

Article

Spectrally Resolved Cell Imaging for Enhanced Production of ϵ -Caprolactone via an Enzyme Cascade Reaction in *E. coli* Immobilized Within Barium–Calcium Alginate Beads Using JetCutter

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Abstract

Jet-cutting—the most powerful immobilization technique—was utilized for the entrapment of recombinant *E. coli* cells expressing a cascade of enzymes, including alcohol dehydrogenase, enoate reductase, and cyclohexanone monooxygenase, within mechanically reinforced barium–calcium alginate beads. Cost-effective alginate beads with entrapped cells were applied in a model process for the production of the industrially relevant ϵ -caprolactone under bioreactor-controlled conditions, enabling parallel repeated biotransformations. Immobilization resulted in a reduced rate of cell deactivation over four biotransformation cycles, leading to overall ϵ -caprolactone yield increases of 36% using 0.55 mm beads and 22% using 0.9 mm beads compared to the use of free cells. Additionally, the model bioprocess was employed to investigate the metabolic adaptation of cells to immobilization and repeated biotransformations using viability assays and spectrally resolved confocal microscopy. These measurements, conducted for the first time throughout the entire cellular life cycle, clearly demonstrated that the cells retained high viability during cultivation, immobilization, and repeated use in biotransformations. Moreover, based on characteristic spectral shifts, advanced analysis via spectrally resolved confocal microscopy revealed distinct mechanisms of metabolic adaptation in entrapped cells versus free cells during repeated cascade reactions in parallel bioreactors.

Keywords: immobilization; entrapment; barium–calcium alginate beads; whole-cell biocatalyst; enzyme cascade reaction; ϵ -caprolactone; cell viability; spectrally resolved confocal imaging



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1. Introduction

Baeyer–Villiger monooxygenases (BVMOs) represent a group of flavin-dependent enzymes that catalyze the oxidation of ketones into ester or lactone products by inserting an oxygen atom. These enzymes are significant in biocatalysis due to their ability to selec-

tively modify substrates, which is especially valuable in the synthesis of pharmaceutical intermediates and fine chemicals [1,2].

In recent years, BVMOs have been increasingly utilized in cascade reactions, either as isolated enzymes or in whole-cell systems, where they work in conjunction with other enzymes to achieve more efficient substrate conversion without the need to isolate intermediate products. Such cascade systems improve process efficiency and reduce costs, which is advantageous for industrial applications [3–5]. One example of such a cascade reaction involves alcohol dehydrogenase (ADH), enoate reductase (e.g., XenB), and cyclohexanone monooxygenase (CHMO) in recombinant *Escherichia coli*. In this system, ADH oxidizes cyclohexanol to cyclohexanone, generating NADPH in the process. XenB then reduces enoate bonds, and CHMO catalyzes the Baeyer–Villiger oxidation of cyclohexanone to ϵ -caprolactone. This cascade is beneficial because it utilizes the NADPH generated in the first step for the subsequent steps, reducing the need for external coenzyme supply (Figure 1) [6]. ϵ -caprolactone is a cyclic ester that serves as a monomer for the production of poly(ϵ -caprolactone), a biodegradable and biocompatible polymer used in tissue engineering and medicine. Industrially, ϵ -caprolactone is traditionally produced with chemical Baeyer–Villiger oxidation of cyclohexanone using peroxy acids, a method that can be less environmentally friendly and less selective than others. Biocatalytic approaches using BVMOs offer a greener alternative with higher selectivity and milder reaction conditions [7–9]. The general utility of cascade enzyme systems can be illustrated through their important industrial applications, including the synthesis of key intermediates of atorvastatin—one of the world’s best-selling cholesterol-lowering drugs [10]. Immobilization of recombinant cells, such as *E. coli* expressing the aforementioned enzymes, offers several advantages. Cell immobilization allows for biocatalyst reuse, cost reduction, and operational stability. Moreover, immobilization allows for better control of reaction conditions and facilitates product separation [11].

