168 and *Pseudomonas putida* KT2440 were obtained from the American Type Culture Collection having accession numbers ATCC 23857 and ATCC 47054 respectively. *E. coli* BL21(DE3) and *E. coli* DH5α were purchased from Invitrogen (Paisley, UK), while the strain *E. coli* BL21(4-OT) bearing the plasmid for the inducible expression of 4-oxalocrotonate tautomerase was previously generated and described by our group [29].

Stock cultures of all strains were maintained at −80 °C in Luria–Bertani (LB) broth with glycerol (20%, v/v). To prepare the whole-cell biocatalyst, cultures were firstly inoculated onto minimal M9 agar plates [30] that contained casamino acids (0.5%, w/v) and ampicillin (50 $\mu g\,mL^{-1}$) when recombinant *E. coli* BL21(4-OT) was used. 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) and casamino acids (0.5%, w/v) which was incubated at 30 °C for 24 h with shaking at 200 rpm. As described previously, in the case of *E. coli* BL21(4-OT), medium contained ampicillin (50 $\mu g\,mL^{-1}$) was used and the recombinant protein expression was induced by addition of IPTG (0.1 mM) [29]. Optical density of the cultures was measured at 600 nm (Spectophotometer Ultrospec 3300pro, Amersham Biosciences) and the cells were harvested by centrifugation 5000 × *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 approximately 2.5 $g\,L^{-1}$ (OD_{600} = 10) in ice-cold 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 cells suspension at 28 °C with shaking at 150 rpm. Each substrate (**1**–**13**; Fig. 2) was added to a final concentration of 2 mM from a 200 mM stock solution in ethanol. Samples (800 μL) were withdrawn from the reaction over time, ethyl acetate (200 μL) was added and the samples were vigorously mixed and centrifuged at 13,000 × *g* for 3 min and organic layer analyzed by thin layer chromatography.

Biotransformations were stopped by addition of the equal volume of ethyl acetate after the reaction mixture was saturated with solid NaCl. Extraction with ethyl acetate was carried out twice and the organic fractions were combined, dried with anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. These crude extracts were purified further using flash chromatography.

2.4. Generation of E. coli BL21ΔnemA knockout mutant of N-ethylmaleimide reductase

Gene sequence analysis and assignment was assisted using KEGG database resources (http://www.genome.jp/kegg/). All general molecular biology techniques were performed following the procedures described by Sambrook et al. [30]. Isolation of plasmid DNA from *E. coli* was carried out using mini-prep plasmid isolation procedure as specified by QIAGEN (Hilden, Germany). PCR was performed using DNA Engine Thermal Cycler (Bio-Rad, Hercules, USA) and the following parameters: 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 55 °C for 40 s, 72 °C for 1–3 min depending on the length of the product, with final extension at 72 °C for 10 min. PCR products were resolved on the 1% (w/v) agarose gel and visualized using ethidium bromide staining. Identity of PCR products was confirmed by sequencing (GATC Biotech, Konstanz, Germany).

PCR amplification of *nemA* gene was carried out using primers designed for *E. coli* BL21 (DE3) *nemA* gene namely nemA-BL21 (F): 5′-TCATCTGAAAAACTGTATTCCCC-3′ and nemA-BL21 (R): 5′-AACGTCGGGTAATCGGTATAG-3′ primers set. Set of degenerate primers around conserved regions of *nemA* genes from *E. coli* BL21 (Gen Bank accession number YP_003054249), *P. putida* KT2440 (Gen Bank accession number NP_745317.1) and *Bacillus thuringiensis* Bt407 (Gen Bank accession number YP_006928343.1) was designed and utilized in PCR reaction using genomic DNA from *E. coli* BL21, *P. putida* KT2440 and *B. subtilis* 168 as templates. Primer sequences were nemA-gen (F): 5′-ATGGCACCGCTGACCC-3′ and nemA-gen(R): 5′-AGGTCNGGTTNGCAAT-3′. Sequence alignment of *nemA* genes was carried out using multiple sequence alignment tool ClustalW available from http://www.ebi.ac.uk/Tools/msa/clustalw2/ (Supplementary Information).

Gene inactivation procedure was carried out using pKNOCK insertional mutagenesis procedure [31]. Plasmid for *nemA* gene inactivation was generated by amplifying internal region of 503 bp nemA int (F): 5′-CACCGCTGGCGTTCATGCT-3′ and nemA int (R): 5′-GCATCGGCTTCTTCATTCG-3′primer pairs. PCR amplified product was cloned into pGEM-T Easy vector taking advantage of protruding A overhangs following manufacturer's procedure [32]. *Eco*RI fragment from pGEM-T:nemA derivative was further subcloned into pKNOCK-Km vector designed for insertional mutagenesis [31] containing unique *Eco*RI restriction site generating pKNOCK:nemA plasmid that was introduced into *E. coli* BL21(DE3) cells via electroporation and knockout mutants were selected for kanamycin resistance (Km^r) (Fig. 5b). Insertional mutation of *nemA* gene in *E. coli* BL21ΔnemA strain was confirmed by PCR.

