

Alcohol dehydrogenase catalyzed hexanol oxidation in microreactor

¹A. Šalić, ²A. Tušek, ²Ž. Kurtanjek, ¹B. Zelić

¹University of Zagreb, Faculty of Chemical Engineering and Technology, Marulićev trg 19,
HR-10000 Zagreb;

²University of Zagreb, Faculty of Food Technology and Biotechnology, Pierottijeva 6, HR-
10000 Zagreb,

E-mail: asalic@fkit.hr, atusek@pbf.hr, zkurt@pbf.hr, bzelic@fkit.hr

Abstract

Alcohol dehydrogenase catalyzed hexanol oxidation was carried out in continuously operated tubular glass microreactors with two inflows (“Y”-shape) and one outflow. A microreactors and a micoreactor equipped with micromixes were used and results were compared.

Conversion of 13.5 % was achieved in a “Y”-shaped tubular microreactor without internal mixers and at residence time of 72 s and ten fold lower initial concentration of enzyme and coenzyme, which was significant improvement in comparison with results obtained in conventional macro-scale batch macroreactor (conversion of 5.3 % after 180 s). Introducing two immiscible liquids in “Y”-shaped microreactor and superficial velocity in the range 5 – 200 $\mu\text{L min}^{-1}$ at 25 °C slug (plug) flow was developed. In order to analyze influence of flow patterns and interphase area, ADH catalyzed hexanol oxidation was performed in microreactor equipped with micromixers. Due to the mixing effect, smaller slugs were developed in microreactor equipped with micromixers comparing to those formed in “Y”-shaped tubular microreactor at the same flow rates. Comparing conversions achieved in both types of the microreactors for the same residence times higher conversions were observed in microreactor equipped with micromixers indicating that mixing of phases has a great effect on mass transfer.

Key words: microreactor, multiphase flow, hexanol oxidation, alcohol dehydrogenase

1. Introduction

In recent years, more and more effort is made on development of natural friendly technologies. In this context it is important to develop chemical processes based on sustainable technology (Kashidi, 2007). The engineering solution for this problem is to improve mass and heat transfer, reduce necessary amount of chemicals and amount of process waste stream, enhance safety (i.e. when working with reactions that are highly explosive and demand use of toxic reagents), reduce the total energy consumption and to achieve better yield, selectivity and process control (Ehrfeld et al., 2000). Microreactor technology offers potential benefits due to well defined high specific interfacial area available for heat and mass transfer, which increases transfer rates and enhances safety (Kashidi, 2007). Parallel scale-out, so called numbering – up present in microreactors eliminates scale – up problems present in conventional processes. Considering this, application of microreactors could be the next generation of producing processes. Biotransformation in microreactors could be good alternative to classical chemical synthesis. Comparing microreactors with macroreactors, they offer many advantages. For example, in macroreactors processes are mainly operated batch or semi-batch way, but using microreactors there is possibility to make those processes continuous. This fact could benefit pharmaceutical and fine chemical industry. Roberage et al. (2005) claim that 50 % of reactions in those industry could prosper from a continuous process based mainly on microreactor technology.

Nowadays, one of most interesting pharmaceutical, agrochemical and aroma compounds are so called “green notes” (Shade et al., 2003.). They are high value molecules widely used in flavor to impart both the green character and the impression of freshness (Akacha and Gargouri, 2009.) and their market is estimated at USD 20 – 40 million annually (Whitehead et al., 1995). Majority of them are six-carbon alcohols and aldehydes such as hexanal. They are being produced by fermentation, extraction from plants or by enzyme-

catalyzed reactions (Márczy et al., 2002, Shade et al., 2003, Brunerie and Koziol, 1997.). However, traditional methods like extraction from plant leaves cannot give sufficient amount of those components so alternative natural ways and new techniques have to be developed. Considering all benefits of microreactors, biotransformation in them could be a solution.

The aim of this work was to investigate the effect of different types of microreactors on hexanal production. Two phase hexane – water system with dissolved yeast alcohol dehydrogenase was selected as a media for reaction. Reaction was performed in three different types of microreactors equipped with one outlet channel and two “Y”-shaped inlet channels, one for the inflow of enzyme and coenzyme (NAD^+) dissolved in aqueous buffer and one for inflow of hexanol dissolved in organic (hexane) phase.