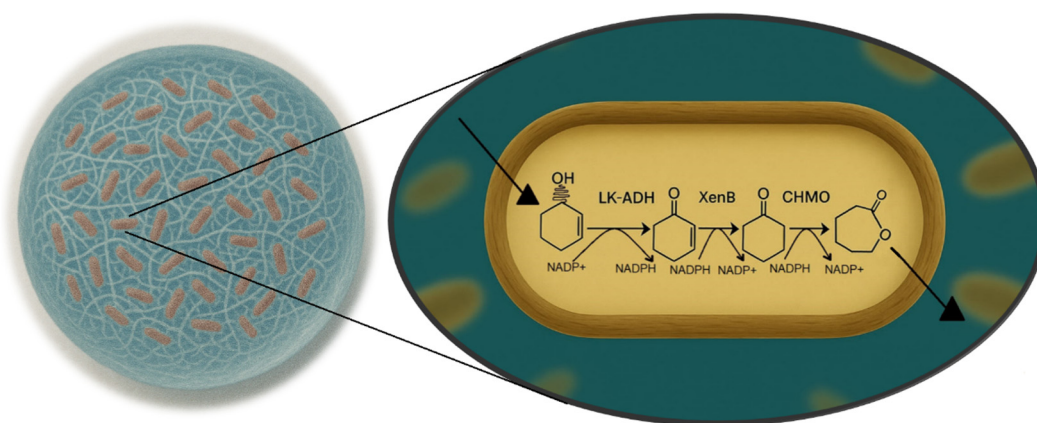


Figure 1. Schematic representation of an alginate bead (left) containing immobilized recombinant *E. coli* cells, which express three enzymes—namely, alcohol dehydrogenase (LK-ADH), enoate reductase (XenB), and cyclohexanone monooxygenase (CHMO)—to catalyze a model biocatalytic cascade reaction. The enlarged section (right) illustrates the model reaction from substrate 2-cyclohexenol, through intermediates 2-cyclohexenone and cyclohexanone, to product ϵ -caprolactone.

Dripping-based techniques constitute a well-established subset of the various methods available for cell immobilization. Among them, coaxial air flow technology, vibrating technology, electrostatic technology, and JetCutter technology operate by breaking a liquid stream into droplets, which then solidify in a gelling bath. These methods differ in droplet formation mechanisms, affecting bead size and uniformity, and also vary significantly in their rates of bead production. Coaxial air flow, vibrating, and electrostatic technolo-

gies typically produce 100–1000 beads per second, while the JetCutter can reach up to 10,000 beads per second [12]. Its high speed results from continuous mechanical cutting and also enables precise control of particle characteristics while ensuring monodispersity, making it an ideal tool for both laboratory and industrial applications [13]. Development in the field of immobilization with entrapment and encapsulation techniques using hydrogel materials is focused on expanding the material base as well as improving the properties of existing systems [14]. Attention is being paid to increasing the resistance and regulation of matrix permeability; for example, by gelling alginate with other cations and combinations thereof or gelling with various guluronate and manuronate subunits [15]. The utilization of high-performance immobilization techniques such as jet-cutting is considered desirable for mimicking industrial conditions [16].

The combination of enzymatic three-step cascades in viable whole-cell biocatalysts utilizing Baeyer–Villiger monooxygenase, along with their immobilization, remains a relevant concept for the intensification of biotransformations [17]. The proposed heterogeneous biocatalysis method combines the reduced carbon footprint of the cascade enzymatic reaction, achieved by minimizing the production of toxic wastes [6], with the low toxicity of immobilization polymers [11], which may also lead to environmental benefits. A high-performance method for immobilizing viable recombinant cells containing an enzymatic cascade using the jet break-up technique was successfully applied for the repeated model production of ϵ -caprolactone [11]. From this perspective, the use of low-cost, biocompatible immobilization materials such as barium–calcium alginate particles entrapping cells with enzymatic cascades, newly prepared using the high-performance jet-cutting technique and applied in bioreactor-controlled biotransformations, may serve as a suitable model for a biotechnological process. At the same time, the latter process is valuable for continuing the study of physiological changes in immobilized whole-cell biocatalysts [11], extended for each cycle during repeated biotransformations through a novel approach employing spectrally resolved confocal imaging techniques. Moreover, researchers have not yet investigated previous techniques for measuring the viability of free and immobilized cells [18], used throughout the entire life cycle of cells from cultivation, through immobilization, to gradual inactivation during repeated biotransformation cycles in bioreactors, as well as spectrally resolved confocal imaging during these same bioreactor cycles. The principal advantages of fluorescence spectroscopy methods include their non-invasive nature, enabling measurements without the need for labeling and thereby avoiding damage to cells in the samples; their capacity to spectrally discriminate between fluorophores in living cells, facilitating a more precise understanding of cellular metabolic states; and their high temporal and spatial resolution, which allows for detailed monitoring of the dynamics of metabolic processes [11,19].

