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# Continuous Flow Chemo-Enzymatic Baeyer-Villiger Oxidation with Superactive and Extra-Stable Enzyme/Carbon Nanotube Catalyst: An Efficient Upgrade from Batch to Flow

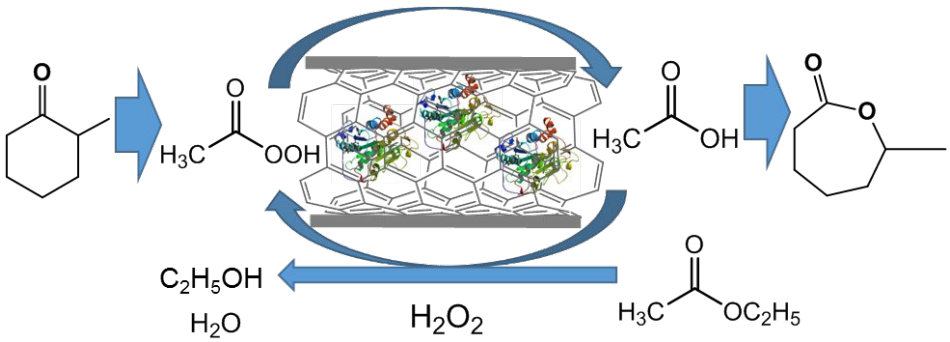
*Anna Szelwicka[a], Przemysław Zawadzki[b], Magdalena Sitko[a], Sławomir Boncel[c], Wojciech Czardybon[b], Anna Chrobok[a]**

[a] Department of Chemical Organic Technology and Petrochemistry, Silesian University of Technology, Krzywoustego 4, 44-100 Gliwice, Poland.

[b]Selvita S.A., Bobrzynskiego 14, 30-348 Cracow, Poland

[c]Department of Organic Chemistry, Bioorganic Chemistry and Biotechnology, Silesian University of Technology, Krzywoustego 4, 44-100 Gliwice, Poland.

## GRAPHICAL ABSTRACT



**ABSTRACT:** Continuous flow chemo-enzymatic Baeyer-Villiger oxidation in the presence of exceptionally active *Candida antarctica* lipase B immobilized *via* simple physical adsorption on multi-walled carbon nanotubes has been investigated. The nanobiocatalyst was used to generate peracid *in situ* from ethyl acetate and 30 wt.% aq. hydrogen peroxide as the primary oxidant. Application of the highly-stable and active nanobiocatalyst in the Baeyer-Villiger oxidation of 2-methylcyclohexanone to 6-methyl-ε-caprolactone after 8 h at 40 °C led to a high product yield (87%) and selectivity (>99%). Environmentally-friendly ethyl acetate was applied as both solvent and the peracid precursor. To determine the most favorable reaction conditions, a series of experiments using various parameters was performed. The main contribution of this work is that it describes the first application of the nanobiocatalyst in a chemo-enzymatic Baeyer-Villiger oxidation in a flow system. Since the process was performed in a flow reactor, many improvements were achieved. First of all, substantially shorter reaction times as well as a significant increase in the product yield were obtained as compared to the batch process. Since peracids are unstable, a large increase in the safety of the process was demonstrated under mild conditions in this work. In summary, this work shows a particularly efficient upgrade in the studied processes by transfer from a batch to a flow system.

KEYWORDS: (nano)biocatalyst, carbon nanotubes, flow chemistry, lipase, chemo-enzymatic Baeyer-Villiger oxidation

## INTRODUCTION

Environmental regulations concerning the safety and waste disposal of industrial processes have caused a considerable acceleration in research involving the design of sustainable processes. A continuous flow reactor strategy is one of the modern tools that offers greener approaches to organic synthesis. Flow systems using microreactors have several advantages over the traditional batch processes, such as lower waste generation and safer experimental conditions. Additionally, more efficient mass and heat transfer, as well as precise temperature control to avoid hazardous exothermic side reactions are among the other benefits. Another advantage is a rapid early stage reaction optimization and a direct scale-up. Three different approaches in flow chemistry to produce larger amounts of product can be applied: running the process longer (scaling-out), reactors-in-parallel (numbering up), or larger continuous reactors (scaling-up).[1-3]

### *Heterogeneous catalysts for flow systems*

Catalytic continuous flow systems with homogenous or heterogeneous catalysts have been reported in the literature.[4] When the homogeneous catalyst is employed, it flows through the reactor together with the reactants, and the additional step to separate the product from

the catalyst is necessary. In the case of heterogeneous catalyst, the catalyst is confined in the reactor, the reagents pass through the system, and no separation of the product from the catalyst is required. However, catalyst leaching is the main limitation of the flow systems. And so, transition metal catalysts such as palladium, copper, ruthenium, and nickel have been immobilized on silica, monoliths, magnetic nanoparticles and polymer supports. These catalytic systems have been applied in a range of reactions including Heck, Sonogashira, Suzuki, Kumada, olefin metathesis, hydrogenation and benzannulation reactions.[1-4]

Apart from the metal catalysts, enzymes have been used as biocatalysts in micro enzyme-reactors.[5-7]Among others, lipases, Novozyme-435 (which is commercially available *Candida antarctica* lipase B (CALB) physically immobilized on a mesoporous poly(methyl methacrylate) support), protease and dehydrogenases have been used to catalyze oligosaccharide synthesis, polymerization, dehalogenation reactions, esterification and hydrolysis or transesterification reactions.[8] Enzyme micro-reactors have been constructed either in the solution phase or by immobilization of enzymes. Enzyme immobilization reduces the overall costs of the process since the biocatalyst can be easily reused, often multiple times.[9,10] Organic, inorganic or hybrid materials – based mainly on polymers and silica – can be used as solid supports for enzyme immobilization. The selection of the support may be a critical step in the design of the biocatalyst.[11] An interesting example is shown in the application of a micro-reactor for the polymerization of ε-caprolactone catalyzed by Novozyme-435. Using a flow system enabled the reaction to be performed at elevated

temperatures in organic media; additionally, faster polymerization and higher molecular weight polymers were achieved compared to the batch reactors. Therefore, continuous biotransformations using immobilized enzymes are becoming more important.[12] Another important example is flow chemo-enzymatic epoxidation of olefins mediated by Novozyme-435. After the recently discovered hydrolase-catalyzed synthesis of peroxycarboxylic acids, the chemo-enzymatic methods for oxidation were developed.[13] For example, Novozyme-435 was very active and stable in flow chemo-enzymatic epoxidation in the packed-bed flow reactor over 24 h.[14]

Nevertheless, the critical limitation of enzyme catalyzed reactions is leaching of the enzymes from solid beds into the products which reduces the life-time of the biocatalyst. In general, however, enzyme leaching is lower in flow systems than in batch reactors.[15]

*Chemo-enzymatic Baeyer-Villiger oxidation*

A one-pot chemo-enzymatic approach for Baeyer-Villiger reaction involves the oxidation of long-/medium-chain carboxylic acids or ethyl acetate with $H_2O_2$ to generate peracids. Then, peracids oxidize cyclic ketones to lactones. In this method, organic peracids are generated *in situ* hence one avoids direct handling of those hazardous (shock sensitive) materials[16].

