Small scale biotransformation of food additive *trans*-2-hexenal to *trans*-2-hexenol by recombinant alcohol dehydrogenase and formate dehydrogenase produced in *Escherichia coli*

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Summary

Aromatic compounds, such as green note volatiles, belong to highly demanded chemicals in food and cosmetics industries. Their production, isolation and purification often represents a difficult challenge. The natural sources are often scarce and difficult to process, while chemical synthesis produces forms that do not occur naturally or even burden the environment. Production of aromatic compounds by recombinant enzymes have proven to represent the best of two worlds, as it enables preparation of economically sufficient quantities of the product while keeping its quality at required levels. The aim of our work was to develop sustainable laboratory-scale processes allowing production of two key enzymes used in biotransformation of highly valued, but volatile and unstable, aromatic compound *trans*-2-hexenal to a more stable *trans*-2-hexenol. We also demonstrated the ability of alcohol dehydrogenase coupled to formate dehydrogenase to catalyse this conversion at desired levels in a one-pot reaction at conversion rates above 90 %.

Keywords

alcohol dehydrogenase; biotransformation; formate dehydrogenase; green aroma; *trans*-2-hexenol

Six-carbon aldehydes and alcohols, such as *trans*-2-hexenal and *trans*-2-hexenol, are characterized by their green leaf odour, due to which they are often used in food and cosmetic industries. Green note is a collective term for predominantly C6 alcohols and aldehydes. These include mainly hexanol, hexanal, *trans*-2-hexenal, *trans*-2-hexenol, *cis*-2-hexenal and *cis*-3-hexenol. Green notes are present in nature in a wide range of fruits, vegetables and leaves and are used commercially to enhance organoleptic properties of food and perfumes. They are often produced by fermentation, extraction from plants or biocatalysis [1–4]. The examples of use of these compounds in food include chewing gum, gelatins and puddings, hard and soft candy as well as non-alcoholic beverages [5]. As a "green" alternative to their synthesis, various biotransformation systems were proposed and developed ensuring the natural origin of the compounds [3, 4, 6–9].

The separation and purification procedures of these volatile substances often require large quantities of fresh leaf material and suffer from various difficulties, rendering their preparation laborious and largely ineffective. The main drawbacks are the need for separation of the target compounds from complex mixtures of plant and microbial origin or a sequence of isolation and separation steps

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as well as loss of material due to metabolic consumption of the cells. The use of whole cell catalyst and/or fusion enzymes is a promising solution to this problem, ensuring the bioprocess effectivity increase while retaining relatively low cost of production [9].

An alternative approach is the utilization of purified recombinant enzymes in optimized reaction mixtures ensuring as low as possible number of potential contaminants and controllable environment in bioreactors. One of the examples, also the subject of this work, is the bioconversion of volatile *trans*-2-hexenal to *trans*-2-hexenol using *Saccharomyces cerevisiae* alcohol dehydrogenase (EC 1.1.1.1.) [6, 10] teamed with formate dehydrogenase (EC 1.2.1.2.) from *Candida boidinii* [11] to ensure nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) regeneration, thus allowing a lower need of expensive co-factor addition. In our previous work, we successfully demonstrated the procedure for expression of soluble alcohol dehydrogenase (ADH) in *E. coli* and its application in oxido-reduction bioconversions [12]. In this work, we further optimized the fermentation conditions along with those of formate dehydrogenase (FDH). We also applied both enzymes in conversion of *trans*-2-hexenal to *trans*-2-hexenol and achieved high conversion rates.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, Missouri, USA) unless otherwise stated.

Plasmids and microbial strains

Two expression plasmids carrying ADH and FDH genes fused to 6× histidine tag (His tag) were used. The construction of ADH gene-carrying plasmid pRSF-ADH was described in a previously published work [12]. The FDH expression vector was constructed by polymerase chain reaction (PCR) amplification of FDH locus using site-specific primers and *Candida boidinii* chromosome as template. The amplified region was then cloned through *Nde*I/*Xho*I restriction sites on pET29b+ vector yielding pET29-FDH construct. Both plasmids were subsequently sequenced to confirm than no mutations occurred during the cloning process. *E. coli* strains used in this study are described in Tab. 1.