E. coli BL21ΔnemA strain was grown and utilized in biotransformation procedures using substrates 1 and 2 in as described in Section 2.3 adding kanamycin (30 $\mu g\,mL^{-1}$) to the growth medium.

2.5. Analytical methods

2.5.1. Preparative and thin layer chromatography (TLC)

Ethyl acetate extracts of biotransformation mixtures were filtered and after the removal of the solvent under reduced pressure, the residue was purified by flash column chromatography (silica gel), eluting with petroleum ether/Et_2O mixture (9:1) to afford pure compounds. Solvents were purified by distillation before use. Flash chromatography employed silica gel 60 (230–400 mesh), while thin layer chromatography was carried out using alumina plates with 0.25 mm silica layer (Kieselgel 60 F_{254}, Merck).

2.5.2. NMR and IR spectroscopy

The NMR spectra were recorded on a Bruker Avance III (500 MHz) or Varian Gemini 2000 (200 MHz) spectrometer. Chemical shifts are given in parts per million (δ) downfield from tetramethylsilane as the internal standard and deuterochloroform was used as a solvent unless otherwise stated. IR spectra were recorded on an IR Termo Scientific NICOLET iS10 (4950) spectrometer. Spectral data were as follows:

4-Nitro-1-phenylbutene (1a) [33] Yellow oil, yield 54%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.34–7.23 (m, 5 H), 6.52 (d, J = 15.5 Hz, 1 H), 6.10 (dt, J = 15.5, 7.0 Hz, 1H), 4.49 (t, J = 7.0 Hz, 2 H), 2.89 (dt, J = 7.0, 7.0 Hz, 2 H): ^{13}C NMR ($CDCl_3$, 125 MHz) δ 136.4, 134.0, 128.6, 127.8, 126.3, 122.9, 75.0, 30.7.

2-Nitropropylbenzene (2a) [19] Yellow oil, yield 45%. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.36–7.26 (m, 3H), 7.19–7.14 (m, 2H), 4.87–4.70 (m, 1H), 3.33 (dd, J = 14.0, 7.5 Hz, 1H), 3.01 (dd, J = 14.0, 6.8 Hz, 1H), 1.55 (d, J = 6.7 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 135.5, 129.0, 128.8, 127.4, 84.4, 41.1, 18.8.

1-Fluoro-4-(2-nitropropyl)benzene (3a) [19] Pale yellow oil, yield 47%. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.18–7.08 (m, 2H), 7.04–6.96 (m, 2H), 4.80–4.69 (m, 1H), 3.28 (dd, J = 14.0, 7.2 Hz, 1H), 2.99 (dd, J = 14.0, 6.8 Hz, 1H), 1.55 (d, J = 6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 164.6, 159.7, 130.6, 130.4, 115.9, 115.5, 84.4, 40.3, 18.8.

1-Methoxy-4-(2-nitropropyl)benzene (4a) [19] Pale yellow oil, yield 56%. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.10–7.04 (m, 2H), 6.86–6.81 (m, 2H), 4.78–4.68 (m, 1H), 3.79 (s, 3H), 3.25 (dd, J = 14.0, 7.6 Hz, 1H), 2.95 (dd, J = 14.0, 6.6 Hz, 1H), 1.53 (d, J = 6.6 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 158.7, 130.0, 129.9, 114.2, 84.7, 55.2, 40.4, 18.6.

N-(1-(4-methoxyphenyl)propan-2-yl)acetamide (4c) [34]. White solid, yield 71%. ^{1}H NMR ($CDCl_3$, 200 MHz) 7.11 (m, 2H), 6.85 (m, 2H), 5.42 (br, 1H), 4.20 (m, 1H), 3.78 (s, 3H), 2.82–2.59 (m, 2H), 1.93 (s, 3H), 1.09 (d, J = 6.6 Hz, 3H). δ ^{13}C NMR ($CDCl_3$, 50 MHz) δ 169.3, 158.2, 130.3, 129.9, 113.7, 55.2, 46.1, 41.4, 23.4, 19.8. The enantiomeric excess was determined by HPLC with CHIRALPAK IA column at 230 nm (heptane/iPrOH in the ratio of 95/5, flow rate = 1.0 mL min^{-1} tr = 21.61 min, tr = 23.05 major), e.e. 3%.