To date, the former reactions have demonstrated that enzymes could serve as efficient catalysts in a batch system in various configurations: CALB (Novozyme-435),[16-21] CALB

immobilized as cross-linked enzyme aggregates (CLEA),[22] perhydrolase immobilized as CLEA,[23] CALB immobilized on multi-walled carbon nanotubes (MWCNTs),[24] CALB incorporated into mesoporous silica materials[25] or in the native form of acyltransferase and CALB[26] The stability of the above heterogeneous biocatalysts in the recycle tests was studied in our group only in a batch system. By this, we intended to illustrate the practical applications, using mesoporous silica materials[25] and MWCNTs[24] as CALB carriers. Additionally, we have applied ionic liquids in order to improve the stability of lipase.[27]

The aim of this paper is to investigate the chemo-enzymatic oxidation of a model ketone using a microreactor in the continuous flow mode. Lipase has been non-covalently immobilized on MWCNTs and used as the innovative, highly-active and stable nanobiocatalyst. The nanobiocatalysis has been indeed recently emerged as the critical innovation involving synergy of nanotechnology and biotechnology. By definition, nanobiocatalysis embraces amalgamation of enzyme molecules and nanocarriers toward significantly enhanced and desirable kinetics and selectivity, including stereoselectivity, for the substrates.[24]

To the best of our knowledge, this is the first attempt to apply flow system to perform Baeyer-Villiger oxidation of ketones to lactones. It is worth to emphasize that the reaction system is complex and characterized by many issues to overcome. One of them is biphasic system and the application of aqueous solution of hydrogen peroxide which can deactivate the enzyme. The hereby proposed environmentally benign, chemo-enzymatic process avoids

the direct use of unstable peracid which is generated *in situ* during the enzymatic stage in the reaction system of the proposed method. To determine the most promising recycling strategy, catalytic reactions were carried out with lipase immobilized on MWCNTs. The tests performed using the batch system have produced a recyclable catalytic system. Upgrading to the flow system resulted in the enhanced activity. This advantage constitutes the basis for the future design of continuous flow fixed-bed reactors for industrial applications. Importantly, lactones belong to the group of key substances in the *fine chemical* sector[26] applicable in pharmaceutical, food, cosmetic, perfume and polymer industry. The *fine chemical* market is projected to grow at a CAGR of 5.76%, and to reach $201.57 billion by 2023.[28] And here, by developing the chemo-enzymatic continuous process we demonstrate that production of those compounds *via* alternative, green technology using the flow reactor is conveniently scalable.

## EXPERIMENTAL

### *Materials and methods*

Ketones, *n*-octanoic acid, potassium phosphate buffer (pH=7), Novozyme-435 and an aqueous solution of *Candida antarctica* lipase B were purchased from Sigma-Aldrich. Nanocyl NC7000 MWCNTs were purchased from Nanocyl (Belgium). Industrial grade CheapTubes MWCNTs are available from Cheap Tubes Inc. (United States of America). Toluene, ethyl

acetate, dichloromethane and chloroform were obtained from Chempur and were used without additional purification.

NMR spectra of the products were recorded using a Varian 500 spectrometer at the following operating frequencies: $^{1}H$ 600 MHz and $^{13}C$ 150 MHz. Chemical shifts are reported as parts per million (ppm) in reference to tetramethylsilane (TMS) for 0.020 g of the sample.

Gas Chromatography analyses were performed using SHIMADZU 2010 chromatograph equipped with SPB (30 m × 0.25 mm × 0.25 mm) column. The parameters of analysis were set as follows: injection temperature 250 °C, a FID detector temperature of 280 °C, the initial temperature of 40 °C (hold for 2 min) with an increasing temperature rate of 20 °C/min and the final temperature of 280 °C (hold for 10 min), with a linear flow set as 30 mL/min, a split ratio of 25:1 and a run time of 24 min. The qualitative analyses were performed using Agilent Technologies 7890C connected to a mass spectrometer Agilent Technologies 5975C detector. The GC-MS chromatograph was equipped with an HP 5MS column (30 m × 0.25 mm × 0.25 μm film), and electron impact (EI) ionization at 70 eV was used. (for retention times see SI, Table S1).

Lipase loadings on the surface of the carbon materials were determined by thermogravimetric analysis (TGA) using a Mettler Toledo TGA851e thermobalance. A sample of approximately 0.010 g was heated from 25 °C to 800 °C at a rate of 10 °C/min in standard 70 μL $Al_2O_3$ crucibles under a dynamic nitrogen flow of 60 mL/min.

Nitrogen adsorption/desorption isotherms for MWCNTs were obtained using a Micrometrics ASAP 2420M instrument at –196 °C to calculate their specific surface area ($S_{BET}$) and pore volume. The size of the pores was obtained using the Barrett–Joyner–Halenda (BJH) method with the Kruk–Jaroniec–Sayari correction. Prior to the experiments, the carbon nanocarrier samples were outgassed at 200 °C and $1.33\times10^{-3}$ Pa for 5 h.