Bacterial cell transformation, selection of recombinants and cultivation conditions

The DH5α strain was used for general cloning and plasmid maintenance, whilst BL21(DE3) and C41(DE3) were used as host strains for protein expression. All transformations were performed by the heat-shock method. Cells were shaken at 37 °C for 1 h and streaked on lysogeny broth (LB) plates supplemented with 50 mg·l^{-1} kanamycin. Positive colonies were then transferred into liquid LB medium. Cultivation conditions in shake-flask cultures were as follows: 20–100 ml of LB medium (10 g·l^{-1} tryptone, 5 g·l^{-1} yeast extract, 5 g·l^{-1} NaCl (CentralChem, Bratislava, Slovakia); pH 7.2) in 50–250 ml Erlenmeyer flasks supplemented with antibiotic was inoculated with a single colony and incubated on a rotary shaker at 3.33 Hz at 37 °C. For inducible expression of ADH or FDH, cells were grown to optical density at a wavelength of 600 nm (OD_{600}) of 0.5–1 and expression was induced with 1 mmol·l^{-1} isopropyl β-D-1-thiogalactopyranosid (IPTG). Induction was maintained for 4–6 h; samples were taken every hour and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Cells were harvested by centrifugation at 7650 ×*g* at 4 °C.

Bioreactor (Biostat B Plus; Sartorius, Goettigen, Germany) cultivation conditions for batch mode cultivation were as follows: 1 litre of LB medium supplemented with 1 g·l^{-1} glucose) and 0.01–0.1 mmol·l^{-1} $ZnCl_2$ was inoculated with 50–100 ml of an overnight culture grown in the same medium supplemented with ampicillin and a higher concentration of glucose of 10 g·l^{-1}) and grown at 28 °C. Culture pH of 7.2 was automati-

Tab. 1. *Escherichia coli* strains used in this study.

Strain	Genotype	Reference
DH5α	*F⁻ ϕ80lacZ ΔlacZYA-argF) U196 endA1 recA1 hsdR17 (rk⁻, mk⁺) supE44 thi-1 gyrA96 relA1 phoA*	Invitrogen (Carlsbad, California, USA)
BL21(DE3)	*F⁻ompT gal dcmlonhsdS$_B$(r$_B$⁻ m$_B$⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	Novagen (Madison, Wisconsin, USA)
C41(DE3)	*F⁻ompThsdSB (r$_B$⁻ m$_B$⁻) galdcm(DE3)*	Lucigen (Middleton, Wisconsin, USA)

cally maintained with 10% HCl and 10% NaOH (CentralChem), O_2 saturation gradually decreased as a result of metabolic activity of cells to 3% and was maintained at this level by culture stirring (3.33–8.33 Hz) and aeration (0.6–1 l·min^{-1}). The temperature of the batch culture was gradually decreased to 18 °C over the period of 6 h. At this point, the expression of ADH or FDH was induced by the addition of 1 mmol·l^{-1} IPTG and maintained for 18 h. Samples were taken at regular time points and analysed by SDS-PAGE. Cells were harvested by centrifugation and pellet was used for distribution determination and purification of ADH or FDH.

Purification of the enzymes

After the cells had been harvested by centrifugation, pellets were resuspended in sonication buffer (50 mmol·l^{-1} Tris.HCl, pH 8, 1 mol·l^{-1} NaCl, 5 mmol·l^{-1} imidazole and 1× Protease inhibitor cocktail set III (EDTA free; Calbiochem, San Diego, California, USA)). Cells were disrupted on ice by 10–15 cycles of 30 s sonication followed by 30 s cool down (Sonopuls HD3200 with KE 76 probe; Bandelin, Berlin, Germany).

