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Production of green note component on a microscale – From idea to integrated systems

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Abstract

In comparison with macro level, batch production processes development of continuous, micro level production processes presents a certain challenge since a lot of new physical phenomena emerge. Different heat and mass transport phenomena, different diffusion time, enhanced surface roughness impact, different flow profiles etc. are just some of the challenges that have to be overcome in order to develop a functional production system on a microscale. As a case study for this statement production of hexanal has been investigated. Different experimental approaches for production in a microreactor were necessary in order to transfer already established microscale batch production. While "one pot" principle was working perfectly for batch production, it was necessary to develop an integrated system with several connected microchips for successful and effective hexanal production on a microscale. In this paper, basic steps that led from idea to integrated system of hexanal production on a microscale are demonstrated.

Keywords: *hexanol oxidation, hexanal, microreactor, alcohol dehydrogenase, coenzyme regeneration, immobilization*

1. Introduction

Hexanal is part of the group of natural volatile chemicals, the so-called "*green notes*". "*Green notes*" (aldehydes and alcohols) are high-value molecules widely used in the aroma industry to impart the green character associated with freshness. Hexanal has a pleasant grassy odor and its organoleptic fresh note is what makes it so interesting for consumers [1].

Nowadays, several methods are applied for hexanal production based on fermentation, extraction from plants, and enzyme-catalyzed reactions [2]. Main problem of these traditional methods is small amount of produced hexanal. Additionally, fermentation processes and extraction from plants result with formation of large amounts of unwanted by-products and a lot of waste. Main problem of the enzyme catalyzed synthesis is low yield. The general opinion is that the demand for hexanal would increase if it could be produced in a more economical way. In addition, although the yield is low, the use of enzymes for catalysis compared to classical chemical catalysts is highly desired in food industrial processes, because the resultant products would be classified as "natural" by food regulatory agencies, a feature that increases their public acceptance as ingredients for food [3].

Following that idea Vrsalović-Presečki [4] demonstrated that hexanal can be produced by oxidation of hexanol using NAD(H) dependent alcohol dehydrogenase (ADH) from baker's yeast as catalyst in a batch reactor (V = 10 mL). Using equimolar concentration of hexanal and coenzyme 5.3% conversion was obtained during 3 min. Results indicated that coenzyme regeneration is necessary, not only to reduce process costs, but also to shift the production in the direction of products.

When enzyme regeneration system based on acetaldehyde reduction was introduced into the process (Fig. 1) conversion in a batch reactor increased to 11% (t = 25 min). Although the results demonstrated that it was possible to produce hexanal by this approach, conversion was still too low.

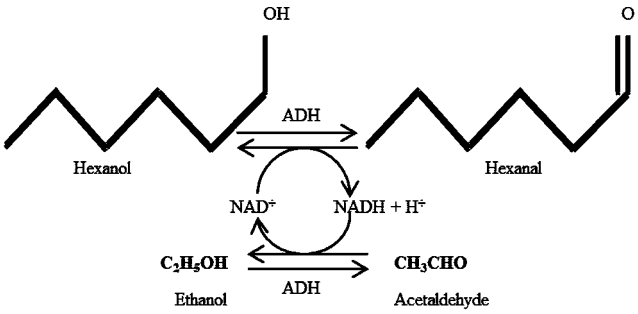

Fig. 1. Reaction scheme of ADH-catalysed hexanal oxidation with NADH coenzyme regeneration based on acetaldehyde reduction

By combining enzymatic biocatalysis and microreactor technology, a new way for hexanal production was proposed. Microreactor technology is a new interdisciplinary technology. Benefits of this new technology pose a vital influence on chemical industry, biotechnology, the pharmaceutical industry and medicine. A high surface to volume ratio, faster diffusion dominated transport, enhanced heat transfer and thus reduced energy demands, good process control, high throughput, usage of minimal (microlitres) of reagent volumes, etc., are some of a microreactor advantages that are usually stressed [5].