## *Synthetic procedures*

*Synthesis of pristine MWCNTs:* Pristine MWCNTs were synthesized *via* catalytic chemical vapor deposition (c-CVD) using a slightly modified protocol from the literature.[29]

*Immobilization of Candida antarctica lipase B on nanocarbon solid supports:* The immobilization step was carried out according to a literature procedure.[30] Into a 100 mL round bottom flask, a solution of CALB (4 mL), the adequate nanocarbon solid support (1.000 g), and a potassium phosphate buffer at pH = 7 (30 mL) were added. The immobilization was carried out for 3 h at 20 °C in a thermostatic shaker (180 rpm). After this time, the mixture was filtered under vacuum and the nanobiocatalyst was washed with 200 mL of potassium phosphate buffer at pH = 7. Then, the nanobiocatalyst was dried over anhydrous $P_2O_5$ for 3 days in a desiccator at 5 °C.

*Batch Baeyer-Villiger process:* Into a 25 mL round-bottom flask, biocatalyst (0.080 g / 1 mmol of ketone), 2-methylcyclohexanone (0.25 mmol, 0.028 g), *n*-octanoic acid (0.5 ml), *n*-

decane (an internal standard, 20% wt. of ketone) and solvent (0.5 mL) were added. Next, 30% aqueous solution of hydrogen peroxide (0.50 mmol, 0.057 g) was added dropwise at 40 °C. The reaction mixture was stirred in a thermostatic shaker (40 °C, 250 rpm) for 6 h, and samples were taken for GC-FID analysis (10 μL of sample diluted in 0.5 mL of dichloromethane). After completion of the reaction, the nanobiocatalyst was filtered off and solvent (10 mL) and water were added (5 mL). The organic phase was washed using a saturated aq. solution of sodium bicarbonate (3×5 mL). Next, the collected water phases were extracted using methylene chloride (3×5 mL) and the organic phase was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure (8 mbar, 25 °C, 1 h). The residue was purified *via* column chromatography using $SiO_2$ as the stationary phase and *n*-hexane:ethyl acetate 8:2 v/v as the eluent yielding 90% of the lactone.

*Flow Baeyer-Villiger process:* To carry out the Baeyer-Villiger reaction, a Vapourtec R2 series two-module flow reactor with two pumps was used. The apparatus was equipped with a back-pressure regulator located in between the reactor and the collection tube, and was set to 8 bar. Pump A pumped an organic phase (ketone concentration: 4.50 mmol (0.504 g)/10 mL ethyl acetate). Pump B pumped 30 wt.% aqueous solution of hydrogen peroxide (20-80 molar excess). The total flow was set to 0.040-0.133 mL/min. The process was carried out for 2-24 h at 25-55 °C using various residence times (12-38 min) and the constant amount of (nano)biocatalyst (0.5 g). During the process the samples were taken for GC-FID analysis (100 μL of sample diluted in 0.5 mL of dichloromethane).

For the synthesis of 6-methyl-ε-caprolactone, a dedicated isolation method was developed. The post-reaction mixture – after the oxidation of 2-metylocyclohexanone – was collected (25 mL), and then water was added thereto (25 mL). The organic phase was washed using a saturated aq. solution of sodium bicarbonate (3×25 mL). Next, the collected aqueous phases were extracted using methylene chloride (3×25 mL), the organic phase was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure (8 mbar, 25 °C, 1 h). The residue was purified *via* column chromatography using $SiO_2$ as the stationary phase and *n*-hexane:ethyl acetate 8:2 v/v as the eluent, yielding 90% of the lactone.

*6-methyl-ε-caprolactone:* $^{1}H$ NMR (600 MHz, $CDCl_3$, TMS): δ/ppm 1.36 (d, 3H, J=5.9 Hz), 1.58-1.71 (m, 4H), 1.88-1.93 (m, 2H), 2.59-2.70 (m, 2H), 4.44-4.63 (m, 1H). $^{13}C$ NMR (150 MHz, $CDCl_3$): δ/ppm 22.58, 22.89, 28.28, 35.01, 36.22, 68.38, 175.57.

*6-methyl-ε-caprolactone:* GC-MS (EI) m/z (%): 128 (1), 113 (1), 84 (61), 67 (10), 55 (100), 47 (43), 21 (14).

*ε-caprolactone:* GC-MS (EI) m/z (%): 114 (22), 84 (28), 70 (20), 67 (5), 55 (92), 43 (100), 28 (28).

*γ-valerolactone:* GC-MS (EI) m/z (%): 100 (30), 81 (1), 70 (17), 56 (54), 42 (100), 28 (42), 15 (3).

*4-tert-butyl-ε-caprolactone:* GC-MS (EI) m/z (%): 171 (4), 155 (4), 137 (5), 114 (56), 95 (8), 86 (54), 68 (18), 57 (100), 41 (48), 29 (24).

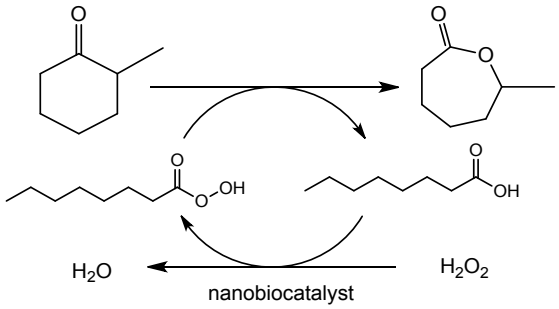
*4-phenyl-ε-caprolactone:* GC-MS (EI) m/z (%): 190 (90), 162 (20), 148 (99), 117 (100), 106 (95), 91 (53), 77 (30), 63 (18), 51 (22), 45 (19) 39 (24), 24 (12).

*4-oxatricyclo[4.3.1.13.8]undecan-5-one:* GC-MS (EI) m/z (%): 166 (2), 122 (5), 107 (18), 93 (18), 80 (100), 67 (16), 53 (28), 41 (17).

## RESULTS AND DISCUSSION

MWCNTs have been described as versatile supports for enzyme immobilization due to their small size, large surface area, mechanical/thermal stability and other unique properties. These characteristics enable, among others, higher enzyme loadings and, more importantly, increased enzyme stability in the presence of hydrogen peroxide without the enzyme leakage.[31-36] Both non-covalent and covalent methods to immobilize various enzymes on MWCNTs have been reported.[37] The simple, direct physical adsorption method is based on hydrophobic, van der Waals and hydrogen bonding interactions between MWCNTs and the enzyme macromolecules.[38]

To demonstrate the catalytic potential of non-covalently immobilized lipase on MWCNTs in a flow reactor, chemo-enzymatic Baeyer-Villiger oxidation of 2-methylcyclohexanone was carried out as the model reaction (Scheme 1).