For affinity chromatography purification, ÄKTA Avant 25 fast protein liquid chromatography (FPLC) system (GE Healthcare, Freiburg, Germany), equipped with 5 ml HisTrap FF column (GE Healthcare) was used. The pH value of the supernatant after cell disruption was adjusted to 8 when needed and it was centrifuged for 30 min at 7500 ×*g* to remove cell debris and aggregates. Before the sample application, the column was washed with 2–5 column volumes (CV) of water and 2–5 CV of equilibration/wash buffer (50 mmol·l^{-1} Tris.HCl pH 8, 1 mol·l^{-1} NaCl). Samples were applied directly onto the column using a pump at a speed of 0.1–1 CV per minute. The column was then washed with 10–15 CV of equilibration/wash buffer together with 10–20 mmol·l^{-1} imidazole to remove the non-specifically bound material. 2–5 CV of elution buffer (50 mmol·l^{-1} Tris.HCl pH 8, 1 mol·l^{-1} NaCl, 0.5 mol·l^{-1} imidazole) were used to elute bound enzymes. Samples were taken by the automatic fraction collector in every step and analysed by SDS-PAGE. The elution fraction was then gradually dialysed against storage buffer (10 mmol·l^{-1} Tris.HCl pH 8, 100 mmol·l^{-1} NaCl, 50% glycerol; 10 kDa molecular weight cut-off membrane). The concentration of enzymes was estimated using Bradford assay [13].

Enzymatic activity assay

The specific activity of recombinant alcohol dehydrogenase (rADH) and recombinant formate dehydrogenase (rFDH) was determined by continuous spectrophotometric measurement at 340 nm and 25 °C. The enzymatic assay was performed in a 500 μl reaction mixture.

To determine rADH activity, the reaction mixture was composed of 100 mmol·l^{-1} sodium phosphate buffer (pH from 7.0 to 7.8 in 0.2 increments), 25 mmol·l^{-1} ethanol, 1.7 mmol·l^{-1} nicotinamide adenine dinucleotide (NAD) and 0.05–0.1 unit in 10 μl of rADH. The activity was measured in a quartz cuvette at 340 nm by UviLine 9400 spectrophotometer (Schott, Mainz, Germany) during a period of 8 min with 30-seconds intervals between measurements. One unit (U) of ADH is defined as the amount of the enzyme capable of converting 1 μmol of ethanol to acetaldehyde in 1 min in the presence of NAD at pH 7.8 and 25 °C [14].

The specific activity of rFDH was determined similarly to that of rADH, the reaction mixture being composed of 100 mmol·l^{-1} sodium phosphate buffer (pH 8), 100 mmol·l^{-1} sodium formate, 1.7 mmol·l^{-1} NAD and 0.05–0.1 unit in 10 μl of rFDH. The assay conditions were identical to rADH measurement. One unit (U) of FDH is defined as the amount of the enzyme capable of converting 1 μmol of formate to CO_2 in 1 min in the presence of NAD at pH 7 and 37 °C [15].

Conversion of *trans*-2-hexenal to *trans*-2-hexenol in a one-pot reaction

The bioconversion reaction mixture contained 0.1 mol·l^{-1} sodium formate, 0.1 mol·l^{-1} sodium phosphate buffer (pH 7.2), 0.5 mmol·l^{-1} NAD, 1.5 μg·ml^{-1} FDH, 0.75 μg·ml^{-1} ADH, 0.344–17.2 mmol·l^{-1} *trans*-2-hexenal. The procedure was as follows. Reaction mixture (without ADH) was pre-incubated for 30 min at 28 °C to allow reduction of NAD to NADH. ADH was then added to the reaction mixture and incubated at 37 °C with shaking. Samples were taken at regular time intervals and mixed with acetone (4:1) to denature enzymes.

Enzyme immobilization

The enzymes were immobilized on 3-aminopropylsilane-functionalized iron oxide magnetic nanoparticles (MNPs) without or with silica coating (APTES-MNP or APTES-MNP-SiO_2) as described by Reza et al. [16] and Morel et al. [17]. Briefly, to the mixture of Fe^{2+}/Fe^{3+} salts heated at 90 °C, oxidation solution was added under argon atmosphere. Black precipitate was separated by neodyme magnet, thoroughly washed with water, isopropranol and vacuum-dried. Then, 200 mg of

MNPs was placed inside a scintillation vial and 8 ml of 8% glutaraldehyde solution was added. The mixture was then sonicated and incubated for 1 h at room temperature with shaking at 11 Hz. MNPs were then washed with 10 ml of phosphate buffered saline (PBS; 137 mmol·l^{-1} NaCl, 10 mmol·l^{-1} phosphate, 2.7 mmol·l^{-1} KCl, pH 7.4) to remove residual glutaraldehyde. Then, MNPs separated by neodymium magnet were sonicated in 5 ml of PBS for 30 s. One millilitre of 5 mg·ml^{-1} enzyme solution was then added to the mixture.