2. Material and methods

2.1. Materials

2.1.1. Chemicals

NAD⁺ and hexanal were purchased from Fluka A.G. (Switzerland). Commercial alcohol dehydrogenase (451 U/mg) from baker's yeast and hexane were from Sigma (Germany). Hexanol was from Merck (Germany) and cyclohexanone was from Carlo Erba (Italy). HCl, glycin and Na₂P₂O₇ · 10 H₂O were purchased from Kemika (Croatia).

2.1.2. Apparatus

A microreactor system (Fig. 1a) was consisted of 3 different microchips with borosilicate glass microchannels (tubular microreactor length:width:depth = 332 mm:150 μm:150 μm with internal volume of 6 μL; tubular microreactor length:width:depth = 676 mm:150 μm:50 μm with internal volume of 13 μL and microreactor equipped with swirl micromixers for high Reynolds number mixing ($Re > 50$) length:width:depth = 53.3 mm:200 μm:150 μm, with internal volume = 2 μL). Each microreactor was quipped with two inlets ("Y"-shaped), so fluids can be injected separately and one outlet (Fig. 1d). Microreactor chips were placed into a stainless steel holder (Fig. 1c), which provided leak-free connection (Micronit Microfluidics B.V., Netherlands). Two syringe pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, USA) equipped with high pressure stainless steel syringes (8 ml, Harvard Apparatus, USA) were used for solution supply. Microreactor chips were connected to pumps with fused silica connection (375 μm O.D., 150 μm I.D., Micronit Microfluidics B.V., Netherlands). Fluid flow in microreactor was observed using microscope (Fig. 1b, Motic B1-220A, binocular Weltzar, Germany) at magnifications of 40x and 100x (eyepiece magnification = 10 x; objective magnification = 4x, 10x).



Figure 1: Experimental set up used to perform hexanol oxidation in microreactor. Snapshot of:

- a) experimental set up
- b) microreactor chip placed into a stainless steel holder under microscope
- c) microreactor chip placed into a stainless steel holder and
- d) microreactpr chip

2.2. Methods

2.2.1. Alcohol dehydrogenase catalyzed hexanol oxidation

Enzyme and coenzyme dissolved in aqueous buffer (75 mmol dm⁻³ glycine-pyrophosphate buffer, pH = 9) were fed from one inflow and substrate (hexanol dissolved in hexane) from another inflow. Inlet concentration of substrate was kept constant (5.5 mmol dm⁻³) through all experiments; inlet concentrations of enzyme and coenzyme were altered. Substrate and enzyme with coenzyme were pumped in equal flow rates (organic phase:water phase = 1:1). Outflows from microreactors, containing substrate, product, enzyme, NAD⁺ and NADH were gathered in 0.1 mol dm⁻³ HCl to stop the reaction by enzyme deactivation.

2.2.2. Analytics

Samples for analysis were prepared by mixing the equal volume of sample with internal standard (1 % solution of cyclohexanone in hexane) for 1 min. Hexane was used to extract the hexanol and hexanal from water solution. To separate water from organic phase samples were centrifuged (Hettich, Universal 320R, Andreas Hettich GmbH & Co. KG, Germany) for 3 min at 4 °C and 9000 min⁻¹. Upper layer, after filtration (Filter Chromafil[®] AO-20/3; 0.2 µm, 3 mmØ100, Macherey, Nagel GmbH, Deutschland) was used for the analysis (Vrsalović Presečki and Vasić Rački 2009). Concentration of hexanol and hexanal in organic phase were analyzed using GC (Shimadzu GC-2014, Kyoto, Japan) with the flame ionization detector. Polar column ZB-WAX (Phenomenex, Torrance, USA) and helium as gas carrier were used. Concentrations of compound were measured under following conditions: split less injector 280 °C, linear velocity 25 cm s⁻¹, detector 240 °C, initial temperature 50 °C, initial time 1 min, rate of 10 °C min⁻¹ to 180 °C, final temperature 180 °C and final time 2 min (Karra-Chaabouni et al., 2003). A sample volume of 1 µL was injected. Observed retention times of hexane, hexanal, cyclohexanone and hexanol were 2.1 min, 5.9 min, 9.2 min and 9.7 min, respectively.