2. State of the art

Within this paper application of microreactor technology for hexanal production is presented. Overall research

was divided in to three phases. *First phase* was focused on hexanal production on a microscale, *second* on coenzyme regeneration and *third* on the development of integrated system for parallel hexanal production and coenzyme regeneration. The idea behind integrated system (Fig. 2) was to introduce hexanol dissolved in hexane as one process phase and enzyme and coenzyme dissolved in buffer as second phase. After introducing the phases, it was necessary to accomplish stable and parallel fluid flow to separate the phases at the end of the first microreactor. At the outlet of the first reactor, an aqueous phase containing enzyme and coenzyme (NADH and NAD^+) dissolved in buffer would be directed in to the second microreactor in order to regenerate the coenzyme. Acetaldehyde dissolved in buffer would be used for coenzyme regeneration.

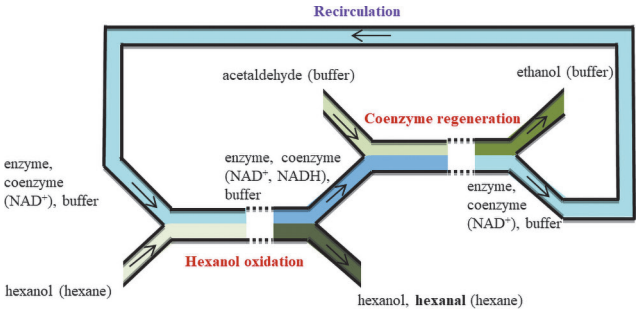

Fig. 2. Process scheme for hexanol oxidation with coenzyme regeneration and recirculation

The outlet of the second microreactor containing regenerated coenzyme and enzyme would then be reused again (recirculation) and fed as the second stream in to the first microreactor. Main goal was to increase the conversion and yield and reduce production time to make the process sustainable.

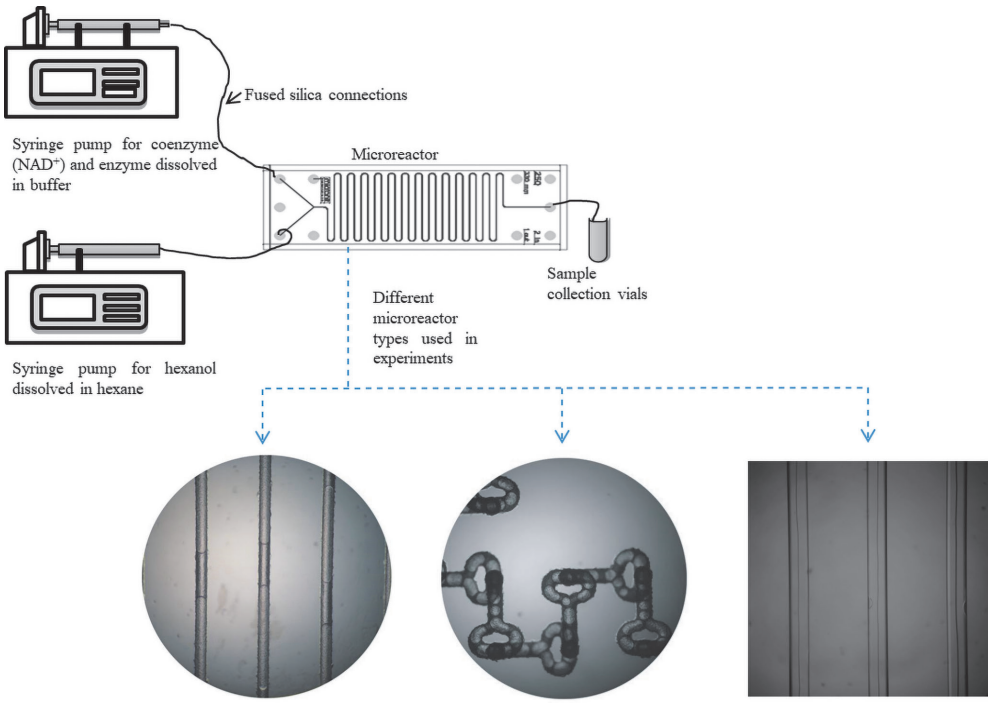

Fig. 3. Process scheme and reactor types used for hexanal production in a microreactor

3. Results

3.1. Hexanal production in a microreactor

As mentioned, first phase was focused on hexanal production on a microscale. Same reaction mechanism as the one described by Vrsalović-Presečki [4] was chosen, meaning hexanol oxidation was performed by using NAD(H) dependent alcohol dehydrogenase (ADH) from baker's yeast. Besides purified enzyme, permeabilized baker's yeast cells were also used as a potential source of ADH.