Gas chromatographic analysis

Samples from the biotransformation reactions were analysed by gas chromatography-mass spectrometry (GC-MS). Before analysis, samples of reaction mixture were diluted with 4-fold volume of acetone. GC-MS measurements were performed on a 6890N gas chromatograph with a 5973 Network mass-selective detector (Agilent Technologies, Santa Clara, California, USA). The injection port was maintained at 250 °C, and the sample of 1 μl was injected in splitless mode, followed by a purge for 0.7 min after injection. The samples were separated using a 30 m × 0.25 mm (inside diameter) capillary column coated with a 0.25 μm film of 50% cyanopropyl-methylpolysiloxane DB-23 as stationary phase (J&W Scientific, Folsom, California, USA). The column temperature was 40 °C initially for 2 min, then increased to 190 °C at a ramp rate of 15 °C·min^{-1}, and held at the final temperature of 190 °C for 2 min. Helium carrier gas with a constant flow of 1.5 ml·min^{-1} was used. The transfer line temperature was set at 200 °C. The mass spectrometer detector conditions were 5 min solvent delay, electron energy of 70 eV and ion source temperature of 230 °C. The target biotransformation analytes were measured based on SCAN mode in the range of 33–200 atomic mass units (amu). Data handling was carried out using MSD ChemStation software E.02.02.1431 (Agilent Technologies).

RESULTS

Expression and purification of rADH and rFDH

Both enzymes were produced in relatively high quantities (150–250 mg per litre of culture), but the differences arose in their solubility. While rFDH retained its solubility and activity when expressed at 27 °C, the rADH-producing strain had to be cultivated at 18–20 °C in order to achieve similar results. Shake flask cultures produced