**Scheme 1.** Chemo-enzymatic Baeyer-Villiger oxidation of 2-methylcyclohexanone.

### *Chemo-enzymatic Baeyer-Villiger oxidation of 2-methylcyclohexanone in the batch system*

The activity of CALB non-covalently immobilized on MWCNTs of various morphologies and surface chemistry has been previously studied in the chemo-enzymatic oxidation of cyclic ketones to lactones in a batch reactor at 20 °C.[24] The most promising results were achieved, when unmodified, pristine MWCNTs – synthesized according to the literature[29] – were used as the CALB support.

In order to apply this nanobiocatalyst in a continuous flow chemo-enzymatic Baeyer-Villiger process, which in fact can be dedicated for the industry, it was necessary to choose commercially available support to increase stability of the enzyme. Therefore, two inexpensive, unmodified, commercially available supports of CALB: MWCNTs from Nanocyl (100 EUR/1 kg) and MWCNTs from CheapTubes (100 USD/1 kg) were tested under pre-optimized reaction conditions at 40 °C. The results were compared with pristine (in-house) MWCNTs as a CALB support synthesized *via* catalytic chemical vapor deposition (c-CVD) in our laboratories as well as with the native form of CALB and Novozyme-435.

CALB was immobilized on carbon nanomaterials using a physical adsorption technique in accordance with the procedure described in the literature.[24] All carbon nanomaterials were characterized (Table 1) and the amount of immobilized CALB was determined using TGA.

Based on previous studies,[24,38] the model reaction system of the chemo-enzymatic oxidation of 2-methylcyclohexanone in toluene consisted of: (1) heterogeneous nanobiocatalyst to form the peracid, (b) 30 wt.% aq. $H_2O_2$ as the primary oxidant, and (3) *n*-octanoic acid as the peracid precursor. The conversion of ketone after 3 and 4 h was measured by GC/FID and compared with the model reaction carried out in presence of CALB-MWCNT nanobiocatalyst (Table 2).

**Table 1.** Characterization of carbon nanotube-based supports and CALB loadings.

| Support | $S_{BET,}$ $m^2/g$ | Pore volume, $cm^3/g$ | Length, μm | Outer diameter, nm | Inner diameter, nm | CALB loading, wt.% |
|---|---|---|---|---|---|---|
| Nanocyl NC7000 | 253 | 1.06 | 1.5 | 9.5 | 7 | 19.8 |
| CheapTubes MWCNTs | 89 | 0.49 | 10-30 | 20-40 | 5-10 | 15.7 |
| In-house MWCNTs | 34 | 0.11 | 130-370 | 35-85 | 15-40 | 6.0 |

**Table 2.** The influence of the carbon-based support on the biocatalyst activity in chemo-enzymatic Baeyer-Villiger oxidation curried out in the batch reactor.

| Biocatalyst | Time, h | α, % |
|---|---|---|
| CALB-MWCNTs | 3 | 98 |
| | 4 | 99 |
| CALB-Nanocyl NC7000 MWCNTs | 3 | 90 |

| | | |
|---|---|---|
| | 4 | 93 |
| CALB-CheapTubes MWCNTs | 3 | 86 |
| | 4 | 89 |
| Novozyme-435 | 3 | 87 |
| | 4 | 93 |
| CALB native | 3 | 68 |
| | 4 | 78 |

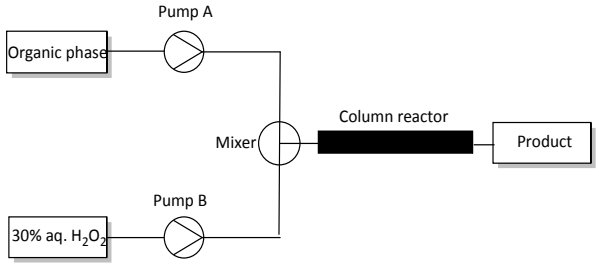
*Reaction conditions:* 40 °C; 250 rpm, 2-methylcyclohexanone (0.25 mmol, 0.029 g); (nano)biocatalyst 0.080 g/ 1 mmol of ketone; toluene 0.5 mL; *n*-octanoic acid 0.5 mL; 30% aq. $H_2O_2$ (0.50 mmol, 0.057 g), the conversion of ketone (α) was determined by GC with 95% selectivity toward the lactone. All experiments were performed in triplicate and the results for each of the three measurements differed by less than 1%.

The activity of nanocarbon-supported lipase was higher than the native one. Among the commercially available CALB supports, Nanocyl NC7000 MWCNTs emerged as the support the most capacious one. This support was characterized by the highest specific surface area and the lowest aspect ratio (length-to-diameter ratio) (Table 1). Hence, the highest amount of CALB was immobilized on the surface of Nanocyl 7000 MWCNTs and, at the same time, it was prone to catalyze the peracid formation. High conversion of the ketone 93% after 4 h was reached, which was only slightly lower than in the case of in-house MWCNTs. CALB anchored on the surface of CheapTubes MWCNTs was also comparatively active to the relatively expensive Novozym-435 (30,000 EUR/1 kg). The lowest activity demonstrated the native CALB (the reaction courses are presented in SI, Fig. S7) which was used for the reaction in the same amount as for immobilized on the support.

***Chemo-enzymatic Baeyer-Villiger oxidation of 2-methylcyclohexanone in the flow system***

Based on the most promising results from the batch reactor (including economy aspects), we selected [CALB Nanocyl NC7000 MWCNTs] system to generate peracid *in situ* and further oxidize ketones[24] in a series of continuous system experiments.

Vapourtec R2 (SI, Scheme S1) flow reactor equipped with two HPLC pumps (one pumping the organic phase: a mixture of *n*-octanoic acid with substrates and solvent and the second one pumping aqueous solution of $H_2O_2$). A column reactor with a 4 mL-working volume charged with 0.500 g of [CALB-Nanocyl NC7000 MWCNTs] was used (Scheme 2). The apparatus was equipped with a back-pressure regulator set to 8 bar.




**Scheme 2.** Diagram of the flow system used in this study. Organic phase: a mixture of *n*-octanoic acid with substrates and solvent.