Permeabilized baker's yeast cells were used as inexpensive and easy to obtain. Additionally, in order to overcome some disadvantages of suspended biocatalyst usage, like decrease of stability during storage, complicated catalyst-product separation, harder operation and impossible reuse, biocatalyst immobilization was investigated for both types of biocatalysts, purified enzyme and permeabilized baker's yeast cells, respectively.

Different types of microreactors:

(*i*) tubular microreactors with rough walls with internal volume of 6 μL and 13 μL,
(*ii*) tubular microreactor with smooth walls and
(*iii*) microreactor equipped with micromixers,

different inlet concentrations of substrate and biocatalyst as well as different flow rate ratios of the organic and aqueous phase were investigated in order to propose the best reactor type and best process conditions for hexanal production.

Comparison of results for different process conditions are shown in Table 1. Simplified process scheme and different microreactor types are presented in Fig. 3.

Obtained results, for every investigated microreactor system, indicated that microreactors could be a good choice for hexanal production in comparison to traditional (batch) production process where, as mentioned, conversion of 5.4% was achieved in 3 min. Best results in microreactors (Table 1) were obtained by using suspended enzyme in microreactor with rough walls (experiment 13) and equimolar concentration of substrates and in microreactor with smooth walls with concentration of coenzyme 10 fold lower than the concentration of the hexanol (experiment 16).

As already mentioned, in order to develop integrated system it was necessary to establish stable and parallel fluid flow that will allow phases separation at the end of the microreactor.

Using the microscope and staining the aqueous phase with blue dye, formation of the flow patterns was observed. It was noticed that in the microchannel with rough walls formation of segmented flow is more characteristic while in microchannel with smooth walls a stable and parallel flow is developed (Fig. 4).

Table 1. Comparison of the highest conversion of hexanal under different reaction conditions

Experiment	Experimental conditions										Results		Reference
	catalyst form	inlet concentration			flow			microreactor			τ (s)	X (%)	
		$c_{i,\text{hexanol}}$ (mmol L^{-1})	$c_{i\text{NAD}^+}$ (mmol L^{-1})	$\gamma_{i,\text{ADH}}$ (g L^{-1})	ratio aqueous	ratio organic	profile	type	V (µL)	surface roughness			
1-4	suspended enzyme	5.5	0.55	0.92	1	1	segmented	tubular	6	rough	7.2	7.8	[6]
		5.5	0.55	0.092	1	1					72	11.8	
		5.5	1.1	0.092	1	1					4.8	14.3	
		5.5	11	0.92	1	1					4.8	9.1	
5	suspended enzyme	5.5	0.55	0.092	1	1	segmented	tubular	13	rough	78	11.3	
6	suspended enzyme	5.5	0.55	0.092	1	1	segmented	micromixers	2	rough	12	10.9	
7-9	suspended enzyme	5.5	0.55	0.092	1	3	segmented	tubular	6	rough	90	11.3	
					1	5					60	9.7	
					1	10					81.8	11.5	
10-12	suspended enzyme	5.5	0.55	0.092	3	1	segmented	tubular	6	rough	90	11.4	
					5	1					60	10.4	
					10	1					81.8	7.9	
13	suspended enzyme	4.4	4.4	0.092	1	1	segmented	tubular	6	rough	72	80	[7]
14	immobilized cells	5.5	–	–	–		–	tubular	6	rough	20	8	[8]
15	suspended cells	5.5	–	–	–		–	tubular	6	rough	2	24	
16	suspended enzyme	5.5	0.55	0.092	1	1	parallel	tubular	6	smooth	20	53	[2]
17	suspended enzyme	5.5	0.55	0.092	3	1	parallel	tubular	6	smooth	10	19	