3. Results and discussion

3.1. Flow profile in microchannels

In order to distinguish two phases in microchannel, the water phase was stained with a blue dye (brilliant blue) to appear darker than the colorless organic phase. The experimental results show that introducing two immiscible liquids in “Y”-shaped microreactor at 25 °C (both tubular and microreactor equipped with micromixers) slug (plug) flow is developed. In those conditions the water phase forms convex shaped slugs while organic phase exhibits a concave geometry (Fig. 2a and Fig. 2b). According to different authors (Kashid and Agar,

2007, Harries et al., 2003, Burns and Ramshaw, 2001) mass transfer occurs at fluid interface. Kashidi and Agar, 2007 claim that the mass transfer in biphasic system where slug flow is formed, intensifies as a result of internal circulation in slugs (Fig.1c) enhancing interface diffusion penetration and consequently increasing the reaction rates. Based on that fact, it could be assumed that when number of segments is increased, better and more effective mass transfer is achieved. Comparing slugs (Fig. 1a and Fig. 1b) in both types of reactors for the equal flow rate, smaller slugs are formed in microreactor equipped with micromixer. Comparing slug formation in tubular microreactor when velocity of both phases was altered in same ratio, it was noticed that the slugs of both phases have a length greater than their diameter and that the pattern was equal and stable along the microchannel at low total velocities. Increasing velocity of both phases, stability of flow was preserved despite the fact that length of slugs was decreasing. Smallest slugs of length:diameter ratio of approximately 1:1 were observed at total velocity of $200 \mu\text{L min}^{-1}$. In microreactor equipped with micromixers slug length:diameter ratio of organic phase was approximately 1:1 and for water phase 0.3:1 for all velocities. As in tubular microreactor, flow pattern was stable in microreactor equipped with micromixers.

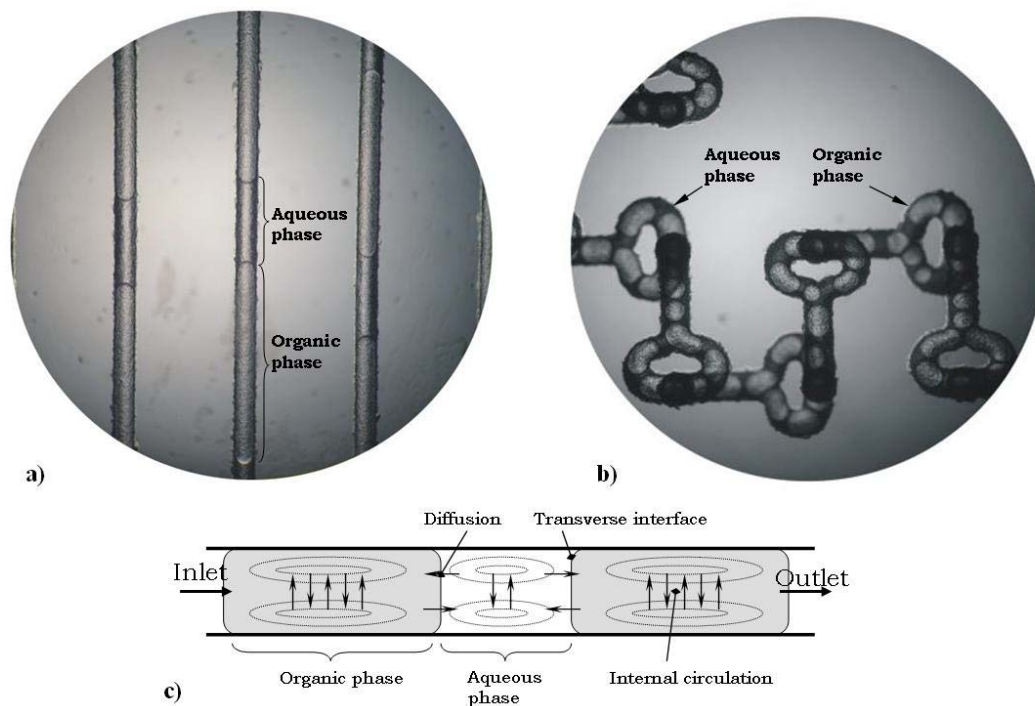


Figure 2: Experimental snapshot of liquid- liquid slug flow in

- a) tubular microreactor
- b) microreactor equipped with micromixers and
- c) schematic view of slug flow internal circulation and transversal diffusion in microchannel

