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Functional expression of a valencene dioxygenase from *Pleurotus sapidus* in *E. coli*

Kateryna Zelena *, Ulrich Krings, Ralf G. Berger

Gottfried Wilhelm Leibniz University Hannover, Institute of Food Chemistry, Callinstr. 5, D-30167 Hannover, Germany

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ABSTRACT

Valencene dioxygenase (ValOx) from the edible basidiomycete *Pleurotus sapidus* converted the sesquiterpene (+)-valencene to the valuable grapefruit flavour (+)-nootkatone and to nootkatols through intermediate hydroperoxides. Expression of the enzyme was carried out in the cytosol and periplasm of *Escherichia coli*. The heterologous production led to high yields of inclusion bodies. The poor yield of soluble recombinant protein was improved by various strategies including cold shock expression, chaperone co-expression, and employment of mutant *E. coli* strains. Up to 60 mg of the biologically active, soluble ValOx was produced by cold shock under control of the *cspA* promoter at 8 °C in the BL21(DE3)Star strain and co-expression of the *E. coli* trigger factor. The recombinant enzyme, purified using the N-terminal His tag, showed the catalytic properties of the wild-type enzyme, as was confirmed by the LC-MS analysis of hydroperoxide intermediates and GC–MS analysis of the volatile products.

1. Introduction

The bicyclic sesquiterpene ketone (+)-nootkatone is a natural flavour compound which was first isolated from cedar wood (*Chamaecyparis nootkatensis*, Erdtman and Hirose, 1962) and later found in trace amounts in grapefruit, mandarin, and pummelo oil. With a characteristic grapefruit-like flavour and a low odour threshold of about 1 μg L^{-1} water (+)-nootkatone represents a valuable ingredient in food and cosmetic applications. Apart from the sensory properties, a wide range of biological activities of (+)-nootkatone was reported (Fraatz et al., 2009a). Ongoing research is devoted to the insecticidal properties of this sesquiterpene and some derivatives (Zhu et al., 2010; Flor-Weiler et al., 2011). Medicinal applications of nootkatone and its microbial transformation products (Jin et al., 2011; Tassaneeyakul et al., 2000), such as the antiproliferative activity towards cancer cell lines (Gliszczynska et al., 2011) and anti-platelet effects (Seo et al., 2011) were discussed.

Most synthetic routes to (+)-nootkatone rely on the C2 allylic oxidation of (+)-valencene, an abundant sesquiterpene constituent of orange and mandarin oil, and, thus, a convenient bioresource (Moshonas and Shaw, 1979; Fraatz et al., 2009a).

Classical chemical methods involve organic solvents and strong oxidants, such as the carcinogenic chromic acid, increasingly raising ecological concerns (Shaffer et al., 1975; Salvador and Clark, 2002). As a result, numerous biotechnological attempts have also been undertaken (Fraatz et al., 2009a). A recent approach investigated 125 cytochrome P450 enzymes from bacteria for the regio-selective oxidation of (+)-valencene, and cytochrome CYP109B1 from *Bacillus subtilis* was found most suitable (Girhard et al., 2009). Even with the best P450 activity, however, only minor concentrations of (+)-nootkatone and many over-oxidized products were found.

In contrast to submerged cultured cells, lyophilisates of the edible mushroom *Pleurotus sapidus* produced more than 250 mg of natural nootkatone L^{-1} (Fraatz et al., 2009a). Lyophilisates, however, not only turned out to be a difficult source for the isolation of the target protein because of the abundant presence of peptidases (Schmidt et al., 2011), but are also inapplicable to larger-scale processes. First steps towards the heterologous expression of the putative ValOx were the purification and partial molecular characterization (Fraatz et al., 2009b). Electrophoretically pure native ValOx generated hydroperoxides of valencene, nootkatone and nootkatols in the absence of any redox co-factor (Krügener et al., 2010), evidencing the potential lipoxygenase character of the dioxygenase.

The expression of a recombinant protein in *E. coli* results in several advantages, such as ease of cultivation and rapid growth, and may result in a larger yield of active enzyme. A successful expression strategy is still largely based on a concerted screening of expression hosts and genetic tools. Upon intracellular expression, the product will either accumulate in a soluble form or precipitate as inclusion bodies. If a secretion system for *E. coli* is used, and the protein is secretable, the gene product will typically accumulate in the periplasm (Jonasson et al., 2002).

The present study describes the amplification, cloning, and functional gene expression of the *P. sapidus* valencene dioxygenase

* Corresponding author. Tel.: +49 511 762 17257; fax: +49 511 762 4547.
E-mail address: kateryna.zelena@lci.uni-hannover.de (K. Zelena).

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in *E. coli* using a cold shock inducible promoter, and supported by co-expression of the *E. coli* trigger factor acting as a chaperone maintaining the newly synthesized protein in an open conformation.

2. Methods

2.1. Chemicals

Chemicals used were either purchased from Carl Roth (Karlsruhe, Germany), Sigma (Neu-Ulm, Germany), or Merck (Darmstadt, Germany). Solvents (p. a. grade) were rectified before use.

2.2. Strains and culture media

Pleurotus sapidus (DSM 8266) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cultivation and subsequent lyophilisation of biomass was performed as published elsewhere (Fraatz et al., 2009b).

Vector propagation and heterologous expression of recombinant protein were accomplished in *E. coli* strains TOP10, LMG194 and BL21(DE3)Star (Invitrogen, Karlsruhe, Germany). *E. coli* cells were cultivated in LB medium (Carl Roth), MDGA-135 minimal medium (Studier, 2005) or RM minimal medium containing 50 mg L^{-1} ampicillin as a selection marker. RM medium consisted of 2% casamino acids, 0.2% glucose, 1 mM $MgCl_2$, 0.1 mM thiamin, 6 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl and 1 g L^{-1} NH_4Cl. In case of chaperone co-expression the medium contained 20 mg L^{-1} chloramphenicol. The recombinant bacterial colonies were grown at 37 °C on LB agar (Roth) plates containing either 50 mg L^{-1} ampicillin or both 50 mg L^{-1} ampicillin and 20 mg L^{-1} chloramphenicol for selection.

2.3. Cloning of the ValOx full-length cDNA

For RNA isolation, 100 mg mycelium of the 4th culture day were ground under liquid nitrogen. Total RNA was extracted using the NucleoSpin® RNA Plant Kit (Macherey–Nagel, Düren, Germany), following the manufacturer's instructions. The integrity of the RNA was checked by denaturing formaldehyde agarose gel electrophoresis. A cDNA library was constructed using the RNA as template with the SMART PCR cDNA Library Construction Kit (Clontech, Heidelberg, Germany). For PCR amplification of *P. sapidus* valencene oxygenase cDNA fragments from the cDNA library, the TrueStart™ Hot Start *Taq* DNA Polymerase (Fermentas, St. Leon-Roth, Germany) was employed. PCR primers were deduced from amino acid sequences of tryptic peptides. Amplification experiments of the specific cDNA fragments were performed in a Master Cycler gradient (Eppendorf, Hamburg, Germany). For the amplification of the *cDNA*, the primers (5'-ATGGTCCACAACATATCGTTGTCG-3')/(5'-TCAAATTACAATCGCATTTGCGAGTTGC-3') were used at an annealing temperature of 62 °C. The PCR conditions were as follows: 95 °C 5 min, 95 °C 1 min, 62 °C 1 min, 72 °C 2 min 30 s, 35 cycles and finally 72 °C for 10 min. After electrophoretic separation of the PCR amplificates and extraction from the agarose gel (1%) using the NucleoSpin Extract II Kit (Macherey–Nagel), the fragments were ligated into the pCR2.1-TOPO vector (TA Cloning Kit, Invitrogen, Karlsruhe, Germany). The plasmid DNA was propagated in *E. coli* TOP10, isolated with a NucleoSpin Plasmid Kit (Macherey–Nagel), and sequenced by MWG-BIOTECH AG (Ebersberg, Germany).