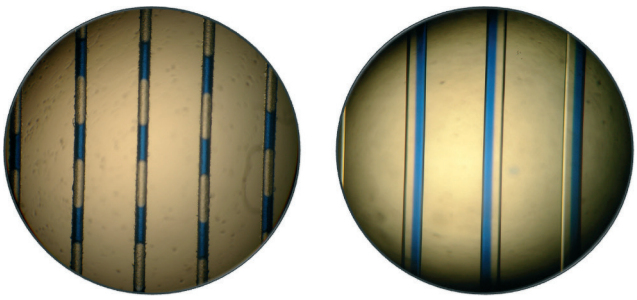

Fig. 4. Microscopic observation of the flow pattern formation in microreactor with rough and smooth channel walls (blue – aqueous phase, colorless – organic phase)

Another effect was noticed when both phases enter the reactor with same velocity. In that case less viscous hexane occupies a much smaller part of the channel and the interphase area between two phases is stable but not formed in the middle of reactor (Fig. 5). This presented a problem for phase separation at the exit of the microreactor. Phase separation was necessary for enzyme regeneration and recirculation as well as for product separation.

In order to resolve this problem, hexane flow velocity was elevated and the new flow ratio of aqueous:organic phase of 1:3 was proposed. Unfortunately, this lead to decrease of conversion but obtained result was still higher than those obtained in batch reactor (Table 1, experiment 17) and phase separation was now possible.

3.2. Coenzyme regeneration in a microreactor

Since the price of coenzyme, which is essential for ADH functionality, coenzyme regeneration process in a microreactor was second phase of process development. Coenzyme must be added in reaction in a stoichiometric amount and may not be replaced by more economical synthetic products. Enzyme ADH used as the biocatalyst in the hexanal production process was also used for regeneration of coenzyme. Other substrate, acetaldehyde was used as a substrate for coenzyme regeneration because of its low price and the high specificity of ADH towards it. Disadvantages of selected reaction system were the possibility of the enzyme deactivation both by substrate, acetaldehyde, and the product, ethanol, instability (possibility of self-condensation in the solution) and volatility of acetaldehyde [9].

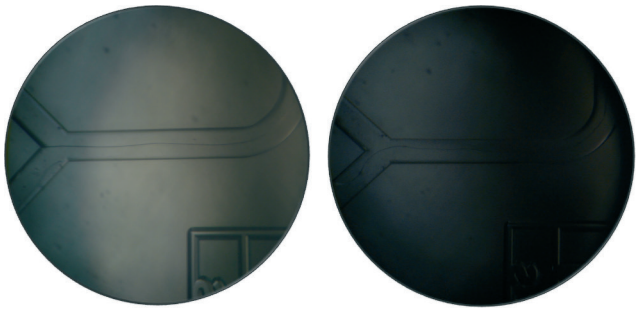

Fig. 5. Microscopic observation of the flow patterns at the exit of the microchannel for the flow ratio of aqueous and organic phase 1:3 and 1:1