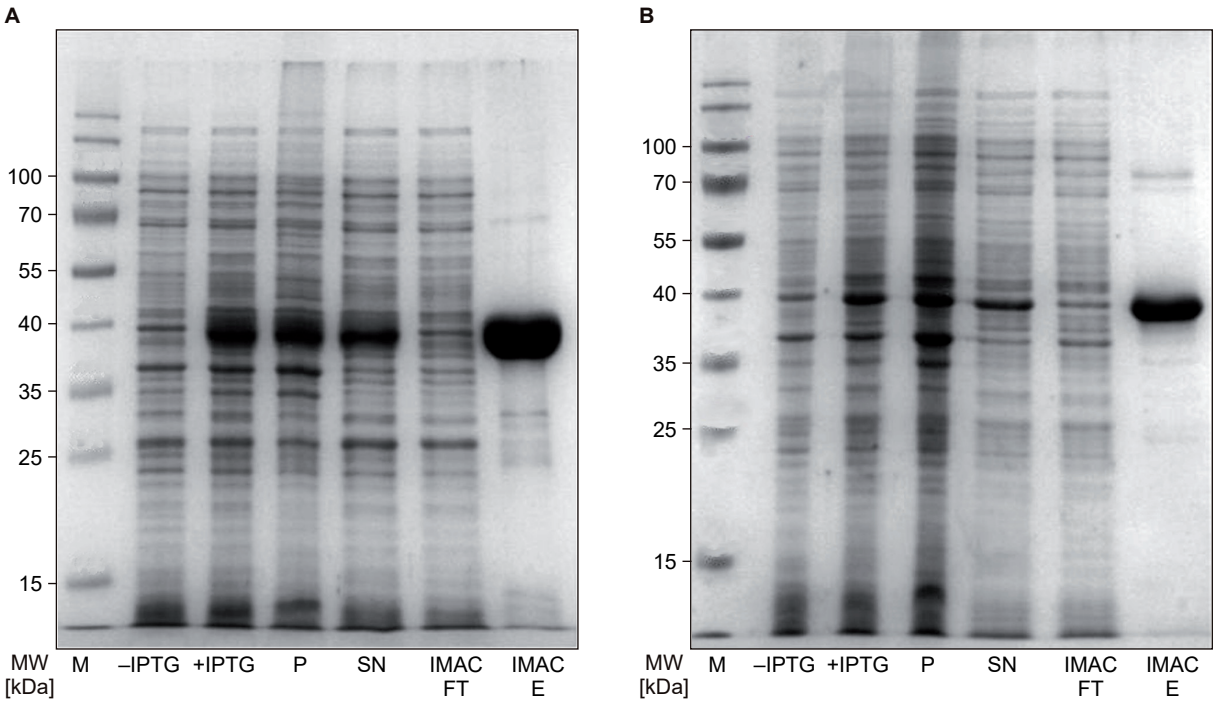

Fig. 1. Electrophoretic analysis of expression and purification of the recombinant enzymes.

A – recombinant alcohol dehydrogenase, B – recombinant formate dehydrogenase.
MW – molecular weight, M – molecular weight marker, (–IPTG) – culture sample prior to induction, (+IPTG) – culture sample after 18 h of induction, P – insoluble protein fraction after cell disruption, SN – soluble protein fraction after cell disruption, IMAC FT – flow-through column fraction after affinity chromatography, IMAC E – proteins eluted from the column.

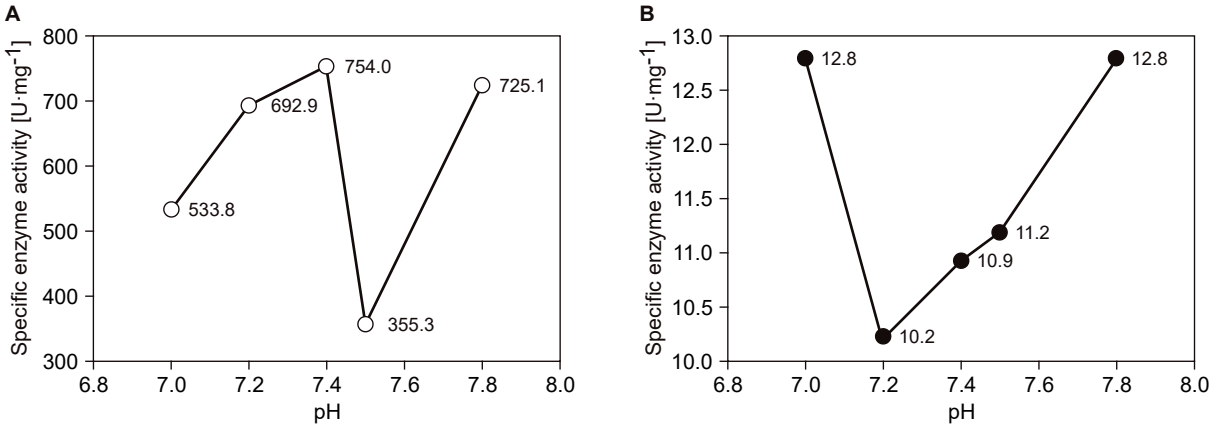

Fig. 2. Specific activities of recombinant enzymes at different pH values.

A – recombinant alcohol dehydrogenase, B – recombinant formate dehydrogenase.

slightly better results in terms of solubility, but batch fermentation was superior in terms of overall amount of soluble enzyme available. Following the protocol described in material and methods section, we were able to purify (73 ± 9) mg of ADH per litre of culture and (68 ± 8) mg of FDH per litre of culture (Fig. 1).

Specific activity of the enzymes

The activity of purified rADH from various enzyme preparations was higher than the activity of the commercially available enzyme (480–680 U·mg^{-1} at optimal dilution and pH of reaction mixture, Fig. 2A). The activity of rFDH from various enzyme preparations at optimal dilution and pH of reaction mixture was in the range of 10–13 U·mg^{-1} of protein (Fig. 2B), which is comparable to the commercially available enzyme. The enzymes rapidly lost their activity when freeze-dried (results not shown), therefore they were stored in 50% glycerol buffer at –20 °C and remained stable for more than a year without a significant loss of activity.