2.4. Vector construction

The vector pCR2.1-TOPO*ValOx* was used as a template to construct the required expression vectors. The plasmids pBAD/gIII A (Invitrogen) and pCold I (TaKaRa, Göttingen, Germany) were used for expression of the target protein in *E. coli*. The vectors pGro7, pG-Tf2 and pTf16 (TaKaRa) were applied for chaperone co-expression.

ValOx cDNA equipped with necessary restriction sites was amplified from a pCR2.1- TOPO*ValOx*-vector with a *Pfu* DNA polymerase (Fermentas) via standard PCR method using primers from MWG (Ebersberg, Germany). For the insertion of the *ValOx* sequence into the pBAD/gIII A vector the forward primer 1 (5́-GTGCTGGAATTCTCGAGATGGTCCACAA-3́) and the reverse primer 1 (5́-CTGCAGAATTCTCTAGAAAAATTACAATCGC-3́) with a cleavage sites for *XhoI* and *XbaI* (underlined) were applied, respectively. In the case of the pCold I plasmid the forward primer 2 (5́-TGGAATTCGGCATATGGTCCACAA-3́) included a site for *NdeI*, and the reverse primer 2 (5́-CTGCAGAATTTCTAGATTCAAATTACAATCGC-3́) contained a cleavage site for *XbaI* (underlined). The thermal cycler profile was as follows: after denaturation for 5 min at 95 °C, 35 cycles (94 °C for 1 min, 62 °C for 1 min and 72 °C for 4 min, and a terminating step at 72 °C for 10 min) were carried out. After purification of PCR fragments by means of gel electrophoresis and extraction from the gel using the NucleoSpin Extract II Kit (Macherey–Nagel, Düren, Germany), the amplificates as well as the plasmids were digested with *XhoI* and *XbaI* or *NdeI* and *XbaI* (Fermentas), respectively. The ligation resulted in the expression vectors pBAD*ValOx* and pCold*ValOx*. To verify the amplified sequence, the plasmid-DNA, propagated in *E. coli* TOP10 and isolated with a NucleoSpin Plasmid Kit (Macherey–Nagel), was sequenced (MWG Operon, Ebersberg, Germany).

2.5. Transformation and ValOx expression

E. coli cells were transformed by heat shock for 30 s at 42 °C using a standard protocol (Sambrook and Russell, 2001) and grown on LB plates in the presence of appropriate antibiotics. In the case of the pBAD/gIII A and pCold I based vectors (empty or recombinant) the plates contained exclusively 50 mg m L^{-1} ampicillin. For the chaperone co-expression *E. coli* cells were first transformed with a chaperone encoding plasmid and selected on the agar plates containing 20 mg mL^{-1} chloramphenicol. The recombinant clones developed were then used for the production of chemically competent cells (Sambrook and Russell, 2001) and subsequently transformed with pBAD*ValOx* or pCold*ValOx* vector. The selection was carried out on LB plates containing both 50 mg m L^{-1} ampicillin and 20 mg m L^{-1} chloramphenicol. The pilot expression with *E. coli* cells containing expression plasmids was performed using 5 mL liquid cultures at 220 rpm. Depending on the production strain LB-Amp, LB-Amp-Cam, RM-Amp, RM-Amp-Cam, MDGA135-Amp or MDGA135-Amp-Cam medium was used. The scale-up experiments were realized using 1-L liquid cultures in the 2-L baffled flasks. *E. coli* cultures containing either recombinant or empty vectors were grown over night at 37 °C, then diluted with liquid medium to an OD_{600} of ~0.1 and finally grown for another 2–2.5 h with vigorous shaking at 37 °C. Chaperone expression was induced immediately after inoculation by adding the respective inducer (Table 1), whereas target protein expression was induced with several inducer concentrations, after an OD_{600} of 0.4–0.6 (mid-log) was reached. For the pBAD based periplasmic system, target protein expression was induced by adding l-arabinose for 4 h at 37 °C or for 16 h at 25 °C. For the cold shock based system a cooling step at 15 °C for 30 min preceded IPTG induction of the ValOx production. The induction took place at 8 °C for 48 h; then the cells were harvested by centrifugation at 12,000×*g* and 4 °C for 5 min.

Cell disruption was achieved after incubation in lysis buffer (20 mM Tris–HCl, pH 7.5, 10 U mL^{-1} benzoase, 2 mM $MgCl_2$, and 1 mg mL^{-1} lysozyme) for 2 h at RT. After 10 vortex cycles, each for 10 s alternating with 10 s storage on ice, the crude extract was centrifuged for 15 min at 12,000×*g* and 4 °C for subsequent fractionation.

2.6. SDS–PAGE and Western blotting

To control the expression level *via* SDS–PAGE, cells from 1 mL *E. coli* cultures were re-suspended in 100 μL SDS–PAGE sample buffer. The supernatants, after cell lysis and centrifugation, were diluted 1:2, and the corresponding pellets were mixed with 100 μL SDS loading buffer. The samples were denatured for 10 min at 95 °C. SDS–PAGE was performed according to Laemmli (1970) using 12% (w/v) polyacrylamide gels. Proteins were stained with 0.1% (w/v) Coomassie Brillant Blue R-250 (Serva, Heidelberg, Germany). Unstained standard proteins (Bio-Rad, Munich, Germany) were used for the preparation of a calibration curve for the determination of molecular masses.

For Western blot analysis the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The recombinant dioxygenase was detected using a Penta-His HRP conjugated Kit (5 PRIME, Hamburg, Germany). Staining was carried out using 4-chloro-1-naphtol and H_2O_2 according to the manual instructions.

2.7. Protein concentration

The protein concentration of duplicate samples was determined by the method of Lowry et al. (1951) using *DC*-Protein-Assay (Bio-Rad) and bovine serum albumin as a standard. In the concentration range used (0.2–1.2 mg mL^{-1}) the calibration curve was linear with a regression coefficient of $R^2 = 0.990$.

2.8. Purification of recombinant ValOx

Soluble lysis fractions of 1 L cultures were concentrated using Centricon Plus-70 Ultracel-PL centrifugal filter devices (10,000 MWCO, Millipore, Schwalbach, Germany). For purification of the His tag labeled recombinant ValOx under native conditions, Protino Ni-IDA 2000 Packed Columns (Macherey–Nagel) were used. Concentrated cell lysates containing the recombinant ValOx were diluted 1:2 with equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8) and purified chromatographically. After the target protein was eluted with 250 mM imidazole, the purified samples were washed three times with a ten-fold volume of 20 mM Tris-HCl, pH 7.5 buffer, and the protein concentration was determined as described above. The quality of the purification procedure was controlled via SDS–PAGE.