The influence of residence time on the flow rate was calculated from the exact volume of the reactor which is the difference of the volume of the empty reactor and the volume of catalyst. In the case of the porous catalysts, it was necessary to consider the volume of the pores in the calculations. The application of a solvent with a known density was helpful here. To determine the volume, the reactor was densely packed with 0.500 g of CALB-Nanocyl NC7000 and weighted. Ethyl acetate was pumped through the reactor for 10 minutes to ensure evacuation of air from the catalyst pores. The amount of solvent was established with

the front of the catalytic bed and weighted once again. The exact volume of the reactor was determined to be 1.56 mL (SI).

*Influence of solvent on the conversion of the 2-methylcyclohexanone*

In the beginning, the possibility of using different solvents instead of toluene was investigated. To this aim, chloroform as solvent of low viscosity was also tested. In these two cases, *n*-octanoic acid as peracid precursor was used. A different approach was the use of ethyl acetate which can be used as solvent and the acid precursor at one.

The initial parameters of the reactor operation were determined. The total flow rate was maintained at 0.133 mL/min with a residence time of 12 min. The flow rate ratio of pump A to pump B was maintained at the level 1:3. Pump A pumped the organic phase (2-methylcyclohexanone and solvent (and when necessary *n*-octanoic acid), while a 30 wt.% aq. solution of hydrogen peroxide was introduced through pump B (Figure 1).

The investigations concerning application of various solvent/precursor systems in flow chemo-enzymatic oxidation of 2-methylcyclohexanone indicated the use of ethyl acetate as the most efficient solvent. In the experiment with ethyl acetate, the highest conversion of ketone (60% after 30 min) was observed with 100% selectivity to the corresponding lactone (in the batch reactor the lower selectivity 95% was caused by a partial hydrolysis of lactone to the appropriate hydroxyacid). At the same time, the conversion of ketone in the system with toluene/*n*-octanoic acid was only 39%.

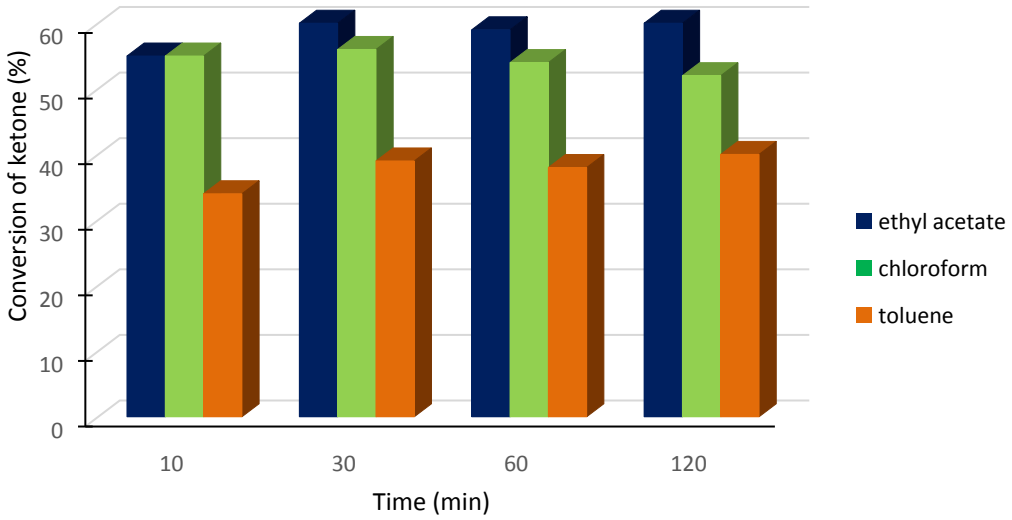



**Figure 1.** The influence of the solvent type on the conversion of ketone in chemo-enzymatic Baeyer-Villiger oxidation carried out in the flow reactor. All experiments were performed in triplicate and the results for each of the three measurements differed by less than 1%.

*Reaction conditions:* 40 °C; 2-methylcyclohexanone (0.05 mol/100 mL, 5.040 g/100 mL); CALB-Nanocyl™ NC7000 MWCNTs (0.500 g); solvent (*n*-octanoic acid was applied as peracid precursor when toluene and chloroform were used as solvents; ethyl acetate was applied as both: solvent and peracid precursor, 2.00 mol/1 mol of ketone, i.e. 288 g/1 mol of ketone); 30% aq. $H_2O_2$ (0.91 mol/100 mL), flow rate ratio: organic phase:oxidant 1:3; total flow 0.133 mL/min; residence time 12 min.; the conversion of ketone (α) was determined by GC with 100% selectivity to the lactone. Reaction time = sampling time after reaching the residence time.

This approach allowed this process to be performed in a non-chlorinated solvent without any additional peracid precursor. Moreover, ethyl acetate can be called environmentally 'preferred solvent' due to non-toxicity and safety reasons as well as the low price.[39] Additionally, the application of ethyl acetate resulted in a lower total pressure in the reactor during the process. Those characteristics limited the potentially detrimental effect of the reaction system on the nanobiocatalyst. Importantly, a use of chloroform is not advisable from the point of view of *green chemistry* and scaling-up. Nevertheless, further improvements in the overall performance of flow system were necessary as the 60% yield of lactone was not satisfying.

***Influence of the residence time on the conversion of the 2-methylcyclohexanone***

The residence time is an important parameter in the continuous processes as it corresponds to the time at which substances are in contact in the reactor. It describes the number of molecules leaving the reactor per unit of time and the contact time of the reagents with the nanobiocatalyst which, in turn, affect the conversion and stability of the enzyme. Hence, the next step was to investigate the influence of the residence time on the substrate conversion (Table 3).

As shown in Table 3, the optimal residence time for this process was found to be 20 min, since this is the time where the highest conversion of 2-methylcyclohexanone as well as the highest stability of nanobiocatalyst were achieved. Under these conditions, 87% conversion of the ketone was obtained after only 5 min, and this conversion was maintained

approximately for 2 h. In contrast, nearly twice lower conversions were observed in the other cases. Moreover, a drop in the conversion of ketone was obtained after only 2 h in both processes which were carried out at the longer residence times. The reason for these phenomena is likely the generation of ethanol and acidic acid *via* ethyl acetate hydrolysis, which are detrimental to the activated enzyme structure and hence its performance. Additionally, prolonged contact of the nanobiocatalyst with the oxidant and the non-polar, hydrolysable solvent, such as ethyl acetate, are other possible reasons for the observed decrease in the enzyme activity.