Table 2. Comparison of the different systems used for NADH regeneration

Experiment	Experimental conditions						Results		Reference
	catalyst form	inlet concentration			microreactor		τ (s)	X (%)	
		$c_{i,\text{acetaldehyd}}$ (mmol L^{-1})	$c_{i,\text{NADH}}$ (mmol L^{-1})	$\gamma_{i,\text{ADH}}$ (g L^{-1})	type	V (µL)			
1	suspended enzyme	5.5	6.9	0.2	glass microreactor	6	2	80	[10]
2	suspended enzyme	44	6.9	0.2	glass microreactor	6	0.8	100	
3	suspended cells	5.5	5.5	0.1	glass microreactor	6	36	65.3	[11]
4	immobilized enzyme	5.5	5.5	–	glass microreactor	6	3.6	12	
5	immobilized cells	5.5	5.5	–	glass microreactor	6	7.5	3.6	
6	suspended cells	5.5	5.5	0.1	PTFE microreactor	273.15	47.1	86.67	
7	suspended enzyme	5.5	5.5	0.1	PTFE microreactor	273.15	47.1	94.35	
8	immobilized enzyme	5.5	5.5	–	PTFE microreactor	273.15	94.3	11.91	
9-11	enzyme loaded on magnetic nanoparticles	5.5	5.5	–	PTFE microreactor with	square magnet	273.15	10 (min)	100	[12,13]
		5.5	5.5	–		cylindrical magnet		6 (min)	96.4	
		5.5	5.5	–		electromagnet		180	100	

These problems could be solved by using continuously operated microchannel system at different flow rates that could provide short contact time between enzyme and the components with inhibition effect. Summary of the conditions and results are presented in Table 2.

Different forms of biocatalyst (suspended and immobilized enzyme and permeabilized baker's yeast cells) in different reactor types (*i*) glass microreactor with smooth walls, (*ii*) PTFE (polytetrafluoroethylene) and (*iii*) microreactor with and electromagnet or oscillating magnetic field that allowed better distribution of biocatalyst immobilized on nanoparticles were tested to find the best solution for coenzyme regeneration.

In order to generate magnetic field to utilize magnetic properties of magnetic nanoparticles as carrier for biocatalyst, a system for magnetic field generation and regulation was developed (Fig. 6). According to Derks et al. [14] in magnetic bead motion within a fluid, the magnetic and drag forces dominate the bead motion, since at the micrometer scale, effects of gravity and inertia become very small. Therefore, a bead will almost instantaneously accelerate to its terminal velocity at which the magnetic and drag force are exactly at balance with each other.

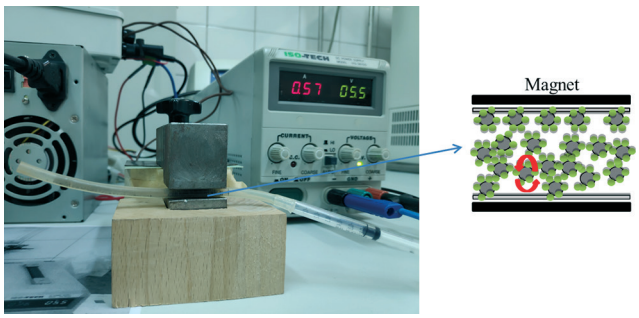

Fig. 6. Experimental set up used for magnetic field regulation developed at University of Zagreb, Faculty of Chemical Engineering and Technology

Using this concept, it was possible to actively move or restrain the particles across the channel. If the oscillating magnetic field is applied constantly, particles will move towards both the north and south poles. Particle oscillation is obtained by switching the poles on and off. When one pole is active, the particles are attracted to it. When the field is switched and the poles are switched, the beads will be attracted towards opposite direction.

When the results of all experiments were compared the best results (Table 2) were obtained by using suspended