2.9. Transformation of (+)-valencene

The transformation of (+)-valencene was carried out in 4 mL glass vials. 25 mg *P. sapidus* lyophilisate was re-suspended in 500 μL Tris–HCl buffer 20 mM, pH 7.5 and deactivated for 30 min at 95 °C. One mL of cell lysate or purified recombinant enzyme was dissolved in Tris–HCl buffer, 1 μL (+)-valencene (Döhler, Darmstadt, Germany) and respective surfactants were added to the deactivated lyophilisate. The transformation was performed over 16 h according to former studies (Fraatz et al., 2009b). A *P. sapidus* lyophilisate sample without deactivation acted as a positive control; as negative controls served soluble fractions of *E. coli* cultures with empty vectors, deactivated *P. sapidus* lyophilisate, and deactivated recombinant protein. In an initial enzyme activity screening, non-induced *E. coli* cultures (*l*-Ara or IPTG for pBAD*ValOx* and pCold*ValOx*, respectively) acted as negative controls as well.

After (+)-valencene bioconversion the products were extracted with 1 mL azeotropic pentane/diethylether (1:1.12). Five-hundred μL of the extracts were dried (Na_2SO_4) and analysed by mass spectrometry (HRGC-MS) and high performance liquid chromatography with UV/Vis and mass spectrometric detection (HPLC-DAD–MS) as described elsewhere (Krügener et al., 2010). Supplementary, 200 μL of a peroxide reagent, containing α-naphtol and N-{2-[N-ethyl-N(4-amino-3-methylphenyl)-amino]-ethyl}-methansulfonamide sesquisulfate (Huber and Fröhlke, 1972) were added to the organic phase, and the samples were then incubated at room temperature for 30 min to complete staining. The presence of peroxides was indicated by the formation of a blue quinone imine pigment.

2.10. Identification of transformation products via GC–MS

One microliter of the organic extracts after (+)-valencene bioconversion was injected cool on-column into a Fisons GC 8000 system (CP Wax 52 CB capillary column, 30 m × 0.25 mm i.d., 0.25 μm film thickness) connected to a Fisons MD 800 mass selective detector (interface: 230 °C, ion source: 200 °C, quadrupole: 100 °C, EI ionisation (70 eV), scan range m/z 33–300 amu) as described (Krügener et al., 2010). The quantification was carried out using thymol as the internal standard. Identification of transformation products was achieved by comparison of EI mass spectra with data from reference compounds or literature (Wiley/NIST 08 spectral libraries).

2.11. LC–MS

The respective concentrates (P/E) were injected onto a EC Nucleodur Pyramid C18, 250 × 4 mm, 5 μm column (Macherey & Nage, Düren, Germany) equipped with a CC Nucleodur Pyramid C18, 8 × 4 mm, 5 μm pre-column (Macherey & Nage, Düren, Germany). HPLC was carried out using two high pressure gradient Varian 212-LC chromatography pumps (Palo Alto, CA, USA), a Varian 4 channel vacuum degasser (Palo Alto, CA, USA) and a Varian Pro Star UV/Vis detector (Palo Alto, CA, USA). The mobile phase was H_2O (A) and MeOH (B) at a flow of 0.5 mL min^{-1}. Samples were injected (5 μL) at 50% B raised to 80% B in 20 min, and finally to 100% B held for 5 min and turned back to start conditions after 5 min. The effluent was consecutively monitored at 210 nm or by MS. MS was carried out using a Varian 320-MS TQ mass spectrometer (Palo Alto, CA, USA) operating in the APCI (+) mode (3.5 μA corona current) with drying gas at 172 kPa and 200 °C, vaporizer gas at 82.7 kPa and 400 °C, 379 kPa nebulizer gas pressure, and API housing at 50 °C. Data were recorded at 30 V capillary voltage.

3. Results and discussion

3.1. Identification of the ValOx gene

Recently, a novel oxidase which catalyzed the selective allylic oxidation of (+)-valencene to the flavour compound nootkatone was characterized on a biochemical and molecular level (Fraatz et al., 2009b; Krügener et al., 2010). However, the concluding proof that the published amino acid sequence indeed corresponded to the dioxygenase activity found in the lyophilisate of *P. sapidus* was missing. A follow-up study (Schmidt et al., 2011) found an unaccountable difference of the molecular mass of the enzyme between Western blot analysis (around 75 kDa) and a mass of 44.2 kDa, calculated from the cloned cDNA (accession number B8ZIU7, Fraatz et al., 2009b). Thus, the PCR screening for the oxygenase encoding cDNA was re-started. Using freshly prepared

Table 1
Host strains and constructs for the heterologous expression of the ValOx from *P. sapidus* in *E. coli*.

Strain	Without chaperones		Chaperones co-expression, vector					
			pGro7groES/groEL chaperone induction l-ara		pG-Tf2 groES/groEL/tig chaperone induction tet		pTf16 tig chaperone induction l-ara	
	Expression vector							
	pBAD/gIIIA*ValOx*	pColdI*ValOx*	pBAD/gIIIA*ValOx*	pColdI*ValOx*	pBAD/gIIIA*ValOx*	pColdI*ValOx*	pBAD/gIIIA*ValOx*	pColdI*ValOx*
BL21(DE3)Star	–[a]	+, i. b.	–	+, s.[d]	–	+, s.	–	+, s.
TOP10	+[b], i. b.[c]	+, i. b.	–	+, s.	+, i. b.	+, s.	–	+, s.
LMG194	+, i. b.	–	–	–	+, i. b.	–	–	–

[a] Strain/chaperone vector / expression vector combination not tried for the recombinant ValOx expression.
[b] Strain / chaperone vector / expression vector combination tried for the recombinant ValOx expression.
[c] Heterologous expression resulted in inclusion bodies.
[d] Heterologous expression resulted in a soluble active enzyme.

cDNA and systematically varied PCR conditions, a cDNA coding for 257 additional N-terminal amino acids was eventually amplified (Schmidt et al., 2011). Two partial sequences found in the preceding ESI-MS/MS analysis (SIPQTFTL and LTQWNQ, Fig. 1; Fraatz, 2007) were also located in the completed amino acid chain. The comparison of the updated ValOx sequence with the obviously homologous protein of the closely related oyster mushroom, *Pleurotus ostreatus* (genome project PC9 v1.0; Joint Genome Institute, protein id: 77103), which recently became publicly accessible, displayed an identity of 93% at the protein level. The analysis of the corresponding DNA revealed the presence of the sequence ACAATGG close to the Kozak consensus sequence A[C/A][A/C]ATG[G/T] indicating the position of the start M in the amino acid chain of the ValOx.

The 1929 bp full-length cDNA of the ValOx gene was cloned from *Pleurotus sapidus* strain 8266. The coding region of *ValOx* encoding 643 aa residues (Fig. 1) with a calculated molecular mass of 72.3 kDa and a theoretical isoelectric point of 5.33 (Compute pI/Mw tool; http://www.expasy.org/cgi-bin/pi_tool).