1-Methoxy-2-(2-nitropropyl)benzene (5a) [35] Pale yellow oil, yield 4%. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.30–6.84 (m, 4H), 5.00–4.83 (m, 1H), 3.84 (s, 3H), 3.27 (dd, J = 13.5, 7.7 Hz, 1H), 3.05 (dd, J = 13.5, 6.5 Hz, 1H), 1.53 (d, J = 6.7 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 157.9, 131.4, 129.3, 124.4, 121.0, 110.7, 83.4, 55.5, 37.4, 19.8.

2,4-Dichloro-1-(2-nitropropyl)benzene (6a) [36] Yellow oil, yield 10%. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.42 (s, 1H), 7.27–7.09 (m, 2H), 4.94–4.84 (m, 1H), 3.37 (dd, J = 14.0, 8.0 Hz,1H), 3.18 (dd, J = 14.0, 6.0 Hz, 1H), 1.62 (d, J = 6.0 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 134.8, 134.3, 132.1, 132.0, 129.7, 127.6, 82.5, 38.2, 19.17.

2-(2-Nitropropyl)pyridine (7a) Colourless oil, yield 44%. IR: 2159, 2028, 1976, 1544, 713 cm^{-1}. NMR ($CDCl_3$, 200 MHz) δ 8.55–8.47 (m, 2H), 7.53–7.47 (m, 1H), 7.29–7.23 (m,1H), 4.84–4.74 (m, 1H), 3.31 (dd, J = 14.0, 8.0 Hz, 1H), 3.07 (dd, J = 14.0, 6.0 Hz, 1H), 1.60 (d, J = 6.0 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 150.2, 149.0, 136.4, 131.1, 123.6, 83.8, 38.1, 18.86. HRMS (ESI): calculated for $C_8H_{10}N_2O_2$ $(M+H)^+$ 167.08150, found 167.08190. The enantiomeric excess was determined by HPLC with CHIRALPAK IA column at 210 nm (heptane/iPrOH in the ratio of 80/20, flow rate = 1.0 mL min^{-1} tr = 9.02 min, tr = 10.28 major), e.e. 29%.

2-(2-Nitropropyl)naphthalene (8a) Yellow amorphous solid, yield 46%. IR: 2159, 2028, 1976, 1541, 713 cm^{-1}. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.82–7.69 (m, 3H), 7.62 (s, 1H), 7.53–7.42 (m, 2H), 7.31–7.25 (m, 1H), 4.93–4.83 (m, 1H), 3.48 (dd, J = 14.0, 7.4 Hz, 1H), 3.18 (dd, J = 14.0, 6.8 Hz, 1H), 1.53 (d, J = 6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 133.4, 132.9, 132.6, 128.6, 127.9, 127.7, 126.8, 126.4, 126.0, 84.3, 41.3, 18.8. HRMS (ESI): calculated for $C_{13}H_{13}NO_2$ $(M+NH_4)^+$ 233.12845, found 233.12720.

1-(2-Nitroethyl)benzene (9a) [37] Colourless oil, yield 30%. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.65–7.01 (m, 5H), 4.63 (t, J = 7.3 Hz, 2H), 3.31 (t, J = 7.3 Hz, 2H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 136.6, 129.4, 129.1, 127.9, 76.9, 33.9.

2.5.3. High performance liquid chromatography and mass spectroscopy

Mass spectral data were recorded using Agilent MSD TOF spectrometer coupled with Agilent 1200 HPLC or Agilent Technologies 5975C MS coupled with Agilent Technologies 6890N GC. The enantiomeric excess was determined by HPLC with CHIRALPAK IA (250 mm × 4.6 mm, 5 μm Analytical Column; Chiral Technologies Europe).

3. Results and discussion

Selective reduction of conjugated nitroalkenes represents one of the most direct routes for synthesis of nitroalkanes. Therefore considerable efforts have been made in development of the numerous reduction methodologies for this purpose including hydride catalysis [19,38,39], organocatalysis [40–42], and biocatalysis [43,44]. During this study we have identified *E. coli* BL21 (DE3) cells developed for recombinant protein expression as potent biocatalyst for chemoselective reduction of a range of nitroalkenes. This finding is in agreement with recent findings of Burda and coworkers that noticed 'background activity' when assessing the activity of recombinant ene reductases from *Gluconobacter oxydans* [13,45]. Likewise Oberleitner and coworkers described interfering reduction activity in *E. coli* BL21(DE3) host during the generation of biocatalytic toolbox for redox cascade reactions [46].