Conversion of *trans*-2-hexenal to *trans*-2-hexenol by free enzymes

Using the reaction mixture and the procedure described in material and methods section, we were able to obtain almost complete conversion of the aldehyde to alcohol. The GC analysis showed that more than 91% of *trans*-2-hexenal (in reactions with up to 17.6 mmol·l^{-1} of the compound) was efficiently converted to *trans*-2-hexenol in 24 h (Fig. 3). Given the low water solubility of *trans*-2-hexenal (5.3 g·l^{-1}; 54 mmol·l^{-1}), higher volume ratios (volume of substrate vs reaction volume) are, in water-based buffers, achievable only with difficulties.

Conversion of *trans*-2-hexenal to *trans*-2-hexenol using immobilized enzymes

We investigated the possibility of utilization of immobilized enzymes, as this approach allows repeated use and/or higher stability of the enzymes in biotransformation reactions [18]. In both cases, the enzymes showed decreased specific activity and decreased ability to convert *trans*-2-hexenal to *trans*-2-hexenol compared to free enzymes (Fig. 4 and Fig. 5).

DISCUSSION

Production of naturally occurring aromatic compounds using biotransformation is a viable and preferred method of production compared to chemical synthesis or isolation from native organisms. It allows higher production yields and higher purities, while keeping the quality and

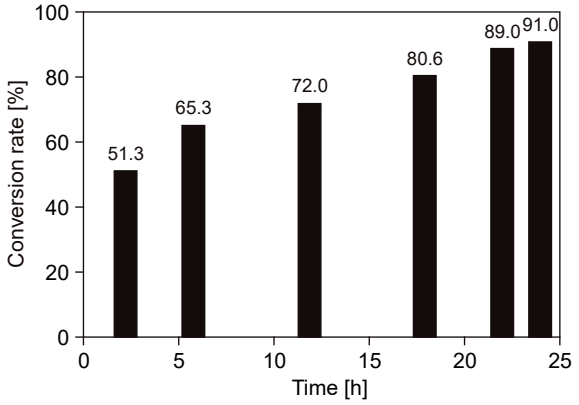

Fig. 3. Conversion rate of *trans*-2-hexenal to *trans*-2-hexenol in biotransformation reaction with free enzymes at different time points.

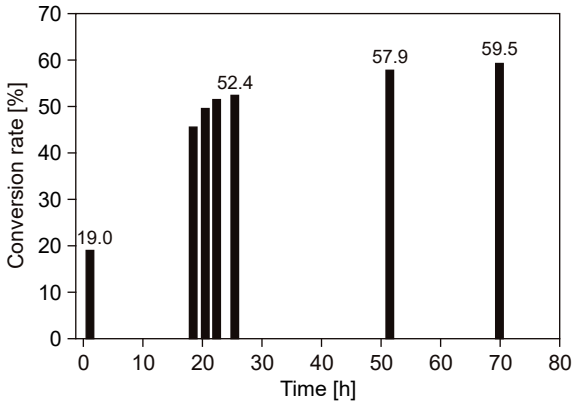

Fig. 4. Conversion rate of *trans*-2-hexenal to *trans*-2-hexenol in biotransformation reaction with immobilized recombinant alcohol dehydrogenase and free recombinant formate dehydrogenase at different time points.

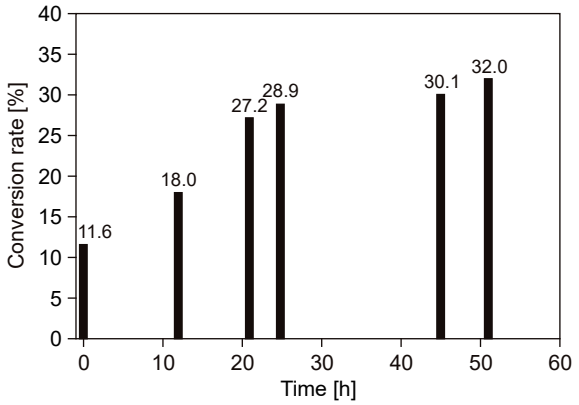

Fig. 5. Conversion rate of *trans*-2-hexenal to *trans*-2-hexenol in biotransformation reaction with free recombinant alcohol dehydrogenase and immobilized recombinant formate dehydrogenase at different time points.

structure of the compounds on a par with the naturally occurring ones. With the onset of recombinant technology, the process of biotransformation received a boost in terms of availability of often exotic and rare enzymes. These, coupled with vast molecular engineering and enzyme evolution possibilities, ensure improvemnet in the overall process and cost efficiency [7, 19].