3.2. Expression vector construction

Valencene oxygenase is the first basidiomycete dioxygenase characterized on both protein and gene level. As no previous experience on the recombinant production of such an enzyme existed, various expression strategies in the heterologous host *E. coli* were chosen: periplasmic expression with the pBAD/gIII A vector and cold shock expression using the pCold I vector. Furthermore, cytoplasmic chaperones GroEL-GroES and trigger factor were co-expressed to investigate possible effects on production level and solubility of the ValOx.

To insert the target gene sequence into the expression plasmids pBAD/gIII A or pCold I, respectively, two sets of primers were designed. The reverse primer either included the native stop codon (pCold system), or the stop codon was omitted in order to clone the *ValOx* gene in frame with a C-terminal tag epitope of the pBAD vector. The forward primer contained a restriction site to ligate the amplified DNA fragments in frame with the N-terminal His tag epitope of the pCold I vector or with the gIII signal sequence of the pBAD/gIII A vector. The recombinant plasmids pBAD*ValOx* and pCold*ValOx* were transformed into the *E. coli* propagation strain TOP10. After confirmation of the correct DNA sequence using automatic sequencing the plasmids were re-transformed into the expression hosts BL21(DE3)Star for pCold*ValOx* and LMG194 for pBAD*ValOx* (Table 1), and the protein production was started. When chaperones were co-expressed, the re-transformation occurred in an appropriate *E. coli* strain including one of the chaperone genes harbouring plasmids. The chaperone genes containing vectors pG-Tf2, pTf16 and pGro7 encoded a chloramphenicol resistance and were compatible with both ValOx-expression vectors encoding an ampicillin resistance. The combination of the *E. coli* strains with the various expression plasmids and chaperone plasmids resulted in 12 different production strains (Table 1).

3.3. Initial steps of recombinant protein production

Proteins expressed in *E. coli* are often prone to precipitate in form of insoluble inclusion bodies. The recombinant protein expression in the current work aimed at a catalytically active soluble ValOx. In the initial phase of the study, the production efficiency with different vector-strain-chaperone combinations (Table 1) was evaluated using 5-mL cultures. Variable culture parameters such as induction timing, aeration conditions, cultivation temperature and duration were optimized on the basis of pCold or pBAD containing strains separately. The pBAD/gIIIA vector (Invitrogen) provides the *ara*BAD promoter, translation initiation signals for optimized *E. coli* expression, and a C-terminal polyhistidine (6xHis) tag for purification of the recombinant product using affinity chromatography. The plasmid pCold I (TaKaRa), in addition to the *cspA* promoter, possesses various expression tools such as the *lac* operator, His-Tag sequence, and Factor Xa cleavage site.

The complete cell extracts of 1 mL cell cultures were examined via SDS–PAGE, and the yield of target protein guided the following steps (data not shown).

3.3.1. Periplasmic expression

One of the possibilities to obtain a soluble, secreted protein is the attachment of a sequence coding for a signal peptide targeting the heterologous product to the periplasm. The periplasmic space is rich in foldases which may catalyze important modifications, such as disulfide bond formation, and shows less peptidase activity compared to the cytoplasm (Fahnert et al., 2004; Jonasson et al., 2002).Furthermore, recombinant protein purification is simpler due to fewer contaminating proteins in the periplasm.

In the current study, the pBAD/gIII A variation of the pBAD/gIII plasmid, a derivative of the pBR322-vector, was employed. This vector family includes the gene III signal sequence, which directs the secretion of the recombinant protein into the periplasm (Boeke and Model, 1982).

High levels of secreted, recombinant protein could be achieved using the *ara*BAD promoter (P_{BAD}) of *E. coli* (Guzman et al., 1995). The pBAD/gIII system allows to influence the recovery of the recombinant protein by varying the inducer (l-arabinose) concentration. It was shown that the promoter is not controllable in individual cells but suffers from "all-or-none" gene expression (Khlebnikov et al., 2002). The concentration of arabinose in the growth medium affects the population of cells that are fully induced compared to non-induced cells (Khlebnikov et al., 2002).

mnttrnpppphldivgtst**M**VHNISLSSRKALHNVHLPYMVQLPKPTGYNVALKNAAEGYDKARRMVAW
LYDIADYES**SIPQTFTL**QQKTDKYTWELSDNFPPHLAVVPPDQSVSAPSIFSPVRLAQTLLIM
SSLWYDDHTDLAPGPEQNTMQK**LTQWNQER**HKDQGWLIKDMFNAPNIGLRNDWYTDEVFAQQF
FTGPNSTTITLASDVWLTAFTSEAKAQGKDKVIALFESAPPNSFYVQDFSDFRRRMGAKPDEE
LFNDSDGAMRYGCAAVALFYLTAMGKLHPLAIIPDYKGSMAASVTIFNKR**TNPLDISVNQAND**
WPWRYAKTCVLSSDWALHEMII<u>H</u>LNNT<u>H</u>LVEEAVIVAAQRKLSPSHIVFRLLEPHWVVTLSLN
ALARSVLIPEVIVPIAGFSAPHIFQFIRESFTNFDWKSLYVPADLESR**GFPVDQLNSPK**FHNY
AYAR**DINDMWTTLKK**FVSSVLQDAQYYPDDASVAGDTQIQAWCDEMRSGMGAGMTNFPESITT
VDDLVNMVTMCIHIAAPQ<u>H</u>TAVNYLQQYYQTFVPNKPSALFSPLPTSIAQLQK**YTESDLMAAL**
PLNAKRQWLLMAQIPYLLSMQVQEDENIVTYAANASTDKDPIIASAGRQLAADLKKLAAVFLV
NSAQLDDQNTPYDVLAPEQLANAIV<u>I</u>*

Fig. 1. Amino acid sequence of the ValOx from *Pleurotus sapidus*. Big M: the start methionine. Small: a part of the UTR. Underlined: amino acids involved in complexing of iron in genetically related lipoxygenases. In bold: amino acid sequences of tryptic peptides determined by ESI-MS/MS (Fraatz, 2007).

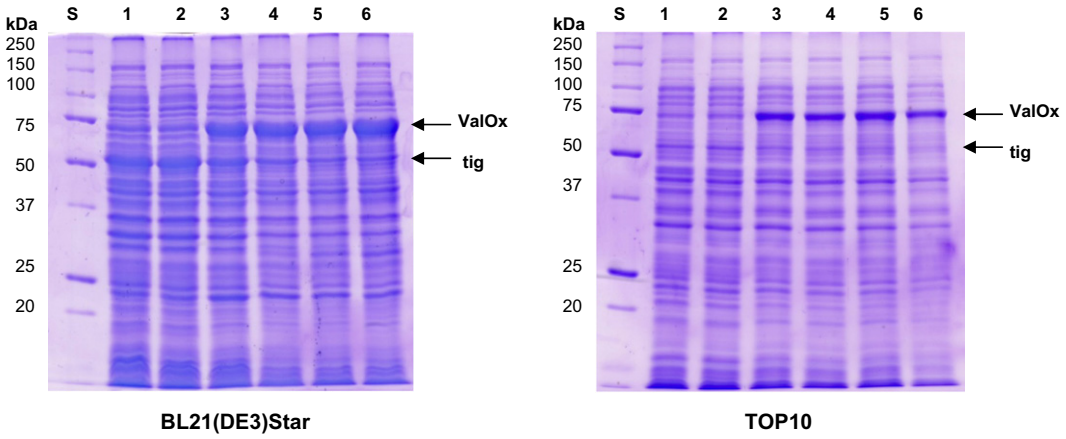

Fig. 2. Expression of the ValOx in *E. coli* strains BL21(DE3)Star and TOP10: Coomassie blue stained SDS–PAGE gels of the total cell extracts. *E. coli* bearing pTf16 and pCold*ValOx* plasmids were induced for 48 h at 8 °C. Expression of the trigger factor tig was induced with 1 mg mL^{-1} L-arabinose. S: M_w protein markers; Lane 1: pTf16 only, negative control 1; Lane 2: pTf16 + pCold*ValOx*, not induced, negative control 2; Lane 3 – Lane 6: pTf16 + pCold*ValOx* induced with 0.05, 0.1, 0.25 and 0.5 mM IPTG respectively.