**Table 3.** The influence of the total flow rate on the conversion of ketone in chemo-enzymatic Baeyer-Villiger oxidation carried out in the flow reactor.

| Time, min | α, % | Flow rate | | | Residence time, min |
|---|---|---|---|---|---|
| | | Pump A, mL/ min | Pump B, mL/ min | Total, mL/ min, (flow rate ratio) | |
| 10 | 55 | 0.033 | 0.100 | 0.133 (1:3) | 12 |
| 30 | 60 | | | | |
| 60 | 59 | | | | |
| 120 | 60 | | | | |
| 5 | 87 | 0.019 | 0.057 | 0.076 (1:3) | 20 |
| 15 | 87 | | | | |
| 60 | 90 | | | | |
| 120 | 93 | | | | |
| 5 | 37 | 0.013 | 0.039 | 0.052 (1:3) | 30 |
| 15 | 76 | | | | |
| 60 | 93 | | | | |
| 120 | 76 | | | | |
| 5 | 27 | 0.010 | 0.030 | 0.040 (1:3) | 38 |

| | |
|---|---|
| 15 | 45 |
| 60 | 84 |
| 120 | 78 |

*Reaction conditions:* 40 °C; 2-methylcyclohexanone (0.05 mol/100 mL, 5.040 g/100 mL); CALB-Nanocyl NC7000 MWCNTs (0.500 g); ethyl acetate as solvent; 30% aq. $H_2O_2$ (0.91 mol/100 mL), the conversion of ketone (α) was determined by GC with 100% selectivity to the lactone. All experiments were performed in triplicate and the results for each of the three measurements differed by less than 1 %. Reaction time = sampling time after reaching the residence time.

*Influence of the flow rate ratio on the conversion of the 2-methylcyclohexanone*

In the next step, the influence of the flow ratio on the conversion of ketone was investigated. The total flow rate was maintained at 0.075-0.076 mL/min with a residence time of 20 min. The influence of the flow rate ratio of pump A to pump B within the range of 1:1 to 1:4 was investigated. Pump A pumped the organic phase (2-methylcyclohexanone and ethyl acetate), while a 30 wt.% aq. solution of hydrogen peroxide was introduced through pump B (Table 4).

**Table 4.** The influence of the flow rate *ratio* on the conversion of ketone in chemo-enzymatic Baeyer-Villiger oxidation carried out in the flow reactor.

| Time, min | α, % | Flow rate | | |
|---|---|---|---|---|
| | | Pump A, mL/min | Pump B, mL/min | Flow rate ratio |
| 5 | 29 | 0.038 | 0.038 | 1:1 |
| 15 | 60 | | | |

| | | | | |
|---|---|---|---|---|
| 60 | 40 | | | |
| 120 | 36 | | | |
| 5 | 35 | 0.025 | 0.050 | 1:2 |
| 15 | 38 | | | |
| 60 | 48 | | | |
| 120 | 40 | | | |
| 5 | 87 | 0.019 | 0.057 | 1:3 |
| 15 | 87 | | | |
| 60 | 90 | | | |
| 120 | 93 | | | |
| 5 | 43 | 0.015 | 0.060 | 1:4 |
| 15 | 45 | | | |
| 60 | 50 | | | |
| 120 | 57 | | | |

*Reaction conditions:* 40 °C; 2-methylcyclohexanone (0.05 mol/100 mL, 5.040 g/100 mL); CALB-Nanocyl NC7000 MWCNTs (0.500 g); ethyl acetate as solvent; 30% aq. $H_2O_2$ (0.91 mol/100 mL); total flow 0.075-0.076 mL/min; residence time 20 min; the conversion of ketone ($\alpha$) was determined by GC with 100% selectivity to the lactone. All experiments were performed in triplicate and the results for each of the three measurements differed by less than 1 %. Reaction time = sampling time after reaching the residence time.

The highest conversion of the model ketone was achieved when the flow rate ratio of the organic to the aqueous phase of 1:3 was used. Below this value, a twice lower conversion of the substrate (at the comparable reaction time) was obtained due to an insufficient amount of the oxidant to generate the necessary concentration of the peracid. On the other hand, a higher amount of oxidant led to deactivation of the enzyme causing a lower conversion of the ketone.

*Influence of temperature on the conversion of 2-methylcyclohexanone*

In order to determine the influence of temperature on the continuous flow chemo-enzymatic Baeyer-Villiger oxidation of 2-methylcyclohexanone, a series of experiments at three various temperatures: 25, 40 and 55 °C were performed. CALB is sensitive to higher temperatures, especially in the presence of hydrogen peroxide. Unfortunately, proceeding at lower temperature slowed down the reaction and, even if the residence time was relatively long (20 min), the conversion of ketone reached only 20%. The increase of temperature to 55 °C have accelerated the reaction (with almost a full conversion of ketone after 15 min) but, on the other hand, the conversion decrease was visible after 1 h, probably caused by a partial deactivation of lipase.

*Influence of the primary oxidant concentration on the conversion of 2-methylcyclohexanone*

Another crucial factor influencing the nanobiocatalyst stability is concentration of hydrogen peroxide in the aqueous phase. In the preliminary studies in the batch system,[24] the possibility of application in chemo-enzymatic continuous process urea hydrogen peroxide UHP – anhydrous form of hydrogen peroxide – was eliminated due to the solid state of this complex and practical insolubility in the reaction system. For comparison, the experiment involving application of 30% aq. $H_2O_2$ as the primary oxidant was performed (Figure 3).