In comparison presented results with results of [Žnidaršič-Plazl and Plazl \(2009\)](#) it was noticed that slug formation also depends on operating parameters, like flow rate and mixing element ("Y"-junction) geometry and capillary dimension ([Kashid and Agar, 2007](#)). Mentioned authors, also working with hexane (organic phase) and water (aqueous phase) in tubular microreactor with "Y"-shaped inlet and outlet (length:width:depth=332 mm:220 μm :50 μm), achieved not slug flow but parallel, laminar flow. They observed different positions of interface area (in the middle of channel or less viscous hexane occupied much smaller part of channel) in microchannel depending on inlet flow rate of both phases. As mentioned, this could be a consequence of different channel dimension and roughness.

Between those two microchannel of the same length (332 μm), microchannel used in our work is approximately 1.5 times narrower, 3 times higher and has 10 times higher relative roughness when compared. According to [Kashid and Agar, 2007](#) formation of slug flow could be advantage. Authors claim that due to relatively low interfacial area and mass transfer only by diffusion, the parallel flow takes long time for higher throughput compared to slug flow and that the slug flow shows very stable behavior compared to the parallel flow.

3.2. Hexanol oxidation in a microchannel at different process conditions

Hexanal production was carried out in three different types of microreactor to compare effect of residence time, microchannel geometry and mixing on conversion and productivity. Influence of inlet initial concentrations of enzyme and coenzyme were also investigated and results are presented in Figures 3-5.

In the first experiment tubular microreactor with internal volume of 6 μL was used. Process conditions ($c_{i,\text{hexanol}} = 5.51 \text{ mmol dm}^{-3}$; $c_{i,\text{NAD}^+} = 5.5 \text{ mmol dm}^{-3}$; $\gamma_{i,\text{ADH}} = 0.92 \text{ g dm}^{-3}$; $T = 25 \text{ }^\circ\text{C}$) were set to be equal to those in batch process conducted by [Vrsalović – Presečki, 2006](#). Conversion of 7.8 % in microreactor (Fig. 3) achieved after 7.2 s was 1.35 times higher than the one obtained in macroreactor (conversion of 5.3 % after 180 s). It is very interesting, when using microreactors very short residence times are necessary for achieving maximum conversions. Longer residence time could not be achieved due to formation of enzyme and coenzyme plugs when experimenting at those initial concentrations. Prolonging residence time even higher conversions could be achieved. To test that assumption experiment with ten fold lower initial concentration of enzyme and coenzyme was conducted ($c_{i,\text{NAD}^+} = 0.55 \text{ mmol dm}^{-3}$; $\gamma_{i,\text{ADH}} = 0.092 \text{ g dm}^{-3}$; $T = 25 \text{ }^\circ\text{C}$). Initial concentration of substrate was kept constant through all experiments. After 72 s, at those initial concentrations conversion of 13.5 % was achieved. Comparing conversions at low residence times for experiments with different inlet

concentrations of enzyme and coenzyme, it was noticed that they are approximately the same indicating that enzyme concentration is not the limiting factor for ADH catalyzed hexanol oxidation in microreactor.