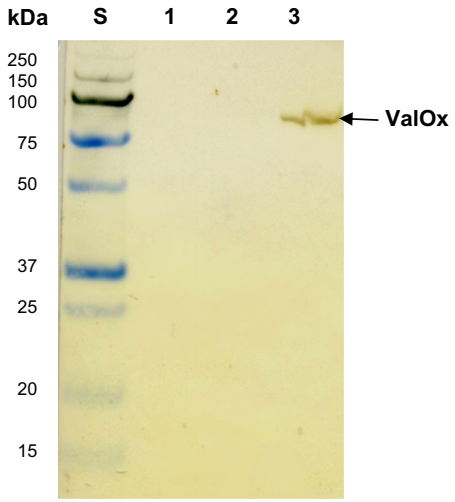

Fig. 3. Detection of the soluble recombinant ValOx using Western blotting. Recombinant production of the ValOx in *E. coli* BL21(DE3)Star strain was induced using 0.05 mM IPTG for 48 h at 8 °C. Expression of the chaperones was induced with 5 ng mL^{-1} tetracycline. S: Mw protein markers; Lane 1, negative control, empty pCold I vector; Lane 2: ValOx production without chaperone co-expression; Lane 3: co-expression of the ValOx with chaperones GroEL, GroES and tig.

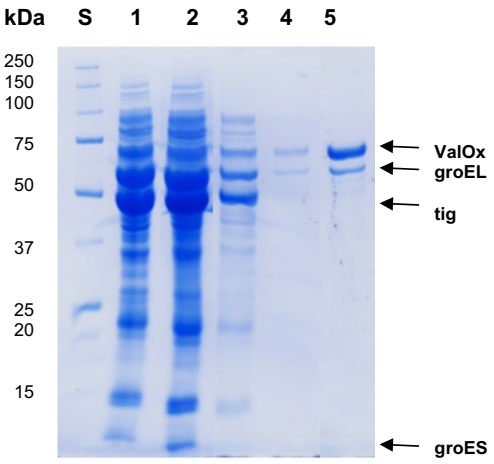

Fig. 4. Purification of the ValOx containing the N-terminal His-tag with a Ni–NTA column, Coomassie blue stained SDS–PAGE gel. S: M_w protein markers; Lane 1: crude cell lysate; Lane 2: break-through; Lanes 3 – 4: washing fractions; Lane 5: elution fraction.

For the heterologous ValOx expression the concentration of l-arabinose was varied in a range from 1×10^{-1}% to 1×10^{-5}% (w/v). The highest yield of heterologous polypeptide was obtained at an inducer concentration of 1×10^{-2}%, which was in good agreement with previous experience (Zelena et al., 2009). The *E. coli* strain LMG194 growing on RM minimal medium containing glucose was employed to possibly repress pBAD. The repression of a basal expression can be advantageous in case of proteins, such as foreign redox enzymes, which are "toxic" to the host cells. However, the majority of the heterologous ValOx formed inclusion bodies in both strains, TOP10 and LMG194, even in the presence of co-expressed folding factors (data not shown). The point of induction was examined from the early to the late logarithmic phase and