The study of the abovementioned model biotechnological process thus formed the basis for defining the original objective of this work, which was to produce barium–calcium alginate beads using the high-performance JetCutter technique with immobilized recombinant *E. coli* cells capable of catalyzing a cascade reaction with three overexpressed enzymes—namely, alcohol dehydrogenase, enoate reductase, and cyclohexanone monooxygenase—ultimately producing the industrially important ϵ -caprolactone. The reaction course and operational stability of immobilized biocatalysts were subsequently examined in a stirred batch bioreactor simulating an industrial biotransformation process at the laboratory scale. The viability of immobilized cells during cultivation, immobilization, and repeated biotransformation cycles was measured. Advanced imaging—in particular, spectrally resolved confocal imaging—was performed to assess the physiology of immobilized cells during repeated cycles of cascade reactions. Additionally, the mechanical resistance of the immobilized biocatalysts was examined.

2. Results and Discussion

2.1. Preparation of Barium–Calcium Alginate Beads with Immobilized *E. coli* with Overproduction of ADH, XenB, and CHMO Using High-Performance JetCutter Encapsulator

The development of an immobilization protocol in the first stage included the examination of coaxial air flow technology, jet break-up, and JetCutter technology to study the effects of these immobilization methods on the viability of cells. The results, summarized in Section 2.4 indicated no significant impact of the immobilization technique on cell viability. From this essential verification result follows the interchangeable applicability of the immobilization techniques in terms of the highly preserved cell viability, as well as the suitability of the high-performance immobilization technique (i.e., jet-cutting) chosen for further experiments. Crucially, this work represents the first application of the JetCutter technique (Figure 2A) for the immobilization of recombinant *E. coli* BL21 (DE3) (pET28_CHMO_XenB and pET22b_LKADH) [6] within barium–calcium alginate beads (hereafter referred to as alginate beads). This approach enabled the formation of uniformly spherical beads in two different sizes: 0.5 ± 0.05 mm and 0.9 ± 0.1 mm in diameter (Figure 2(B1,C1)). A 2% alginate solution containing 10% recombinant cells (cell content is according to [11]), with a resulting viscosity of 6117 mPa·s, was used to form the beads. The beads were formed by dropping a sodium alginate solution with cells using an encapsulator into a stirred beaker with a gelling mixture of BaCl₂ and CaCl₂ in a molar ratio of 1:1. Utilizing a combination of Ca²⁺ and Ba²⁺ for alginate crosslinking enables targeting of different block sequences within the polymer, which synergistically enhances stability and leads to more chemical-resistant and firmer alginate beads. This approach results in gels with improved mechanical strength and appropriate permeability, which enhances their suitability for applications such as cell immobilization [15,20,21]. Preliminary experiments aimed at achieving maximum mechanical resistance demonstrated the highest mechanical strength of alginate particles when using the Ca²⁺/Ba²⁺ combination, as determined by measuring the force at 60% compression after exposure to Tris buffer ($F = 7.6 \text{ g} \pm 0.7 \text{ g}$), compared to Ca²⁺ alone ($F = 5.1 \text{ g} \pm 0.7 \text{ g}$) and Ba²⁺ alone ($F = 5.8 \text{ g} \pm 0.8 \text{ g}$). The biocatalytic productivity of cells in the compared particles was practically the same. Therefore, the mechanically strongest particles prepared with the Ca²⁺/Ba²⁺ mixture were selected as optimal.