3.1. Biotransformation of 1-nitro-4-phenyl-1,3-butadiene using E. coli BL21(4-OT) and E. coli BL21(DE3) whole cells

We have recently described whole-cell biocatalytic system based on recombinantly expressed 4-oxalocrotonate tautomerase that was effective for the asymmetric Michael addition of acetaldehyde to β-nitrostyrenes [29]. To expand the substrate range of this system, we wished to include derivatives with extended conjugation, so we have performed biotransformation reaction using 1-nitro-4-phenyl-1,3-butadiene **1** and acetaldehyde (Fig. 1a). Surprisingly, after 4 h incubation, only 12% (w/w) of the product was expected Michael addition product, 3-(nitro methyl)-5-phenyl-4-pentanal, while the 71% (w/w) of the product was actually reduced 4-nitro-1-phenylbutene (Fig. 1a). Latter was the sole product when acetaldehyde was omitted from the biotransformation (data not shown).

High-level protein production (such in the case of *E. coli* BL21(4-OT)) could have lead to modifications in catabolism and anabolism of this strain, including adjustments of the energy generating system, the protein producing system, and cellular fluxes [47,48]. To determine the possible involvement of the 4-oxalocrotonate tautomerase or specific stress-inducing unfavourable conditions of *E. coli* BL21(4-OT) cells, we have performed the same reaction using *E. coli* BL21(DE3) cells again obtaining the reduction product as the only product regardless of the acetaldehyde presence (Fig. 1b). Therefore it was concluded that neither 4-oxalocrotonate tautomerase nor acetaldehyde were required for the biotransformation of 1-nitro-4-phenyl-1,3-butadiene **1** to 4-nitro-1-phenylbutene **1a** and that *E. coli* BL21(DE3) cells were efficient biocatalyst for the chemoselective reduction of activated C=C bond. Intrigued by this highly chemoselective reductions we decided to explore it in more detail.

3.2. Chemoselective reduction of activated C=C bond in a range of nitroalkenes using E. coli BL21(DE3)

To study the scope of the observed chemoselective reduction, additional compounds were used in biotransformation by *E. coli* BL21(DE3) whole cells (Fig. 2). In order to explore the influence of various stereo-electronic effects on the reaction course, several unsaturated nitro derivatives were selected as substrates (**2**–**8**; Fig. 2). Variation of the aryl substituent including the ring substitution pattern was examined while the conjugated nitro moiety was kept constant. In addition, structurally different compounds **9**–**13** were also included hoping to generate information about the substrate structural features necessitated by the cell line specificity.

E. coli BL21(DE3) reduced 9 different substrates with different efficiency and reaction times between 4 h and 48 h (Table 1). Product yields were mostly between 44% and 56% with an exception of **5a** and **6a** when low yields of 4% and 10% were observed (Table 1). Substrate depletion rates were between 0.2 and 3.8 μmol min^{-1} g CDW^{-1} with the highest depletion rate observed in the case of **3**

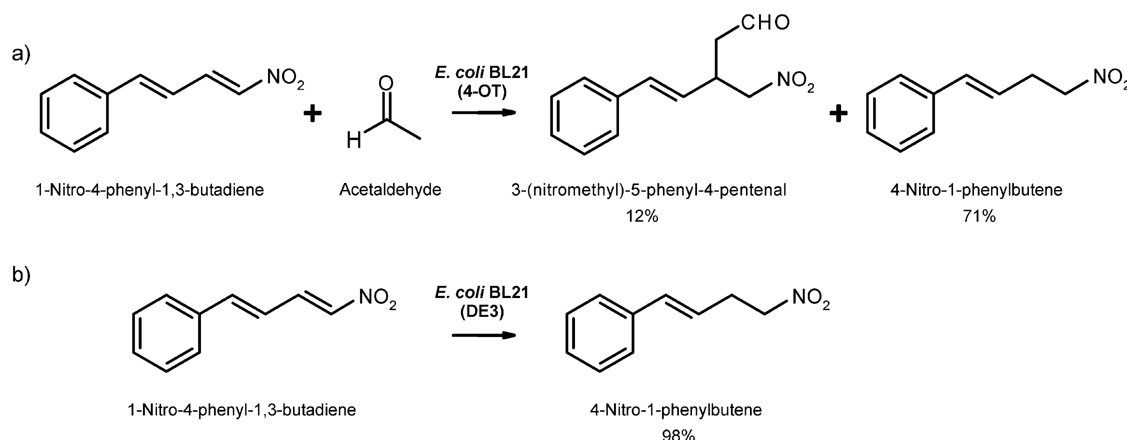

Fig. 1. Biotransformation of 1-nitro-4-phenyl-1,3-butadiene (a) in the presence of acetaldehyde using *E. coli* BL21(4-OT) cells and (b) using *E. coli* BL21(DE3) cells yielding 3-(nitromethyl)-5-phenyl-4-pentanal and 4-nitro-1-phenylbutene and 3-(nitromethyl)-5-phenyl-4-pentanal as products respectively.

(1-fluoro-4-(2-nitro-1-propenyl)benzene) and the lowest in the case of **6** (2,4-dichloro-1-(2-nitro-1-propenyl)benzene).