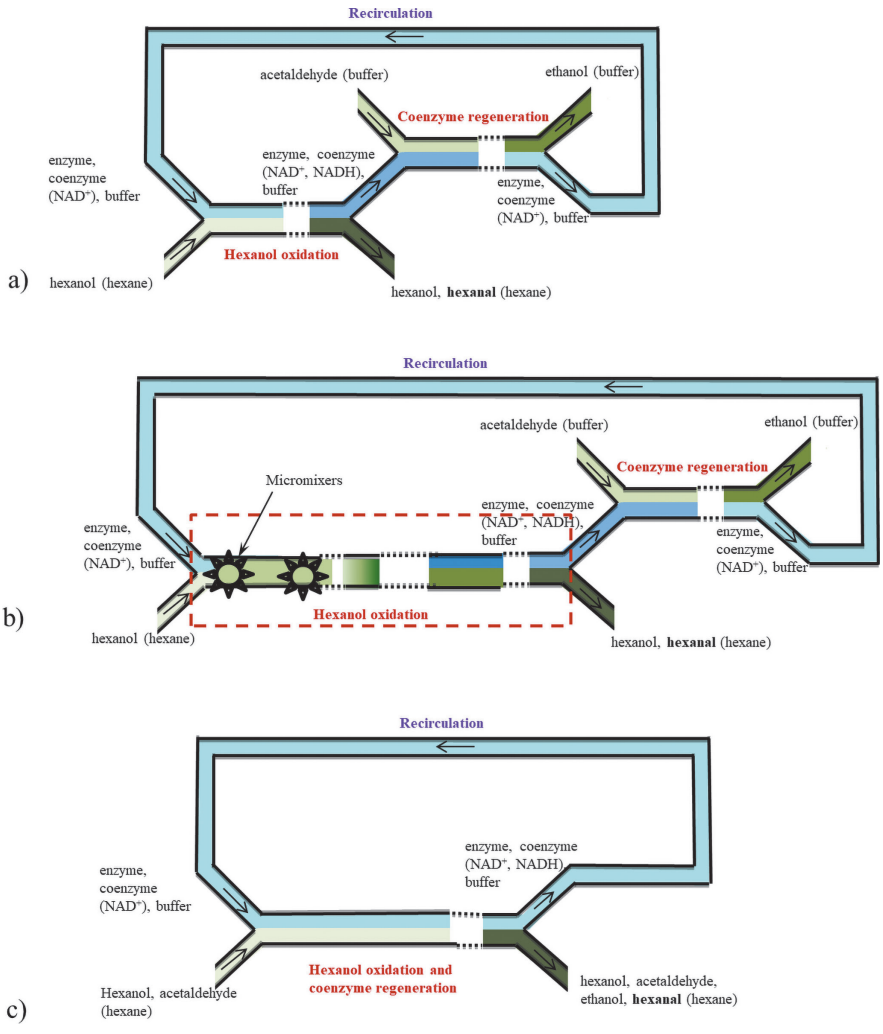

Fig. 7. Different process schemes for hexanal production in integrated systems

enzyme in a microchannel with smooth walls. A conversion of 100% was obtained for the residence time less than 0.8 s when acetaldehyde was in excess. Therefore, this type of the reactor and excess of acetaldehyde in reaction mixture were proposed as the best reaction system for further development of completely integrated system for hexanal production.

3.3. Production of hexanal with in situ product separation – integrated system

In order to develop the production of hexanal with *in situ* product separation all obtained results were analyzed and three different microreactor systems were proposed (Fig. 7).

In the *first system* two microreactors were connected into the series (Fig. 7a). First chip was used for hexanol oxidation and second for coenzyme regeneration. After regeneration, regenerated coenzyme was recirculated into the first microreactor where oxidation step was continuously performed. During the first 4 hours of continuous production, a maximal conversion of 19.5% was achieved. In that period, amount of produced hexanal didn't change but, prolonging the process time, a significant decrease in production was noticed. In 3rd day, process stopped since the activity of the enzyme decreased and only 1% of hexanal was measured at the entrance of microchannel. The biggest advantage of this system was that no additional enzyme and coenzyme were added in the process for all 3 days.

Based on the results presented in Table 1 (experiment 3), where high conversion [6] of hexanol to hexanal were achieved in the microreactor equipped with micromixers, a *second system* was developed (Fig. 7b). It was basically the upgrade of the first system where one microreactor for hexanol oxidation was replaced with two microreactors connected in to the series. First microreactor was microreactor equipped with micromixer and second one was tubular microreactor. On one of the previous research [15] it was noticed that when microreactor chip with micromixers and microreactor with smooth microchannel were connected in series it was possible to enhance mass transfer and separate flows at the exit of microreactor. This would allow regeneration in the third microreactor chip. Unfortunately, despite the literature and previously obtained results, it was not possible to achieve stable flow with the interphase positioned exactly in the middle of second microchannel proving that this developed system was not sustainable for development of integrated system for hexanol oxidation.