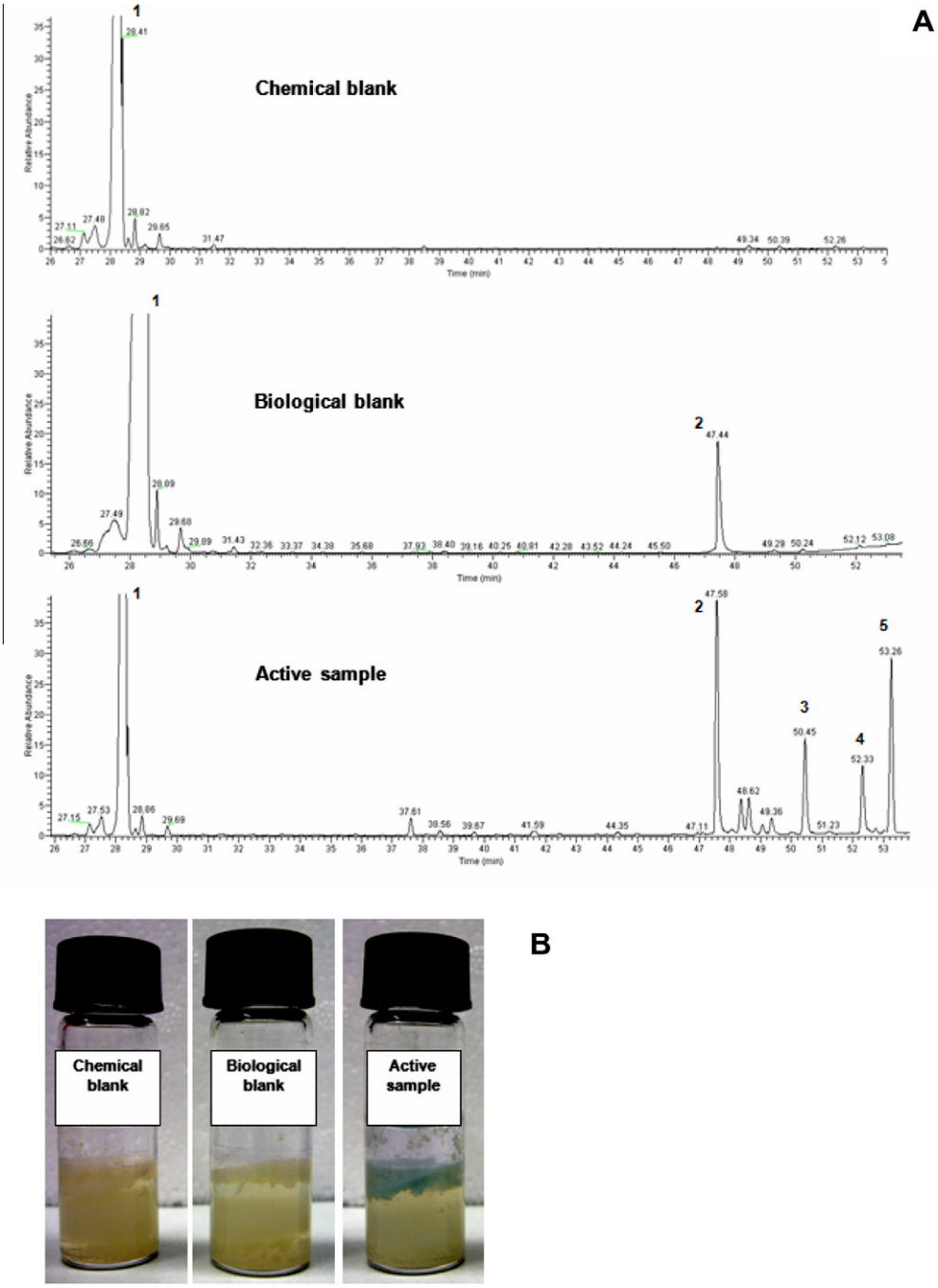

Fig. 5. A: GC–MS-chromatogram of ValOx mediated biotransformation products of (+)-valencene. 1: (+)-valencene; 2:decanoic acid; 3: α-nootkatol; 4: β-nootkatol; 5: (+)-nootkatone. The bioconversion of (+)-valencene was performed in Tris–HCl buffer 20 mM, pH 7.5 for 16 h. For 1.5 mL sample 1 μL (+)-valencene, 1 mg of the recombinant enzyme and 5×10^{-3} mM Na-cholate was used. The chemical blank was prepared without a recombinant enzyme; for the biological blank the recombinant ValOx sample was heat inactivated for 30 min at 95 °C. B: Huber test of the same samples in 4 mL vials.

showed no effect on final protein recovery and solubility. Lowering the cultivation temperature from 37 °C to 25 °C likewise gave no significant rise of the solubility of the expression product as well. The very low yield of soluble protein showed that a correct, permanent folding of the ValOx did not occur in the periplasmic space.

3.3.2. Cold shock expression and chaperone co-expression

The reduction of the rate of protein synthesis by growing the culture at lower temperature may improve target protein solubility considerably (Shirano and Shibata, 1990; Spadiut et al., 2010; Yamanaka, 1999). The pCold I plasmid used in this work is a representative of the pCold vector series that was designed on the background of pUC118 in which protein expression is under the control of the cold shock *cspA* promoter (Qing et al., 2004). Two fungal protein were functionally expressed using this system, and both belonged to oxidoreductases. The successful production of pyranose oxidase from *Trametes multicolor* (Spadiut et al., 2010) and of the MsP2 DyP peroxidase of *Marasmius scorodonius* (Zelena et al., 2011) established an experimental starting point.

However, the cultivation of the production strains TOP10 and BL21(DE3)Star containing the vector pCold*ValOx* alone resulted, as was observed for expression using the pBAD vector, exclusively in the formation of insoluble aggregates, even after elaborate optimization steps and at very slow product formation rates (8 °C).

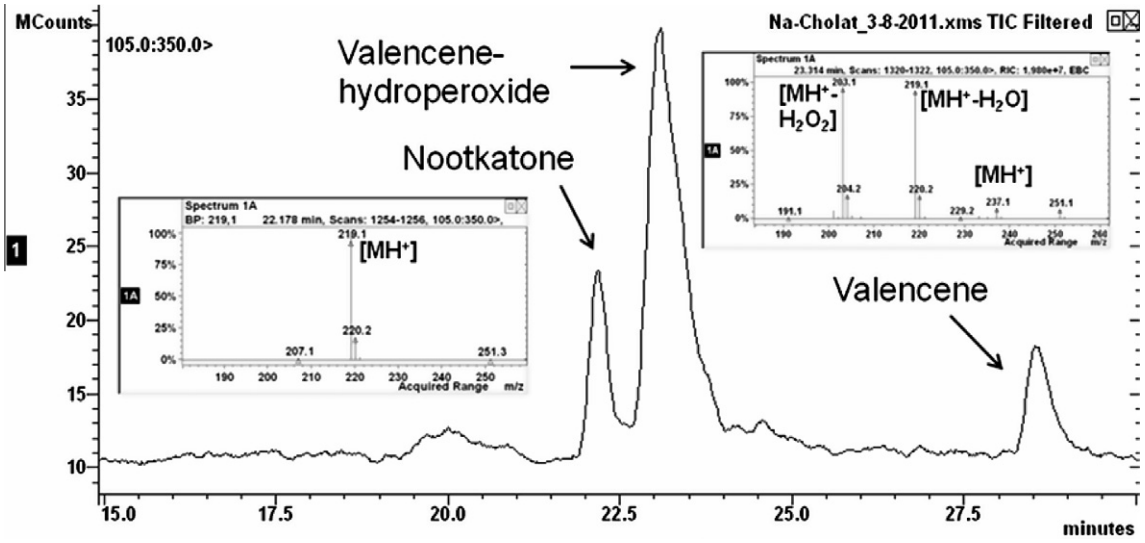

Fig. 6. LC-MS-chromatogram and APCI$^+$ spectra of valencene dioxygenase mediated biotransformation products of (+)-valencene. The bioconversion was investigated in Tris–HCl buffer 20 mM, pH 7.5 for 16 h. For 1.5 mL sample 1 μL (+)-valencene, 1 mg of the recombinant enzyme and 5×10^{-3} mM Na-cholate was used.

Finally, three cytoplasmic chaperones including GroEL-GroES and trigger factor were co-expressed to investigate their possible effect on production level and solubility of the ValOx.

The concentration of the pCold inducer IPTG was varied from 0.05 to 0.50 mM, and the experimental results were compared to negative controls, which were either cells with an empty expression vector, or cells not induced with IPTG. In the case of pCold vectors, a regulatory system based on the *lac* operon is employed additionally to the promoter of a cold-shock protein. Since some inserts could cause a small amount of leakage, it is necessary to add IPTG to avoid any regulation inconvenience.

In the current study, an increase of the IPTG concentration resulted in general in a slightly increased total recovery of the expression product (Fig. 2, lane 3–6). A protein band of the predicted size was not detected in the absence of IPTG (Fig. 2, lane 2) demonstrating, that both cold shock and IPTG induction mechanisms were necessary to obtain the heterologous product. The peptidase deficient *E. coli* expression strain BL21(DE3)Star proved to be the most efficient in terms of resulting cell density and total ValOx production (Fig. 2). An inducer concentration of 0.05 mM IPTG showed the best ratio between the production of chaperone(s) and the target polypeptide. The presence of the target polypeptide in the cell lysis fraction was unequivocally confirmed by SDS–PAGE with a subsequent Western blot (Fig. 3). The expected protein band with a calculated molecular mass of ~75 kDa was detected exclusively in the lysate of the respective *E. coli* culture with chaperones co-expressed.

3.4. Purification of the recombinant ValOx

In order to obtain a pure protein for further biochemical characterization, the concentrated cell lysates from 1 L culture broth (BL21(DE3)Star) were purified via affinity chromatography (2.8). Thereby, the major part of unwanted proteins was successfully removed (Fig. 4). One liter of *E. coli* BL21(DE3)Star culture yielded up to 60 mg soluble ValOx. Thus, the above combination of expression strain, expression vector and chaperone was identified as a high level expression system.