Compounds **2**–**4** showed almost equal reactivity in these biocatalytic reductions, although this may have not been expected considering electronic properties of the rings. Compared to parent compound **2**, derivative **3** possesses electron-deficient aromatic ring due to fluorine substituent (σ_F 0.062), while methoxy-derived **4** has electron rich ring (σ_{MeO} −0.268). This suggested that electronic properties of the aromatic ring, which consequently affect polarity of the double bond, were not influential factors in the reduction process by *E. coli* BL21(DE3) whole-cell biocatalyst. In addition to electronic, compounds **5** and **6**, although related to **3** and **4** regarding the electron density, incorporate steric features. The presence of an *ortho*-substituent destabilizes the planar conformation which is favoured by the electron delocalisation. Our study of the stereochemical properties of these molecules by computational methods suggested almost planar conformation for compound **3**, with dihedral angle between the ring and the C=C bond of 3.4° as opposed to 21.5° and 24.1° for compounds **5** and **6** (Fig. 3). It is possible that these conformational features contributed to low yields of products **5a** and **6a** (Table 1), although the steric hindrance caused by proximity of the *ortho*-substituent and the βC atom of the conjugated double bond, which may slow down the reduction, cannot be excluded.

Pyridine derived **7** and naphthalene derivative **8** afforded products in yields comparable to yields for substrates **1**–**4** (Table 1), supporting the observation that electronic features of the ring and sterics away from the *ortho* do not affect the reaction yield significantly. Interestingly the depletion rate for naphthyl derivative **8** was similar to those of derivatives **5** and **6** although the yield for product **8a**, as opposed to **5a** and **6a**, was comparable to other examples. It shows that extension of the aromatic substituent slows down the reaction but does not affect the yields significantly. Comparison of this result with those for compounds **5** and **6** suggests that the aryl ring subtly influences the reaction pathway.

Table 1
Biotransformation of conjugated nitroalkenes by *E. coli* BL21(DE3) using 2.5 g CDW L^{-1} at 28 °C, pH 7.2 in 60 mL volume.

Entry	Substrate	Product	Reaction time (h)	Substrate depletion rate (μmol min^{-1} g CDW^{-1})[a]	Yield (%)
a	**1**	**1a**	4	2.6	54
b	**2**	**2a**	4	3.2	45
c	**3**	**3a**	4	3.8	47
d	**4**	**4a**	4	2.5	56
e	**5**	**5a**	24	0.6	4
f	**6**	**6a**	48	0.2	10
g	**7**	**7a**	12	1.3	44
h	**8**	**8a**	48	0.3	46
i	**9**	**9a**	4	1.9	30

[a] Standard errors were between 3% and 6%.

In addition to the discussed compounds we also attempted to use derivatives **9**–**13** as substrates in order to get more insight into the reaction. While nitrostyrene **9** afforded the expected product albeit in low yield, the other compounds did not react under the experimental conditions. Based on this evidence it was concluded that nitro functionality was essential for the reaction to proceed, while the role of the aromatic ring in reactivity of the double bond was questionable.

For 7 products (except **7a** and **8a**) routes for chemical synthesis have been described and they usually involve harsh traditional reductive reagents such as hydrogen gas/metal and metal hydrides [49,50]. The synthesis of (4-nitro-1-butenyl)benzene **1a** was described only recently and requires addition of Hantzsch ester and S-benzyl isothiouronium chloride as organocatalyst for 24 h, product yields of 65% were comparable this study [51]. Described products **2a**, **3a** and **4a** were obtained in 99%, 85% and 66% product yields respectively in chemoselective hydrogenation by a rhodium complex [19]. Compound **5a** was synthesized using sodium borohydride reduction in 72% yield in 1 h [52], while **6a** was not obtained via reduction but rather via alkylation procedure [53].

Out of 9 products, 4 have previously been obtained using biocatalytic routes (**2a**, **4a**, **5a** and **9a**). Toogood and coworkers have described biphasic system based on purified pentaerythritol tetranitrate reductase for **2a** and **9a** with excellent yield of 93% and 99% respectively, but it took 3 and 2 days to complete biotransformation and the system required addition of isooctane, cofactors such as NADP, glucose-6-phosphate and the additional enzyme glucose 6-phosphate dehydrogenase [44]. Similarly to our study, low enantioselectivity of 14% e.e. was obtained for **2a** [44]. Compound **2a** was also obtained using baker's yeast reduction in 81% yield and 12% e.e. in 2 h [35] and using *Clostridium sporogenes* extracts with addition of NADH in 10% yield and 30–70% e.e. in 11 h [54]. Previously 1-methoxy-4-(2-nitropropyl)benzene and 1-methoxy-2-(2-nitropropyl)benzene (**4a** and **5a**) were obtained in 65% and 72% yields respectively using baker's yeast in 5 h. Modest enantioselectivity was reported for **4a** (45% e.e.) while **5a** was obtained in almost racemic form (3% e.e.) [35].