In this work, we were able to demonstrate reliable and economically viable procedure for production of recombinant enzymes further applicable to biotransformation of valuable compounds used in cosmetic and food industries. We successfully optimized a laboratory-scale production process of two recombinant enzymes, namely, alcohol dehydrogenase (of *Saccharomyces cerevisiae*) and formate dehydrogenase (of *Candida boidinii*) applicable in a one-pot reaction conversion of *trans*-2-hexenal to *trans*-2-hexenol. Both enzymes have a tendency to aggregate and lose activity while expressed in *Escherichia coli* cells. This issue was addressed by optimized bioreactor cultivation process and decreased cultivation temperature, which allowed purification of satisfactory amounts of both enzymes in a single-step affinity chromatography with subsequent dialysis. The specific enzymatic activities were higher compared to the commercially available enzyme in the case of rADH (> 500 $U \cdot mg^{-1}$ compared to > 300 $U \cdot mg^{-1}$ for ADH from Sigma Aldrich) and comparable to commercially available rFDH (7–12 $U \cdot mg^{-1}$ for FDH compared to 5–15 $U \cdot mg^{-1}$ from Sigma Aldrich).

The reduction of *trans*-2-hexenal to *trans*-2-hexenol catalysed by rADH relies on the regenerative cycling of the co-factor (NAD to NADH) by FDH, which is the rate-limiting step. The reaction requires a pre-incubation step to allow accumulation of sufficient concentration of the reduced co-factor by rFDH, after which rADH is added. The reaction proceeded for another 24 h, during which more than 91 % of substrate was converted to product and remained stable, as determined by gas chromatography. The larger scale application requires more sophisticated bioreactor design, since the agitation of the reaction mixture in volumes > 50 ml causes denaturation and loss of the enzymatic activity.

We also investigated the possibility of use of immobilized enzymes in this reaction. The initial experiments with enzymes immobilized on iminodiacetic acid Sepharose through His-tag (results not shown) or magnetic nanoparticles showed that the conversion rate was around 59 % in case of immobilized rADH and 32 % in case of immobilized rFDH, while the other enzyme was in solution. Experiments with both immobilized enzymes yielded almost no conversion of aldehyde to alcohol and will need to be further investigated and/or optimized to yield satisfactory results.

The demonstrated process represents a convenient method of biotransformation of *trans*-2-hexenal to the less volatile *trans*-2-hexenol by two free recombinant enzymes produced in *E. coli*.