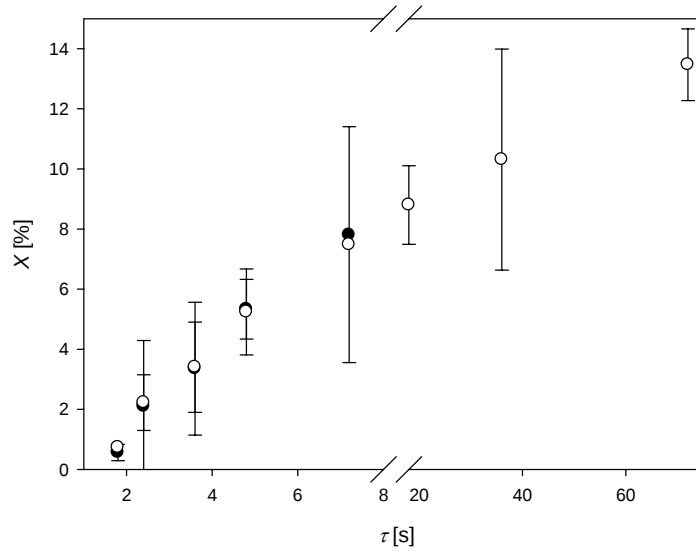


Figure 3: Experimental results of hexanol oxidation with ADH in microreactor with internal volume of 6 μL at different inlet concentrations of NAD^+ and ADH; ($\bullet$) $c_{i,\text{NAD}^+} = 5.5 \text{ mmol dm}^{-3}$, $\gamma_{i,\text{ADH}} = 0.92 \text{ g dm}^{-3}$; ($\circ$) $c_{i,\text{NAD}^+} = 0.55 \text{ mmol dm}^{-3}$, $\gamma_{i,\text{ADH}} = 0.092 \text{ g dm}^{-3}$.

Hexanol oxidation was also performed in 13 μL tubular microreactor where lower residence time could be achieved. Comparing geometry, dimensions of microchannel width and depth were the same for both types of tubular reactors used. Only difference was in channel length. Considering this, not only the effect of residence time but also the effect of channel geometry on reaction could be investigated. Analyzing results (Fig. 4) it was noticed that channel length at the same residence time has no significant effect on conversions, as expected. Obtained conversions were practically the same for the equal residence time in both microreactors. Because of enzyme and coenzyme plugs formation residence time higher than 78 s could not be reached.

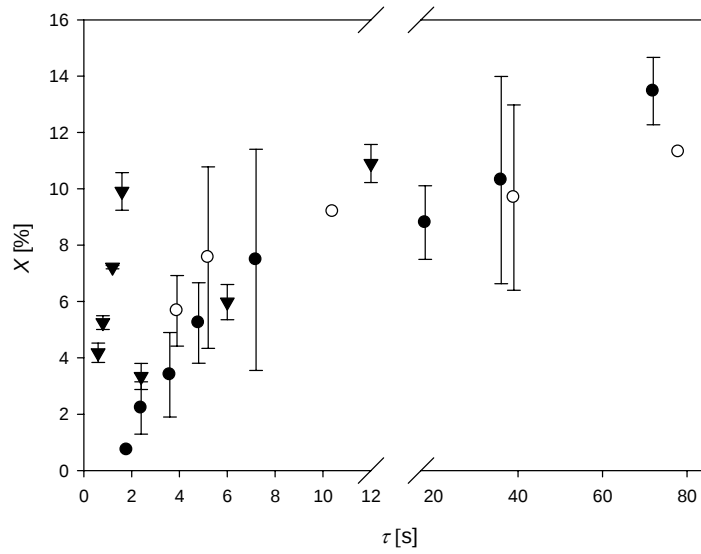


Figure 4: Experimental results of hexanol oxidation with ADH obtained in three different microreactors, (●) microreactor with internal volume of 6 μ L, (○) microreactor with internal volume of 13 μ L, (▼) microreactor equipped with micromixers and internal volume of 2 μ L at different residence times ($c_{i,\text{NAD}^+} = 0.55 \text{ mmol dm}^{-3}$, $\gamma_{i,\text{ADH}} = 0.092 \text{ g dm}^{-3}$, $c_{i,\text{hexanol}} = 5.5 \text{ mmol dm}^{-3}$).

As mentioned before, different total flow of fluids for aqueous and organic phases in tubular microreactor results in slug flow formation. In order to analyze influence of flow patterns and interphase area, ADH catalyzed hexanol oxidation was performed in microreactor equipped with micromixers (Fig 4.). Mixing of fluids in microchannel can be performed in various ways. In general, two different types of passive mixing were distinguish, where the only flow energy is used and active mixing where energy from the exterior is used for mixing effect. In this work stationary or so called Coanda effect micromixers were used. This mixing is based on the redirection of a flow by a special guiding structure which creates new interface within the flow (Hessel, 2005).