The non- or low-stereoselective reduction of α,β-disubstituted nitroalkenes with microbes was previously attributed to racemization of the product because of acidity of the α-proton [55]. During recent study Burda and coworkers also observed that **2**

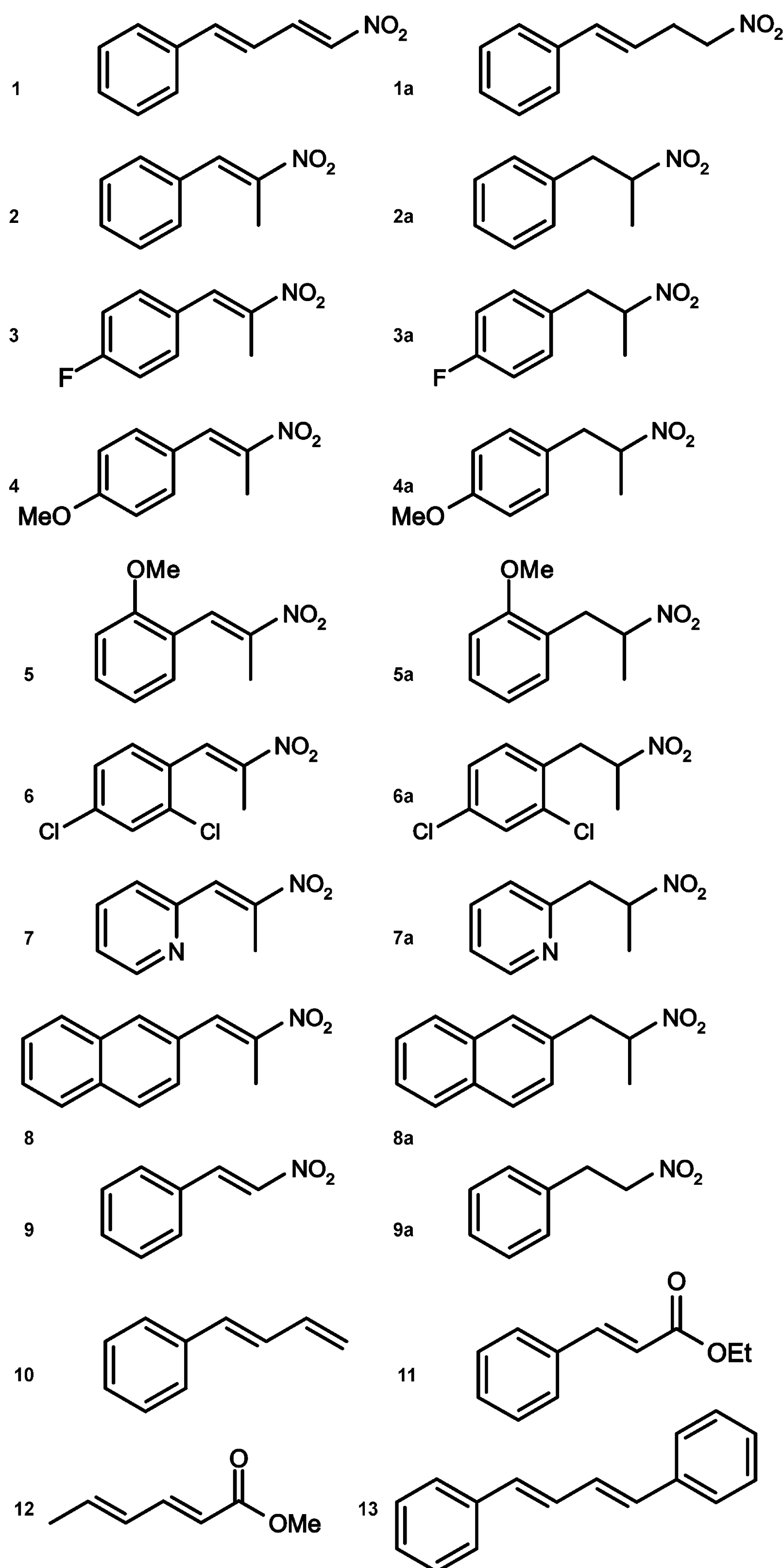

Fig. 2. Substrates and products of biotransformations using *E. coli* BL21(DE3) cells as biocatalyst.

could be transformed by crude cell extracts of *E. coli* yielding **2a** with low enantiomeric excess (<3% e.e.)[13]. Similar results were obtained in our study as well. The e.e. was established on acetamide derivative **4c** which was expected to be configurationally more stable than **4a**. In a standard two-step procedure, comprised of reduction and acetylation, product **4a** was transformed to acetamide **4c** (Scheme 1; Fig. 4). Unfortunately low enantioselectivity was observed (e.e. 3%), which might corroborate the theory of potential racemization due to acidity of the α-CH bond, but this remains to be investigated further. In the case of **7a**,