3.5. Determination of the enzymatic activity of the recombinant ValOx

The soluble recombinant ValOx produced by *E. coli* transformants was tested for its ability to recognize (+)-valencene as a substrate. Cell lysates of 50 mL cell cultures were concentrated to 1 mL, supplemented with (+)-valencene, and assessed by Huber staining (2.9). In agreement with results from SDS–PAGE and Western blotting, the lysis fraction obtained from the expression of the pBAD*ValOx* vector showed no characteristic coloration after incubation. The cell lysates from the pCold*ValOx* containing *E. coli* clones, however, showed a positive Huber reaction when chaperones were co-expressed (data not shown). The combination GroEL-GroES without a trigger factor delivered a weak coloration, while the most intensive staining was observed with the samples from the chaperones combination GroEL-GroES-tig or trigger factor alone. The best-characterized *E. coli* chaperone, the trigger factor, localized at the ribosomal exit site, can potentially interact with most growing polypeptide chains, but may also act independently of its ribosomal association (Hoffmann et al., 2010; Kramer et al., 2009). The heat shock chaperones GroEL–GroES assist in the folding of *de novo* polypeptide chains and in the refolding of misfolded proteins resulting in minimized aggregation and enhanced solubility of the recombinant protein product (Gasser et al., 2008).

It was concluded that the presence of the *E. coli* chaperone trigger factor was crucial for the catalytic activity of the recombinant ValOx. Therefore, *E. coli* BL21(DE3)Star bearing pCold*ValOx* + pG-Tf2 and pCold*ValOx* + pG-Tf16 vector combinations were used for further cultivation on the one liter scale. It should be noted that the samples from the cultures without IPTG induction as well as heat-inactivated *P. sapidus* lyophilisate alone did not show the Huber reaction. The results confirmed that exclusively recombinant ValOx caused the staining, and the possibility of a cross-reaction caused by genuine *E. coli* proteins was ruled out.

A weak positive Huber staining of the reaction buffer provided a preliminary clue for the presence of the active target enzyme. However, the comparison of product concentration of blanks and recombinant enzyme assays using GC–MS showed very low yields (<10 mg L^{-1}; GC–MS; internal standard thymol). Fraatz (2007) found a boosting influence of some surfactants on the formation of (+)-nootkatone when the native oxygenase was used. This effect was likely caused by the better availability of the poorly water soluble substrate (+)-valencene. Thus, a range of surfactants were examined for an effect on the bioconversion of (+)-valencene by the recombinant enzyme. Tween-80, a non-ionic representative, CTAB, a cationic, Na-cholate, an anionic and CHAPS, a zwitter-ionic surfactant were added to separate biotransformation samples in a concentration from ~0.25 to ~1.5 of the critical micellar

concentration (CMC). All of the reagents used, especially Na-cholate and TWEEN-80, executed an enhancing effect on the yield of conversion products. Concentrations corresponding to about one half of the CMC each (6×10^{-3} Tween-80, 0.5×10^{-3} CTAB, 5×10^{-3} Na-cholate, 3×10^{-3} CHAPS) were proven to be sufficient (data not shown). The maximum recovery of (+)-nootkatone reached 80 mg L^{-1} by using 2 mg of the purified recombinant ValOx, whereas traces were found in blanks (Fig. 5).

3.6. GC–MS analysis

The visual selection of the samples was based on Huber staining. The bioconversion products obtained from the positive samples were analyzed by means of GC–MS (Fig. 5). Only with the active sample of the recombinant ValOx the substrate was oxidized, and the catalytic reaction provided the typical (+)-valencene transformation products α- and β-nootkatol and (+)-nootkatone. The ratio between the three substances (80 mg L^{-1} (+)-nootkatone, 32 mg L^{-1} α-nootkatol, 16 mg L^{-1} β-nootkatol) was in good accordance with the results obtained using native enzyme indicating the decay of the same intermediate hydroperoxides (Fraatz et al., 2009a, 2009b; Krügener et al., 2010).

3.7. LC–MS analysis

HPLC-MS in the APCI (+) mode showed little remaining (+)-valencene, and two major oxygenated biotransformation products (Fig. 6). The first at rt 22.2 min was identified as 6(R)-Isopropenyl-4(R),4a(S)-dimethyl-4,4a,5,6,7,8-hexahydro-3H-naphthalen-2-one (nootkatone) and the second at rt 23.1 min as 6(R)-Isopropenyl-4(R),4a(S)-dimethyl-2,3,4,4a,5,6,7,8-octahydro-naphthalen-2(S)-yl-hydroperoxide (valencene hydroperoxide) according to the reference compound nootkatone and to published data (valencene hydroperoxides) (Krügener et al., 2009). The APCI (+)-spectrum of nootkatone showed a pronounced protonated molecular ion, whereas the intensity of the corresponding MH^+-ion in the spectrum of the hydroperoxide was weak. More intensive were the ions at m/z 219 and 203 corresponding to the respective MH^+–H_2O and MH^+–H_2O_2-ions. LC–MS showed valencene hydroperoxide as the predominant reaction product which started to decompose on the column. This confirmed ValOx as a dioxygenase introducing molecular triplet oxygen regio-selectively into the bicyclic skeleton of (+)-valencene. Activation upon addition of surfactants may be either explained by an improved availability of the dispersed substrate, or by the membrane association of the native dioxygenase (Fraatz et al., 2009a, 2009b). An extended substrate screening of this unusual dioxygenase of *P. sapidus* will follow in an attempt to shed light on its true physiological function.

4. Conclusion

A first fungal dioxygenase cDNA coding for a sesquiterpene transforming enzyme, ValOx, was cloned from the basidiomycete *Pleurotus sapidus*. The correct annotation of the *ValOx* gene was verified by functional cold shock expression of the enzyme in *E. coli* supported by co-expression of the chaperone trigger factor. The purified recombinant ValOx converted (+)-valencene to (+)-nootkatone and nootkatols. Compared to previously described Cytochrom enzymes (Girchard et al., 2010; Seifert et al., 2009), ValOx did not require any redox-cofactors, and the yield of (+)-nootkatone was clearly higher. Addition of a surfactant to the recombinant ValOx significantly increased the formation of products.