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REFERENCES

1. Brunerie, P. – Koziet, Y.: Process for producing natural *cis*-3-hexenol from unsaturated fatty acids. Inventors: Brunerie, P. – Koziet, Y. Int. Cl. C07C33/025. US Patent US5620879. 15 April 1997.
2. Márczy, J. S. – Németh, Á. S. – Samu, Z. – Hágerr-Veress, Á. – Szajáni, B.: Production of hexanal from hydrolyzed sunflower oil by lipoxygenase and hydroperoxid lyase enzymes. Biotechnology Letters, *24*, 2002, pp. 1673–1675. DOI: 10.1023/a:1020657618363.
3. Akacha, N. B. – Boubaker, O. – Gargouri, M.: Production of hexenol in a two-enzyme system: kinetic study and modelling. Biotechnology Letters, *27*, 2005, pp. 1875–1878. DOI: 10.1007/s10529-005-3896-x.
4. Akacha, N. B. – Gargouri, M.: Enzymatic synthesis of green notes with hydroperoxide-lyase from olive leaves and alcohol-dehydrogenase from yeast in liquid/gas reactor. Process Biochemistry, *44*, 2009, pp. 1122–1127. DOI: 10.1016/j.procbio.2009.06.006.
5. Burdock, G. A.: Fenaroli's handbook of flavor ingredients. 6th edition. Boca Raton : CRC Press, 2016. ISBN: 9781420090864.
6. Fauconnier, M.-L. – Mpambara, A. – Delcarte, J. – Jacques, P. – Thonart, P. – Marlier, M.: Conversion of green note aldehydes into alcohols by yeast alcohol dehydrogenase. Biotechnology Letters, *21*, 1999, pp. 629–633. DOI: 10.1023/A:1005593821577.
7. Buchhaupt, M. – Guder, J. C. – Etschmann, M. M. W. – Schrader, J.: Synthesis of green note aroma compounds by biotransformation of fatty acids using yeast cells coexpressing lipoxygenase and hydroperoxide lyase. Applied Microbiology and Biotechnology, *93*, 2012, pp. 159–168. DOI: 10.1007/s00253-011-3482-1.
8. Šalić, A. – Pindrić, K. – Zelić, B.: Bioproduction of food additives hexanal and hexanoic acid in a microreactor. Applied Biochemistry and Biotechnology, *171*, 2013, pp. 2273–2284. DOI: 10.1007/s12010-013-0495-5.
9. Jeon, E. Y. – Baek, A. H. – Bornscheuer, U. T. – Park, J. B.: Enzyme fusion for whole-cell biotransformation of long-chain *sec*-alcohols into esters. Applied Microbiology and Biotechnology, *99*, 2015, pp. 6267–6275. DOI: 10.1007/s00253-015-6392-9.
10. Raj, S. B. – Ramaswamy, S. – Plapp, B. V.: Yeast alcohol dehydrogenase structure and catalysis. Biochemistry, *53*, 2014, pp. 5791–5803. DOI: 10.1021/bi5006442.
11. Schute, H. – Flossdorf, J. – Sahm, H. – Kula, M.-R.: Purification and properties of formaldehyde dehydrogenase and formate dehydrogenase from *Candida boidinii*. European Journal of Biochemistry, *62*, 1976, pp. 151–160. DOI: 10.1111/j.1432-1033.1976.tb10108.x.
12. Utekal, P. – Tóth, C. – Illésová, A. – Koiš, P. – Bocánová, L. – Turňa, J. – Drahovská, H. – Stuchlík, S.: Expression of soluble *Saccharomyces cerevisiae* alcohol dehydrogenase in *Escherichia coli* applicable to oxido-reduction bioconversions. Biologia, *69*, 2014, pp. 722–726. DOI: 10.2478/s11756-014-0376-6.
13. Bradford, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, *72*, 1976, pp. 248–254. DOI: 10.1016/0003-2697(76)90527-3.
14. Kägi, J. H. R. – Vallee, B. L.: The role of zinc in alcohol dehydrogenase: V. The effects of metal-binding agents on the structure of the yeast alcohol dehydrogenase molecule. The Journal of Biological Chemistry, *235*, 1960, pp. 3188–3192. ISSN: 0021-9258. <http://www.jbc.org/content/235/11/3188.full.pdf>
15. Bergmeyer, H. U. – Gawehn, K. (Ed): Methods of enzymatic analysis. 2nd edition. Weinheim : Verlag Chemie, 1974. ISBN: 3-527-25597-4.
16. Reza, R. T. – Martínez Pérez, C. A. – Rodríguez González, C. A. – Romero, H. M. – García Casillas, P. E.: Effect of the polymeric coating over Fe_3O_4 particles used for magnetic separation. Central European Journal of Chemistry, *8*, 2010, pp. 1041–1046. DOI: 10.2478/s11532-010-0073-4.
17. Morel, A.-L. – Nikitenko, S. I. – Gionnet, K. – Wattiaux, A. – Lai-Kee-Him, J. – Labrugere, C. – Chevalier, B. – Deleris, G. – Petibois, C. – Brisson, A. – Simonoff, M.: Sonochemical approach to the synthesis of Fe_3O_4@SiO_2 core-shell nanoparticles with tunable properties. ACS Nano, *2*, 2008, pp. 847–856. DOI: 10.1021/nn800091q.
18. DiCosimo, R. – McAuliffe, J. – Poulose, A. J. – Bohlmann, G.: Industrial use of immobilized enzymes. Chemical Society Reviews, *42*, 2013, pp. 6437–6474. DOI: 10.1039/C3CS35506C.
19. ul Hassan, M. N. – Zainal, Z. – Ismail, I.: Green leaf volatiles: biosynthesis, biological functions and their applications in biotechnology. Plant Biotechnology Journal, *13*, 2015, pp. 727–739. DOI: 10.1111/pbi.12368.

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