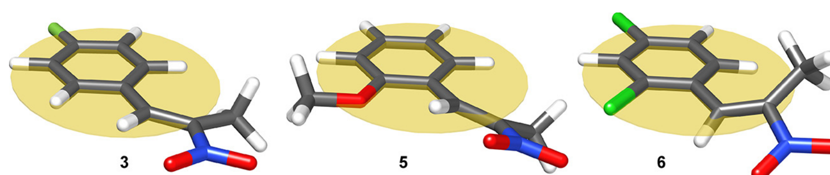

Fig. 3. Dihedral angles between the aryl and the C=C functionalities for biotransformation substrates **3**, **5** and **6**.

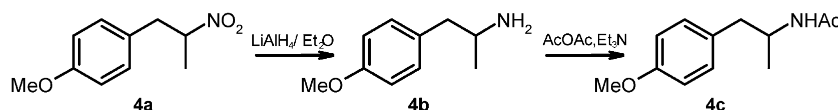

Fig. 4. Scheme of derivatization of biotransformation product **4a** to yield acetamide (**4c**) through chemical reduction and acetylation.

enantiomeric excess of 29% e.e. was observed without prior derivatization. Fryszkowska and coworkers have recently described that active site modifications in pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2 could lead to improved product enantiopurity, decreased by-product formation and altered stereochemical outcome in reactions with α,β-unsaturated nitroolefins [56]. Furthermore, generation of products with improved enantiopurities was achieved through manipulation of the reaction conditions as well [56] leaving space for the improvement of biocatalytic procedure based on whole-cell *E. coli* BL21(DE3) reductions described herein.

3.2.1. Identification and characterization of biotransformation products

Biotransformations (60 mL) containing *E. coli* BL21(DE3) cells consumed completely 9 different substrates (Fig. 2 and Table 1). A single product was observed in each biotransformation (**1a–9a**). The biotransformation products were purified and subsequently identified by their ^{1}H and ^{13}C NMR (Section 2.5.2). Spectral data corresponded well to the data available in the literature. Spectral data for compounds **7a** and **8a** were not available, so complete characterization of these compounds was done, including IR and HRMS (Section 2.5.2).

3.3. Testing other bacterial whole-cell biocatalysts for the bioreduction of *1* and *2*

In order to establish if described chemoselective reduction of conjugated nitro olefins was exclusive for the *E. coli* BL21(DE3) cells, three other bacterial strains were tested for the ability to reduce substrates **1** and **2**. Closely related *E. coli* DH5α could perform the reduction of **1** and **2** with slightly lower efficiency in comparison to *E. coli* BL21(DE3) judged by the overall product yield (Table 2). So far one possible protein from *E. coli* has been shown to catalyze asymetric redutions of activated alkenes such as cyclic conjugated ketones, conjugated esters, aldehydes and nitro-derivatives [11] and to carvone [46], namely NemA (*N*-ethylmaleimide reductase). However, the model Gram-positive strain of *B. subtilis* 168 was not able to transform either of substrates under conditions tested. *P. putida* KT2440 was able to biotransform only substrate **2** ((2-nitro-1-propenyl)benzene) with 1.8-fold lower efficiency in comparison to *E. coli* BL21(DE3). Indeed, Yanto and coworkers have described xenobiotic reductase XenA from this organism and reported it's activity on a range of substrates including **2** [57]. Using purified enzyme, with addition of glucose, $NADP^{+}$ and glucose dehydrogenase, only 13.6% conversion and no enantiomeric excess were achieved [57]. This enzyme exhibited much better activity in biocatalytic conversion of 2-cyclohexanone, ketoisophorone, and 1-nitrocyclohexene [57]. *nemA* gene was also present in the genome of *P. putida* KT2440. While this strain is known for is ability to degrade various substrates gene encoding morphinone reductase (MorR) that was previously shown to be able to catalyze a range of asymetric reductions of activated alkenes [11] was not present. This enzyme was described in closely related *P. putida* M10 that could grow on morphine as a sole source of carbon [58].

In silico search of available genomes of all three strains revealed 84, 101 and 65 hits for oxidoreductases in *E. coli* BL21(DE3), *P. putida* KT2440 and *B. subtilis* 168 respectively (genomes are available through KEGG: Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/catalog/org_list.html). Nevertheless, functional and biochemical annalysis would be required to annotate these genes.