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References

Boeke, J.D., Model, P., 1982. A prokaryotic membrane anchor sequence. carboxyl terminus of bacteriofage f1 Gene III protein retains it in the membrane. Proc. Natl. Acad. Sci. 79, 5200–5204.
Erdtman, H., Hirose, Y., 1962. The chemistry of the natural order Cupressales. Acta Chem. Scand. 16, 1311–1314.
Fahnert, B., Lilie, H., Neubauer, P., 2004. Inclusion bodies: formation and utilisation. Adv. Biochem. Eng./Biotechnol. 89, 93–142.
Flor-Weiler, L.B., Behle, R.W., Stafford 3rd, K.C., 2011. Susceptibility of four tick species, *Amblyomma americanum*, *Dermacentor variabilis*, *Ixodes scapularis*, and *Rhipicephalus sanguineus* (Acari: Ixodidae), to nootkatone from essential oil of grapefruit. J. Med. Entomol. 482, 322–326.
Fraatz, M.A., 2007. Enzymatische Oxidation von Mono- und Sesquiterpenen. Dissertation thesis. Leibniz University Hannover.
Fraatz, M.A., Berger, R.G., Zorn, H., 2009a. Nootkatone – a biotechnological challenge. Appl. Microbiol. Biotechnol. 83, 35–41.
Fraatz, M.A., Riemer, S.J.L., Stöber, R., Kaspera, R., Nimtz, M., Berger, R.G., Zorn, H., 2009b. A novel oxygenase from *Pleurotus sapidus* transforms valencene to nootkatone. J. Molec. Catal. B: Enzymatic. 61, 202–207.
Gasser, B., Saloheimo, M., Rinas, U., Dragosits, M., Rodriguez-Carmona, E., Baumann, K., Giuliani, M., Parrilli, E., Branduardi, P., Lang, C., Porro, D., Ferrer, P., Tutino, M.L., Mattanovich, D., Villaverde, A., 2008. Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview. Microb. Cell Fact. 7, 11.
Girhard, M., Machida, K., Itoh, M., Schmid, R.D., Arisawa, A., Urlacher, V.B., 2009. Regioselective biooxidation of (+)-valencene by recombinant *E. coli* expressing CYP109B1 from *Bacillus subtilis* in a two-liquid-phase system. Microb. Cell Fact. 8, 36.
Girchard, M., Klaus, T., Khatri, Y., Bernhardt, R., Urlacher, V.B., 2010. Characterization of the versatile monooxygenase CYP109B1 from *Bacillus subtilis*. Appl. Microbiol. Biotechnol 87, 595–607.
Gliszczynska, A., Lysek, A., Janeczko, T., Switalska, M., Wietrzyk, J., Wawrzenczyk, C., 2011. Microbial transformation of (+)-nootkatone and the antiproliferative activity of its metabolites. Bioorg. Med. Chem. 19, 2464–2469.
Guzman, L.M., Belin, D., Carson, M., Beckwith, J., 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J. Bacteriol. 177, 4121–4130.
Hoffmann, A., Bukau, B., Kramer, G., 2010. Structure and function of the molecular chaperone trigger factor. Biochim. Biophys. Acta (BBA) – Mol. Cell Res. 1803, 650–661.
Huber, W., Fröhlke, E., 1972. A new spray-reagent for the detection and quantitative estimation of peroxides. Chromatographia 5, 256–257.
Jin, J., Lee, D.U., Kim, Y., Kim, H., 2011. Anti-allergic activity of sesquiterpenes from the rhizomes of *Cyperus rotundus*. Arch. Pharmacal Res. 34, 223–228.
Jonasson, P., Liljeqvist, S., Nygren, P.A., Ståhl, S., 2002. Genetic design for facilitated production and recovery of recombinant proteins in *Escherichia coli*. Biotechnol. Appl. Biochem. 35, 91–105.
Khlebnikov, A., Skaug, T., Keasling, J.D., 2002. Modulation of gene expression from the arabinose-inducible araBAD promoter. J. Ind. Microbiol. Biotechnol. 29, 34–37.
Kramer, G., Boeringer, D., Ban, N., Bukau, B., 2009. The ribosome as a plattform for cotranslational processing, folding and targeting of newly synthesized proteins. Nat. Struct. Mol. Biol. 16, 589–597.
Krügener, S., Krings, U., Zorn, H., Berger, R., 2010. A dioxygenase of *Pleurotus sapidus* transforms (+)-valencene regio-specifically to (+)-nootkatone via a stereo-specific allylic hydroperoxidation. Bioresour. Technol. 101, 457–462.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680–685.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Moshonas, M.G., Shaw, P.E., 1979. Composition of Essence Oil from Overripe Oranges. J. Agric. Food Chem. 27, 1337–1339.
Qing, G.L., Ma, L.C., Khorchid, A., Swapna, G.V.T., Mal, T.K., Takayama, M.M., Xia, B., Phadtare, S., Ke, H.P., Acton, T., Montelione, G.T., Ikura, M., Inouye, M., 2004. Cold-shock induced high-yield protein production in *Escherichia coli*. Nature Biotechnol. 22, 877–882.
Salvador, J.A.R., Clark, J.H., 2002. The allylic oxidation of unsaturated steroids by tert-butyl hydroperoxide using surface functionalised silica supported metal catalysts. Green Chem. 4, 352–356.
Sambrook, J., Russell, D.W., 2001. Molecular Cloning. A laboratory manual, Cold Spring Harbor, New York.
Seifert, A., Vomund, S., Grohmann, K., Kriening, S., Urlacher, V.B., Laschat, S., Pleiss, J., 2009. Rational design of a minimal and highly enriched CYP102A1 mutant library with improved regio-, stereo- and chemoselectivity. Chembiochem. 10, 853–861.
Shaffer, G.W., Eschinasi, E.H., Purzycki, K.L., Doerr, A.B., 1975. Oxidations of valencene. J. Org.Chem. 40, 2181–2185.

Shirano, Y., Shibata, D., 1990. Low-temperature cultivation of *escherichia-coli* carrying a rice lipoxygenase l-2 cdna produces a soluble and active enzyme at a high-level. Febs Lett. 271, 128–130.

Schmidt, K., Fraatz, M.A., Riemer, S.J.L., Zelena, K., Linke, D., Berger, R.G., Zorn, H., 2011. Molecular biological characterization of a nootkatone generating oxygenase from the basidiomycete *Pleurotus sapidus*. In: Hofmann, Th., Meyerhoff, W., Schieberle, P., (Eds.), 9th Wartburg Symposium on Flavor Chemistry & Biology, 2010, Eisenach, Germany. DFA 2011, pp. 235–241.

Seo, E.J., Lee, D.-U., Kwak, J.H., Lee, S.-M., Kim, Y.S., Jung, Y.-S., 2011. Antiplatelet effects of *Cyperus rotundus* and its component (+)-nootkatone. J. Ethnopharmacol. 135, 48–54.

Spadiut, O., Posch, G., Ludwig, R., Haltrich, D., Peterbauer, C., 2010. Evaluation of different expression systems for the heterologous expression of pyranose 2-oxidase from *Trametes multicolor* in *E. Coli*.. Microb. Cell Fact. 9, 68–81.

Studier, F.W., 2005. Protein production by auto-induction in high-density shaking cultures. Protein Expression Purif. 41, 207–234.

Tassaneeyakul, W., Guo, L.Q., Fukuda, K., Ohta, T., Yamazoe, Y., 2000. Inhibition selectivity of grapefruit juice components on human cytochromes p450. Arch. Biochem. Biophys. 378, 356–363.

Yamanaka, K., 1999. Cold shock response in *Escherichia coli*. J. Mol. Microbiol. Biotechnol. 1, 193–202.

Zelena, K., Krügener, S., Lunkenbein, S., Zorn, H., Berger, R.G., 2009. Functional expression of the lipase gene Lip2 of Pleurotus sapidus in *Escherichia coli*. Biotech. Lett. 31, 395–401.

Zelena, K., Lehnert, N., Krings, U., Horváth, G., Molnár, P., Turcsi, E., Deli, J., Berger, R.G., 2011. Degrading of carotenoids by the DyP peroxidase MsP2 from *Marasmius scorodonius* Acta Biol. Cracoviensia 53, 60.

Zhu, B.C.R., Henderson, G., Sauer, A.M., Crowe, W., Laine, R.A., 2010. Structural requirements for repellency: norsesquiterpenes and sesquiterpenoid derivatives of nootkatone against the Formosan subterranean termite (*Isoptera: Rhinotermitidae*). Pest Manag. Sci. 66, 875–878.