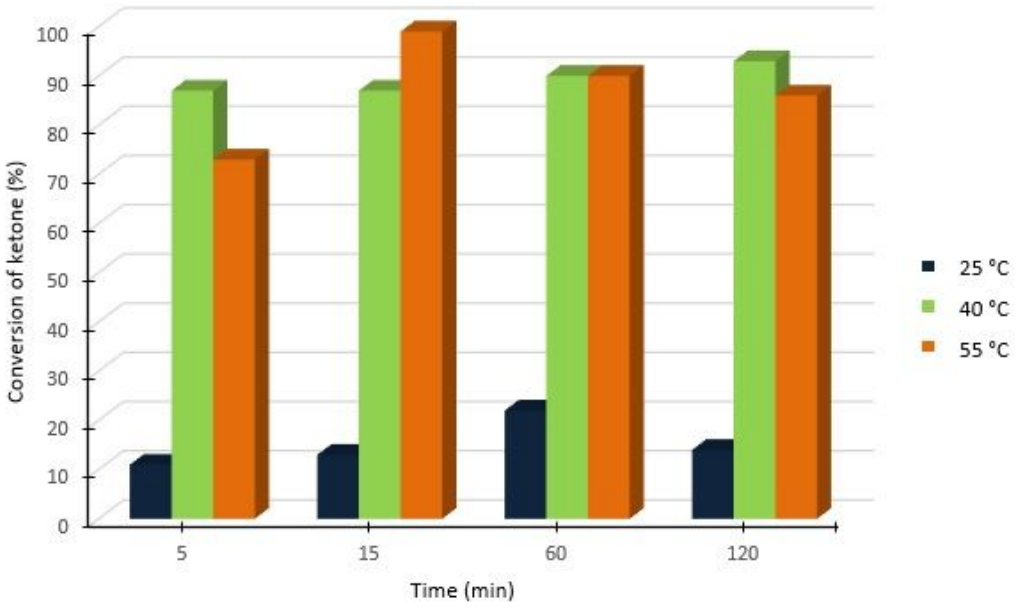



**Figure 2.** The influence of the temperature on the conversion of ketone in chemo-enzymatic Baeyer-Villiger oxidation carried out in the flow reactor. All experiments were performed in triplicate and the results for each of the three measurements differed by less than 1%.

*Reaction conditions:* 2-methylcyclohexanone (0.05 mol/100 mL, 5.040 g/100 mL); CALB-Nanocyl NC7000 MWCNTs (0.500 g); ethyl acetate as solvent; 30% aq. $H_2O_2$ (0.91 mol/100 mL), flow rate ratio organic phase:oxidant 1:3; total flow 0.076 mL/min; residence time 20 min; the conversion of ketone ($\alpha$) was determined by GC with 100% selectivity to the lactone. Reaction time = sampling time after reaching the residence time.

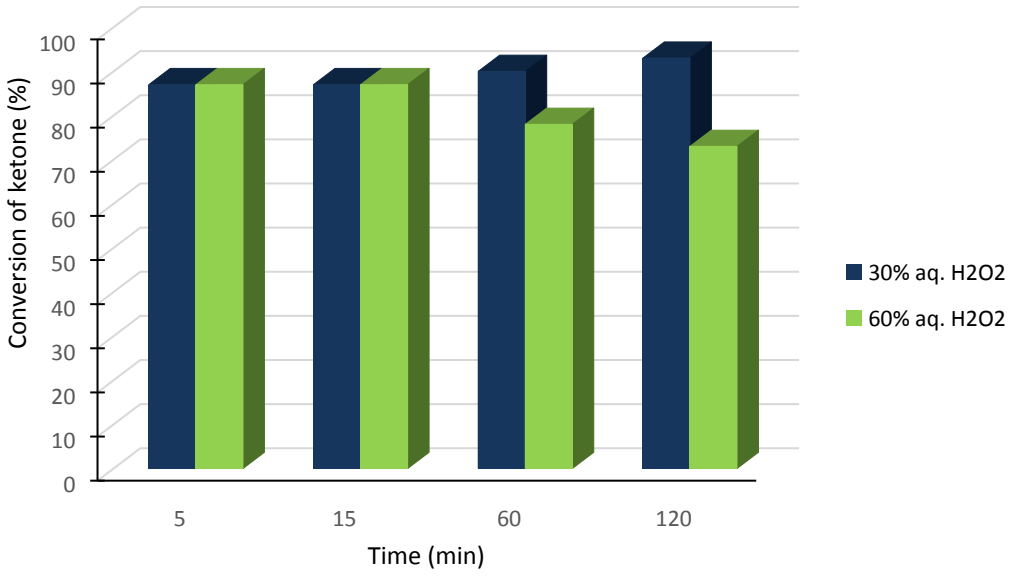



**Figure 3.** The influence of the $H_2O_2$ concentration on the conversion of ketone in chemo-enzymatic Baeyer-Villiger oxidation carried out in the flow reactor. all experiments were performed in triplicate and the results for each of the three measurements differed by less than 1 %.

*Reaction conditions:* 40 °C; 2-methylcyclohexanone (0.05 mol/100 mL, 5.040 g/100 mL); CALB-Nanocyl NC7000 MWCNTs (0.500 g); ethyl acetate as solvent; flow rate ratio organic phase:oxidant 1:3; total flow 0.075 mL/min; residence time 20 min; the conversion of ketone (α) was determined by GC with 100% selectivity to the lactone. Reaction time = sampling time after reaching the residence time.

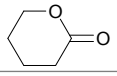
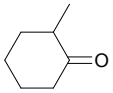
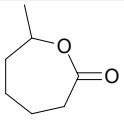
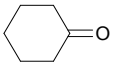
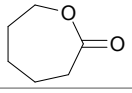
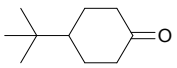
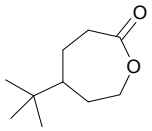
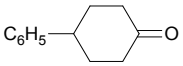
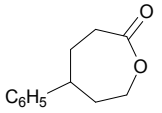
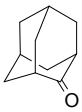
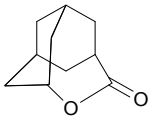
As expected, higher concentration of hydrogen peroxide proved a negative effect on the lipase lifetime. Although, in the beginning, both processes were carried out with the same

reaction rate (conversion of ketone 87% after 5 and 15 min), after 1 h a significant drop in the conversion of ketone was observed while using 60% aq. $H_2O_2$.

### *Substrate scope*

In order to broaden the scope of possible substrates and hence to confirm the practical potential of the proposed approach, a selection of various substrates was subjected to the reaction under so-optimized conditions (Table 5). The experiments were carried out for seven various ketones: 2-methylcyclohexanone, cyclohexanone, 4-phenylcyclohexanone, 4-*tert*-butylcyclohexanone, cyclopentanone and 2-adamantanone.