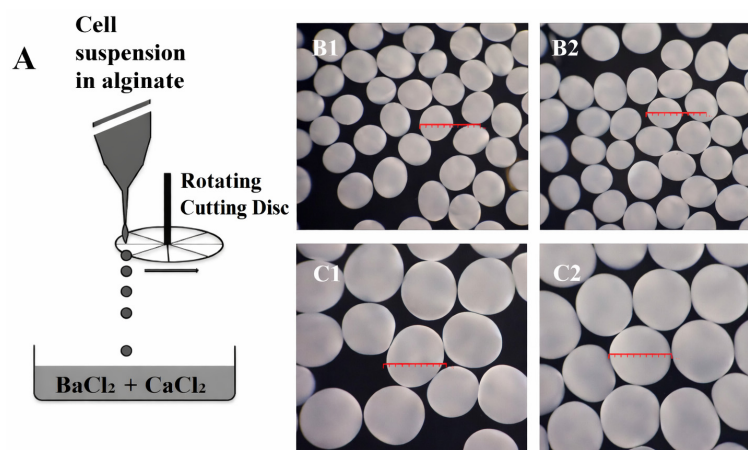


Figure 2. (A) The JetCutter technique, used for preparing alginate beads with immobilized recombinant *E. coli* (10% wt.) containing enzymes LK-ADH, XenB, and CHMO in cascade, utilizes a rotating cutting tool with wires to disrupt the polymer flow, leading to the formation of droplets through surface tension as they fall into the gelling solution. (B1) Alginate beads before the first working cycle with an average diameter of 0.55 ± 0.05 mm. (B2) Alginate beads after four working cycles with an average diameter of 0.6 mm. (C1) Alginate beads before the first working cycle with an average diameter of 0.9 ± 0.1 mm. (C2) Alginate beads after four working cycles with an average diameter of $1.0 \text{ mm} \pm 0.1$ mm. The length of the red scale corresponds to 1 mm.

2.2. Operational Stability of Immobilized *E. coli* with Overproduced ADH, XenB, and CHMO in Bioreactor Immobilized in Different-Sized Barium–Calcium Alginate Beads

The operational stability of immobilized recombinant *E. coli* cells overexpressing the enzymes LK-ADH, XenB, and CHMO, in comparison with the operational stability of free (non-immobilized) cells, was tested in three parallel controlled stirred batch bioreactors (3×0.5 L), which enabled tight control of all necessary parameters, including temperature, pH, oxygen saturation, and stirring frequency. In the preliminary step of operational stability testing in a bioreactor, immobilized cells with 3 h and 20 h induction times were compared in one reaction cycle. Although biocatalytic activity and viability (Section 2.4) remained unaffected, the 20 h induction resulted in approximately double the biomass. This enables one to adjust the cultivation time according to the whole bioprocess priority; namely, considering whether to save bioprocessing time using short cultivation (3 h) or attain the maximum productivity of active biomass (20 h). In addition, during other preliminary tests, it was found that washing the cells after each reaction cycle is not advisable, as it leads to the inactivation of the biocatalytic process. Therefore, the cells were only washed immediately before starting the next cycle, which preserved their biocatalytic activity for the subsequent biotransformation cycle. In the next step, the effect of an alginate bead size of 0.55 mm and 0.9 mm on the performance of immobilized cells was evaluated over four repeated cycles and compared to free cells. The latter comparison of different bead sizes was motivated by the assumption that bead size may influence the effectiveness of the mass transfer of substrates and nutrients to the cells, potentially affecting the reaction rate [22]. During four cycles of the biocatalytic cascade reaction converting 2-cyclohexanone to ϵ -caprolactone, a gradual decline in cell performance was observed, leading to complete inactivation by the fifth cycle in all samples (Figure 3A). The total caprolactone yield after four cycles in the bioreactor was 748 mg for the sample with immobilized cells in 0.55 mm beads, 611 mg for the sample with immobilized cells in 0.9 mm beads, and nearly 550 mg for the sample with free cells (Figure 3B). These results indicate that smaller beads may provide better access to a larger fraction of the immobilized biomass, which could lead to increased reaction efficiency [22]. Moreover, changes in cell activity during 4 biotransformation cycles (Figure S1A) as well as changes in space-time yield of the product (Figure S1B) are depicted in the Supplementary Materials.