3.4. The effect N-ethylmaleimide reductase (NemA) coding gene inactivation in E. coli BL21(DE3) on bioreductions of *1* and *2*

In an attempt to elucidate the active principle during bioreductions of **1** and **2**, we have carried out PCR screen of genomes of *E. coli* BL21(DE3), *P. putida* KT2440 and *B. subtilis* 168 for the presence of *nemA* or genes similar to *nemA* using a set of designed degenerate oligonucleotide primers (Fig. 5a). As predicted from the in silico analysis of the genome sequences, the fragment of the expected size of 956 nt was successfully amplified from the genome of *E. coli* BL21(DE3). The fragment of the corrected size of 926 nt was amplified from the genomic DNA of *P. putida* KT2440 (Fig. 5a). Identity of both fragments was confirmed by sequencing that they were partsof respective *nemA* genes. Additional fragment of approximately 800 bp with 95% sequence similarity to quinone oxidoreductase belonging to YhdH/YhfP family was also amplified from *E. coli* BL21(DE3) (Fig. 5b).

Table 2
Biotransformation of **1** and **2** using different biocatalysts.

Biocatalyst	Substrate **1**			Substrate **2**		
	Product	Reaction time (h)	Yield (%)	Product	Reaction time (h)	Yield (%)
E. coli DH5α	**1a**	4	40	**2a**	4	37
P. putida KT2440	**n.r.**[a]	24	–	**2a**	4	25
B. subtilis 168	**n.r.**	24	–	**n.r.**	24	–

[a] No reaction occurred (no substrate depletion observed).

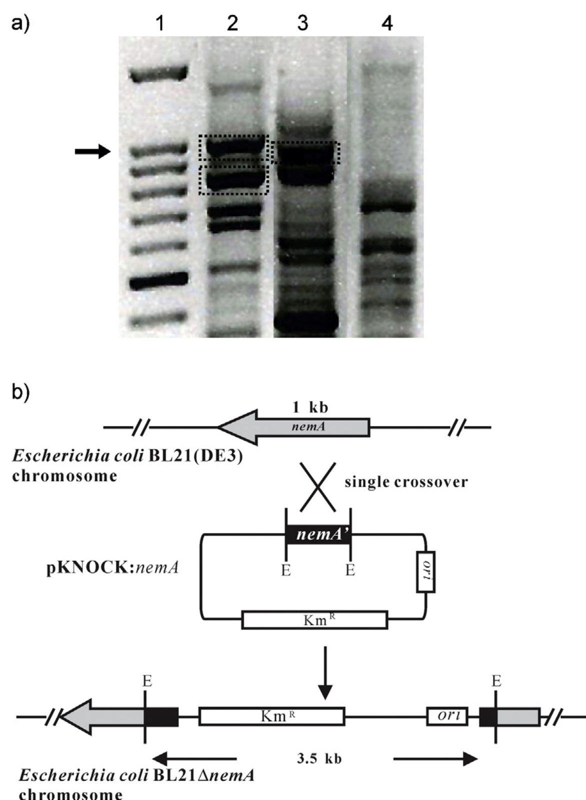

Fig. 5. (A) Polymerase chain reaction screen for the presence of *nemA* reductase gene in *E. coli* BL21 (lane 2), *P. putida* KT2440(lane 3) and *B. subtilis* 168 (lane 4). DNA molecular size marker with arrow pointing to 1 kb fragment (lane 1). Dashed boxes indicate putative reductase fragments confirmed by sequencing. (B) Schematic representation of the generation of the *E. coli* BL21ΔnemA strain.

In order to establish if NemA was responsible for bioreductions of **1** and **2**, gene encoding this protein was insertionally inactivated yielding *E. coli* BL21Δ*nemA* strain (Fig. 5B). This strain was not affected in growth in comparison to wild type *E. coli* BL21(DE3) and it's ability to transform **1** and **2** was still present. **1** was transformed with the same efficiency, while the efficiency to reduce **2** was decreased by 40% in comparison to *E. coli* BL21(DE3) (data nor shown). In contrast, Oberleitner and coworkers lost the interfering reduction activity of carvone upon deletion of *nemA* gene [46]. These finding lead us to conclude that although involved in bioreductions of **1** and **2**, NemA is not the only active reductase present in herein described biocatalyst.

4. Conclusion

E. coli B strains (including the strain BL21(DE3)) are among the most frequently used bacterial hosts for production of recombinant proteins on an industrial scale [21], so it was used as a whole-cell biocatalyst in this study. Selective biocatalytic transformation of multifunctional compounds were achieved using this strain whereby nitro group had stayed intact, offering possibility of further transformation and novel application of this strain. Indeed, transferring this application to an industrial scale would require detailed assessment of physical–chemical phenomena of biocatalyst efficiency and tailored solutions for the large scale process. On the other side, this particular strain is amenable to industrial scale fermentations [59] and has frequently been applied in industrial biocatalytic applications relying on immobilization technologies [60,61].

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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.enzmictec.2014.03.010.

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