Due to the mixing effect, smaller slugs were developed in microreactor equipped with micromixers comparing to those formed in “Y”-shaped tubular microreactor for the same flow rates (Fig. 2a and Fig. 2b). As previously mentioned decreasing slug size, mass transfer is increasing.

Comparing conversions achieved in both types of the microreactors for the same residence times higher conversions were observed in microreactor equipped with micromixers, confirming that mixing had a great effect on mass transfer. Conversion of 9.91 % was achieved in this type of microreactor for approximately 1.6 s. To accomplish this, approximate conversion (9.69 %) in tubular (13 μL) microreactor it took 22.5 time longer residence time. In batch experiment half of this conversion was observed after 180 s. Highest conversion of 10.9 % was achieved for residence time of 12 s. Longer residence time could not be studied due to plugs formation.

Production of hexanal was also monitored through all experiments (Fig. 5). As expected, highest productivity of $2.5 \text{ kg L}^{-1} \text{ d}^{-1}$ was noticed when microreactor equipped with micromixers was used. Interestingly productivity of $0.56 \text{ g L}^{-1} \text{ d}^{-1}$ was obtained from residence time of only 0.6 s. Comparing those results with results obtained in both used tubular microreactors it was obvious that in microreactor equipped with micromixers significantly higher productivities were achieved for all analyzed residence times. Even though tubular microreactors are not as promising as the one with micromixers, productivity of hexanal achieved when they were used, was still more than 10 fold higher than the one achieved in batch process performed in a macroreactor (around $0.05 \text{ kg L}^{-1} \text{ d}^{-1}$).

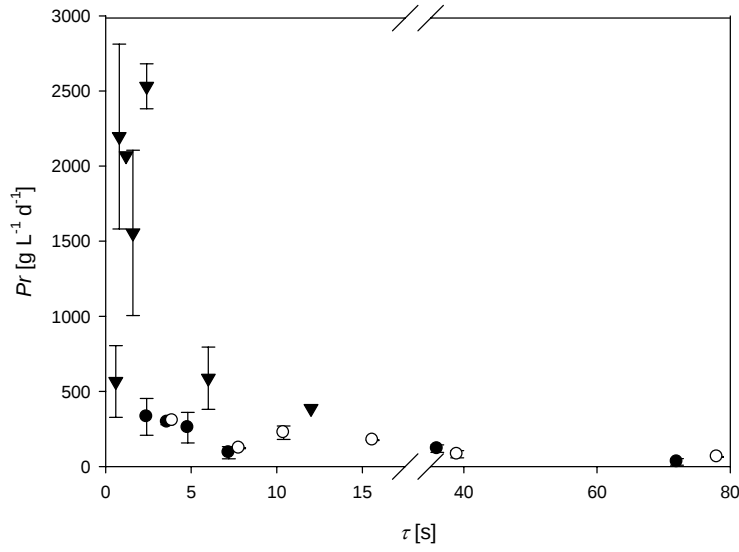


Figure 5: Comparison of productivities for different types of microreactors (●) microreactor with internal volume of 6 μL , (○) microreactor with internal volume of 13 μL , (▼) microreactor equipped with micromixers and internal volume of 2 μL at different residence times ($c_{i,\text{NAD}^+} = 0.55 \text{ mmol dm}^{-3}$, $\gamma_{i,\text{ADH}} = 0.092 \text{ g dm}^{-3}$, $c_{i,\text{hexanol}} = 5.5 \text{ mmol dm}^{-3}$).

Comparing all the results and taking mass transfer, residence time and productivity in consideration it could be concluded that in this case, microreactor equipped with micromixers would be the best solution for hexanal production.