**Table 5.** The influence of the type of the substrate on the conversion of ketone in chemo-enzymatic Baeyer-Villiger oxidation carried out in the flow reactor.

| Ketone | Lactone | α, % |
|---|---|---|
|  |  | *85* |
|  |  | 90 |
|  |  | *83* |
|  |  | *97* |
|  |  | *95* |
|  |  | *99* |

*Reaction conditions:* 40 °C; ketone (0.05 mol/100 mL); CALB-Nanocyl NC7000 MWCNTs (0.500 g); ethyl acetate as solvent; 30% aq. $H_2O_2$ (0.91 mol/100 mL); total flow rate 0.076 mL/min; flow rate *ratio* organic phase:oxidant 1:3; residence time 20 min; reaction time 60 min, the conversion of ketones (α) was determined by GC with 100% selectivity to the lactone. All experiments were performed in triplicate and the results for each of the three measurements differed by less than 1 %. Reaction time: time of sample collection after crossing of residence time.

All ketones were obtained in high to excellent yields (83-99%) and excellent selectivities (100%). We have therefore clearly demonstrated that the developed method is comprehensive and competitive to other non-enzymatic methods of oxidation.

### *Stability of the (nano)biocatalysts in the flow system*

Finally, the studies concerning the stability of nanobiocatalyst in the long-term experiments were performed. Two (nano)biocatalysts were taken into consideration: the one proposed in this work, i.e. immobilized *via* simple, one-step physical adsorption – [CALB-Nanocyl NC7000 MWCNTs] nanobiocatalyst and commercially available Novozyme-435 for comparison (Table 6).

**Table 6.** The stability of the CALB-Nanocyl NC7000 MWCNTs in comparison with Novozyme-435 in chemo-enzymatic Baeyer-Villiger oxidation carried out in the flow reactor.

| (nano)biocatalyst | Time, min | α, % | Flow rate | | |
|---|---|---|---|---|---|
| | | | Pump A, mL/ min | Pump B, mL/ min | Total, mL/ min, (flow rate ratio) |
| Novozyme-435 | 5 | 82 | 0.016 | 0.048 | 0.064 (1:3) |
| | 15 | 96 | | | |
| | 60 | 99 | | | |
| | 120 | 95 | | | |
| | 240 | 90 | | | |
| | 360 | 74 | | | |
| | 480 | 68 | | | |
| | 1440 | 0 | | | |
| CALB-Nanocyl$^{T}$ NC7000 MWCNTs | 5 | 87 | 0.019 | 0.057 | 0.076 (1:3) |
| | 15 | 87 | | | |
| | 60 | 90 | | | |
| | 120 | 93 | | | |
| | 240 | 95 | | | |
| | 360 | 88 | | | |
| | 480 | 87 | | | |
| | 1440 | 16 | | | |

*Reaction conditions:* 40 °C; 2-methylcyclohexanone (0.05 mol/100 mL, 5.040 g/100 mL); CALB-Nanocyl NC7000 MWCNTs or Novozyme-435 (0.500 g); ethyl acetate as solvent; 30% aq. $H_2O_2$ (0.91 mol/100 mL); flow rate ratio organic phase:oxidant 1:3; residence time 20 min; the conversion of ketone (α) was determined by GC with 100% selectivity to the lactone. All experiments were performed in triplicate and the results for each of the three measurements differed by less than 1 %. Reaction time = sampling time after reaching the residence time.

Due to different morphology, specific surface, grain size and other properties, it was necessary to determine the exact volume of the reactor (SI) filled with 0.500 g of Novozym-435. Here,

this value was found equal to 1.25 mL which caused the necessity of the reduction of total flow rate in order to carry out both processes with the comparable residence time.

CALB-Nanocyl NC7000 MWCNTs nanobiocatalyst emerged as more stable than Novozym-435 in the elaborated process of continuous chemo-enzymatic oxidation of 2-methylcyclohexanone to 6-methyl-$\varepsilon$-caprolactone. This approach enabled us to carry out the process with >90% conversion of the ketone after 8 h (480 min). Conversely, a significant drop in the conversion of ketone after 6 h (360 min) was observed in the process with Novozym-435. Moreover, this biocatalyst was completely inactive after 24 h of oxidation in the contrast with 16%-conversion of ketone in the process with [CALB-Nanocyl NC7000 MWCNTs] after identical reaction time. Additionally, the bed of Novozym-435 swollen during the process and the flow resistance of the catalyst bed was higher what caused a dangerous increase of the pressure in the reactor. Another important factor is the possibility to reuse Nanocyl NC7000 MWCNTs after deactivation *via* combustion (burning off) the protein in the furnace (1.5 h, 350 °C, air). This method is simply inaccessible for Novozym-435 and other typical polymer carriers.

Summarizing, albeit a notable decrease in the conversion of ketone was observed after 24 h, a significant improvement in its performance in chemo-enzymatic Baeyer-Villiger oxidation has been achieved.

## CONCLUSIONS

The application of highly efficient flow system in the chemo-enzymatic Baeyer-Villiger oxidation has been demonstrated by comparison with the batch processes. This approach has guaranteed high yield of the product per the reactor capacity and eliminated the need to handle unstable and extremely dangerous peracids. The highly active and stable nanobiocatalyst consisting of *Candida antarctica* lipase B immobilized *via* physical adsorption onto commercially available, unmodified MWCNTs (Nanocyl NC7000) has been demonstrated for the first time using flow chemistry. Moreover, 30% aq. hydrogen peroxide has been successfully applied as the green primary oxidant in the oxidation of 2-methylcyclohexanone to 6-methyl-*ε*-caprolactone. Mild reaction conditions (40 °C) were used to obtain high conversions of substrates (87%) in short reaction times (5 min), while the nanobiocatalyst was stable even after 8 h of performing the process in ethyl acetate.

In summary, this work undeniably represents an extremely efficient method of chemo-enzymatic oxidation toward lactones. A use of superactive and extra-stable nanobiocatalyst in the flow system has been shown as a versatile and scalable method for the synthesis of compounds from the sector of *fine chemicals*.

## ASSOCIATED CONTENT

Supporting Information. TGA curves of nanobiocatalysts; $^{1}$H NMR and $^{13}$C NMR spectra of 6-methyl-ε-caprolactone; GC/MS spectra of lactones; the procedure for determination of the

exact volume of the reactor are attached. This material is available free of charge *via* the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

*Corresponding Author*

* e-mail: Anna.Chrobok@polsl.pl, tel. +48 322372917, fax: +48 322371032.

*Author Contributions*

All authors have given approval to the final version of the manuscript.

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