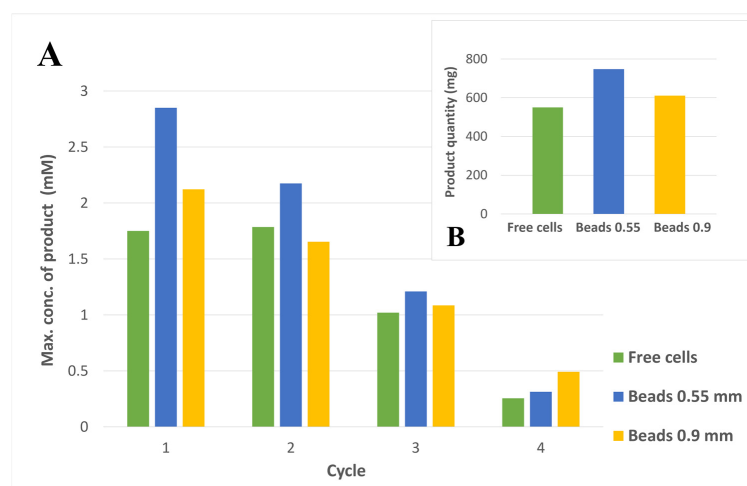


Figure 3. (A) The operational stability of viable *E. coli* cells expressing the enzyme cascade LK-ADH, XenB, and CHMO was evaluated in both immobilized (alginate beads) and free cells over four repeated cycles of a model cascade reaction converting 2-cyclohexen-1-ol to ϵ -caprolactone, represented by the maximum achieved product concentration. Biotransformations, initiated with a substrate concentration of 4 mM, were carried out in 3 parallel controlled bioreactors at 25 °C with a stirrer speed of 90 rpm and a flow of oxygen of 1.5 L/min in Tris-HCl buffer (pH = 7 controlled by automatic addition of 3M NaOH), supplemented with 1% glucose, ampicillin, and kanamycin. (B) Total ϵ -caprolactone yield over four cycles for individual samples.

2.3. Microscopy and Mechanical Stability of Alginate Beads

Microscopy was used to observe the size and shape of alginate beads containing immobilized recombinant cells after production and at the end of each biotransformation cycle. Microscopic observation revealed that the structure of the alginate beads remained intact; however, their size increased by approximately 10% after four cycles of the biocatalytic cascade reaction (Figure 2(C1,C2)). This could be due to the fact that alginate gels gelled with divalent cations, such as Ca^{2+} and Ba^{2+} , can absorb water in buffer solutions, leading to slight swelling and an increase in size, typically around 0.1 mm for beads initially of 0.9 mm, due to factors such as crosslinking ion type, pH, and ionic strength, which influence hydration behavior [23,24]. The mechanical stability is crucial for maintaining the integrity of beads containing biocatalysts during biotransformation, as well as for their recyclability and large-scale applications. Beads in a bioreactor are exposed to various chemical components of the reaction solution that may compromise their structure, along with mechanical stress, such as collisions with reactor components (stirrers), hydrodynamic shear forces, and inter-bead collisions. Texture analyzers are used to assess the mechanical properties of these beads, including their strength and elasticity, by applying controlled compression [25–27].

Measurements of the mechanical strength of alginate beads after each cycle showed that the mechanical strength of smaller 0.55 mm beads decreased, while the mechanical strength of larger 0.9 mm beads fluctuated slightly over the four consecutive cycles in the bioreactor (Table 1). Smaller alginate beads may be exposed to higher effective hydrodynamic and shear stresses during mixing due to their larger surface-to-volume ratio, which leads to a faster degradation of their mechanical strength. In contrast, larger beads remain partially inert to these latter fluctuations and therefore retain their strength.

Table 1. Mechanical strength of alginate beads with immobilized cells measured at 60% compression.

Sample	Force [g] Before	Force [g] After 1st Cycle	Force [g] After 2nd Cycle	Force [g] After 3rd Cycle	Force [g] After 4th Cycle
0.55 mm beads	3.5 ± 0.7	4.0 ± 0.9	4.0 ± 1.0	3.8 ± 0.5	2.9 ± 0.7
0.9 mm beads	10.0 ± 1.3	9.6 ± 1.7	10.5 ± 2.8	9.5 ± 2.6	10.0 ± 2.3

2.4. Viability of Recombinant Cells During Cultivation, Immobilization, and Repeated Cascade Reactions

To assess the cell viability, the intake of propidium iodide (PI, $\lambda_{\text{ex}} = 535 \text{ nm}$, $\lambda_{\text{em}} = 617 \text{ nm}$) into necrotic cells, with simultaneous recording of the autofluorescence (AF) signal as a reference indicator of living cells (Figure 4A,B), was measured. To evaluate the results in a way that will be least affected by possible user errors, segmentation according to Otsu's thresholding algorithm was performed for both channels (see Section 3 for more details), such that only the regions of the image with reasonable spatial features were selected according to the intensity distribution and contrast and identified as cells. Based on the assumption that cell size follows a continuous distribution with approx. $1 \mu\text{m}$ diameter $\times$ $3 \pm 1 \mu\text{m}$ length [28], the total cell viability was evaluated as a ratio of image areas corresponding to living cells vs. all cells (Figure 4D–F). The main reason for using image areas instead of automatic cell counting was that, under our conditions (especially after multiple biotransformation cycles), the autofluorescence from the cells was very low and the signal became noisy at the pixel level, rendering the cell count estimation algorithms unreliable.