Finally, *third system* that was developed was downsizing of the first system (Fig. 7c). The main idea was to make the integrated process as simple as possible, so oxidation and regeneration were placed on the same chip. Maximal conversion obtained at the beginning of the experiment was 17.1% but enzyme activity decreased (deactivation by organic components) rapidly and the process stopped after 1.5 day.

4. Conclusion

Taking all the results into consideration, we believe that with some additional optimization, and further production cost projections, microreactors could serve as the next-generation production process not only for hexanal production but also for the fast and efficient production of different fine chemicals and pharmaceuticals as well as for production of large-scale products.

References

[1] Noordermeer, M.A., Van der Goot, W., Van Kooij, A.J., Veldsink, J.W., Veldink, G.A., Vliegenthart, J.F.G. Development of a biocatalytic process for the production of C6-aldehydes from vegetable oils by soybean lipoxygenase and recombinant hydroperoxide lyase. J. Agric. Food Chem. 50 (2002) 4270-4274

[2] Šalić, A., Zelić, B. ADH catalysed hexanol oxidation with fully integrated NADH regeneration performed in microreactors connected in series. RSC Adv. 4 (2014) 41714-41721

[3] Mugo, S.M., Ayton, K. Lipase immobilized microstructured fiber based flowthrough microreactor for facile lipid transformations. J. Mol. Catal. B: Enzym. 67 (2010) 202-207

[4] Vrsalović Presečki, A. Study of fumarase and alcohol dehydrogenase in biotransformations. PhD Thesis (in Croatian), Zagreb, 2006

[5] Šalić, A., Zelić, B. Synergy of microtechnology and biotechnology: microreactors as an effective tool for biotransformation processes. Food Technol. Biotechnol. 56 (2018) 464-479

[6] Šalić, A., Tušek, A., Kurtanjek, Ž., Zelić, B. Biotransformation in a microreactor: new way for production of hexanal. Biotechnol. Bioproc. Eng. 16 (2011) 495-504

[7] Tušek, A., Šalić, A., Kurtanjek, Ž., Zelić, B. Modelling and kinetic parameter estimation of alcohol dehydrogenase catalyzed hexanol oxidation in a microreactor. Eng. Life Sci. 12 (2012) 49-56

[8] Šalić, A., Pindrić, K., Zelić, B. Bioproduction of food additives hexanal and hexanoic acid in a microreactor. Appl. Biochem. Biotechnol. 171 (2013) 2273-2284

[9] Chenault, H.K., Whitesides, G.M. Regeneration of nicotinamide cofactors for use in organic synthesis. Appl. Biochem. Biotech. 14 (1987) 147-197

[10] Šalić, A., Ivanković, M., Ferk, E., Zelić, B. ADH based NAD+ regeneration in a microreactor. J. Chem. Technol. Biotechnol. 88 (2013) 1721-1729

[11] Šalić, A., Faletar, P., Zelić, B. NAD+ regeneration in a microreactor using permeabilized baker's yeast cells. Biochem. Eng. J. 77 (2013) 88-96

[12] Šalić, A., Pindrić K., Hojnik Podrepšek, G., Leitgeb, M., Zelić, B. NADH oxidation in a microreactor catalyzed by ADH immobilized on γ-Fe2O3 nanoparticles. Green Processing Synth. 2 (2013) 569-579

[13] Šalić, A., Pindrić, K., Hojnik Podrepšek, G., Novosel, N., Leitgeb, M., Zelić, B. NADH oxidation in a microreactor with an oscillating magnetic field. J. Flow Chem. 6 (2016) 27-32

[14] Derks, R., Dietzel, A., Wimberger-Friedl, R., Prins, M. Magnetic bead manipulation in a sub-microliter fluid volume applicable for biosensing. Microfluid. Nanofluid. 3 (2007) 141-149

[15] Šalić, A., Tušek, A., Fabek, D., Rukavina, I., Zelić, B. Aqueous two-phase extraction of polyphenols using a microchannel system – process optimization and intensification. Food Technol. Biotechnol. 49 (2011) 495-501