4. Conclusion

Alcohol dehydrogenase catalyzed hexanol oxidation performed in different types of microreactor demonstrated that use of microreactors could be a good alternative for classical hexanal production processes. Conversion of 13.5 % achieved in a “Y”-shaped tubular microreactor for residence time of 72 s was significant improvement in comparison with results obtained in conventional macro-scale batch bioreactor for the ten fold lower concentrations of reactants. Even better results were achieved in microreactor equipped with micromixers for the same residence time (intensified mass transfer).

For increasing process selectivity and conversion of hexanol in to desirable "green note" component hexanal, separation of phases after reaction and parallel coenzyme regeneration is proposed.

Acknowledgment

This research was financially supported through The National Foundation for Science, Higher Education and Technological Development of the Republic of Croatia.

References

- Akacha, N.B., Gargouri, M. (2009) Enzymatic synthesis of green notes with hydroperoxide-lyase from olive leaves and alcohol dehydrogenase from yeast in liquid/gas reactor. *Process Biochemistry*, **44**, 1122 – 1127.
- Brunerie, P., Koziat, Y., Process for producing natural cis-3-hexenol from unsaturated fatty acids. U.S. Patent 5,620,879 (1997).
- Burns, J.R., Ramshaw, C. (2001) The intensification of rapid reactions in multiphase systems using slug flow in capillaries. *Lab on a Chip*, **1**, 10 – 15.
- Ehrfeld, W., Hessel, V., Löwe, H. (2000) Microreactors: New Technology for Modern Chemistry. Wiley-VCH, Weinheim.
- Harries N., Burns J.R., Barrow D.A. and Ramshaw C. (2003) A numerical model for segmented flow in a microreactor. *International Journal of Heat and Mass Transfer* **46**, 3313 - 3322.
- Hessel, V., Löwe, H. Schönfeld, H. (2005) Micromixers—a review on passive and active mixing principles. *Chemical Engineering Science*, **60**, 2479 – 2501.

- Karra-Chaabouni M., Pulvin S., Meziani A., Thomas D., Touraud D., Kunz W. (2003) Biooxidation of n-hexanol by alcohol oxidase and catalase in biphasic micellar systems without solvent. *Biotechnology and Bioengineering*, **81**, 27 – 32.
- Kashid, M.N. (2007) Experimental and modeling studies on liquid-liquid slug flow capillary microreactors. PhD Thesis, University of Dortmund, Technical University, Department of biochemical and chemical engineering.
- Kashid, M.N., and Agar, D.W. (2007) Hydrodynamics of liquid-liquid slug flow capillary microreactor: Flow regimes, slug size and pressure drop. *Chemical Engineering Journal*, **131**, 1 – 13.
- Márczy, J.S., Németh, A.S. Samu, Z., Hágér-Veress, Á., Szajáni, B. (2002) Production of hexanal from hydrolyzed sunflower oil by lipoxygenase and hydroperoxid lyase enzymes. *Biotechnology Letters*, **24**, 1673 – 1675.
- Roberge, D.M., Durcy, L., Bieler, N., Cretton, P., Zimmermann, B. (2005) Microreactor Technology: A Revolution for the Fine Chemical and Pharmaceutical Industries? *Chemical Engineering and Technology*, **28**, 318 - 323.
- Shade F., Thompson J. E., Legge R. L. (2003) Use of a Plant-Derived enzyme Template for the Production of the Green-Note Volatile Hexanal. *Biotechnology and Bioengineering*, **84**, 265 - 273.
- Vrsalović Presečki, A. (2006) Study of fumarase and alcohol dehydrogenase in the biotransformations. PhD Thesis, University of Zagreb, Faculty of Chemical Engineering and Technology.
- Vrsalović Presečki, A., Vasić Rački Đ. (2009) Mathematical modeling of the dehydrogenase catalyzed hexanol oxidation with coenzyme regeneration by NADH oxidase. *Process Biochemistry* **44**, 54 – 61.

Whitehead, I. M., Muller, B. L., Dean, C. (1995) Industrial use of soybean lipoxygenase for the production of natural green note flavor compounds. *Cereal Foods World*, **40**, 193 - 197.

Žnidaršić-Plazl, P. and Plazl, I. (2009) Modelling and experimental studies on lipase-catalyzed isoamyl acetate synthesis in microreactor. *Process Biochemistry*, **44**, 1115 - 1121.