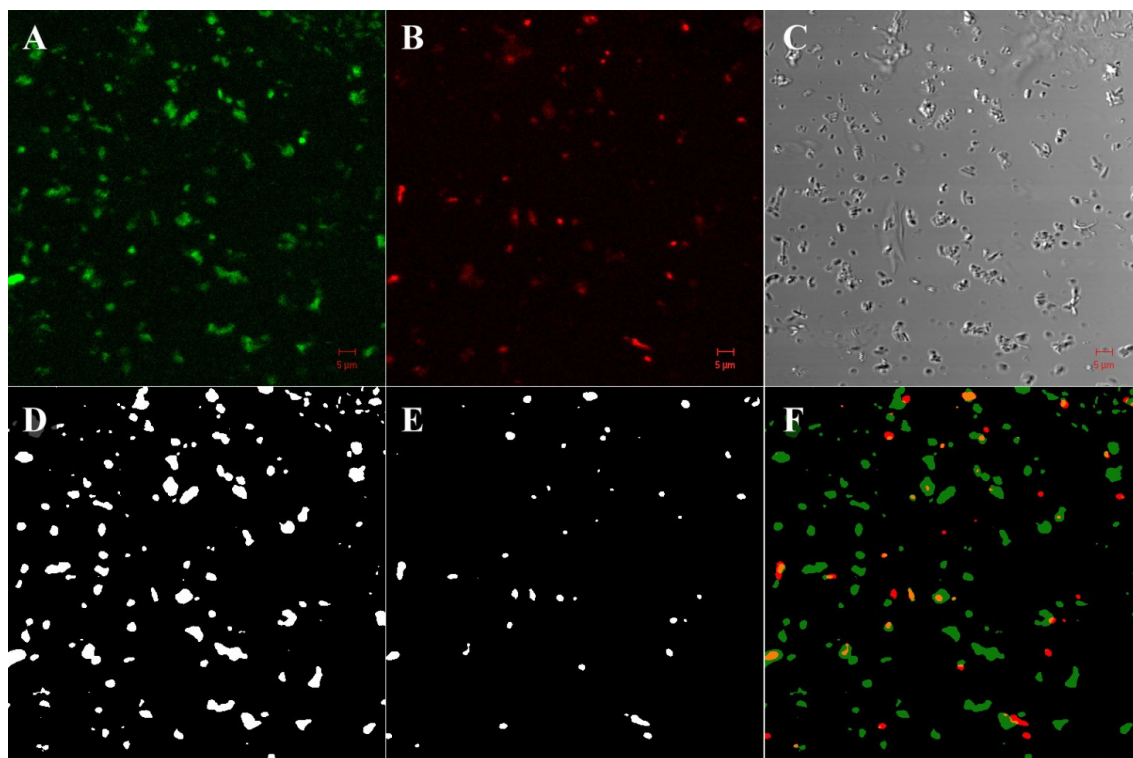


Figure 4. Example of the cell viability assessment for 0.55 mm beads during the second biotransformation cycle. (A): Vital cells (autofluorescence). (B): Necrotic cells (propidium iodide). (C): Transmission image. (D): (A) after segmentation. (E): (B) after segmentation. (F): Merged images (D) + (E) showing final areas used for viability estimation.

Propidium iodide is a DNA-binding stain that cannot cross intact plasma membranes and is used to identify necrotic or late-apoptotic cells. The viability of a large set of cells based on PI fluorescence assay could often be slightly decreased due to various reasons, e.g., some cells are ending their life cycle by being apoptotic, some cells could have damaged plasma membranes, and prolonged exposure to PI itself also leads to an increased portion of dead cells. Moreover, when analyzing bacterial biofilms, PI is known to bind to extracellular DNA (eDNA), creating a “false dead” layer of red-stained cells [29]. Overall, a slight underestimation of viability values is always to be expected in bacterial cell studies. In the following figures, 100% viability is referenced to a (hypothetical) state when no observable PI fluorescence, corresponding to necrotic cells, was detected in the sample.

It should be noted that upon preparation of entrapped cells for microscopy—which includes mechanical disintegration of barium–calcium alginate beads—small pieces of alginate material from particles could remain in the sample. These remains possess intrinsic fluorescence, which is very similar in spectral shape to that of AF of bacteria. This effect was minimized by centrifugation and precise sample handling, such that the overall contribution of these particles was negligible in comparison to the total cell signal. However, a small degree of uncertainty in the viability results was unavoidable under our experimental settings. Automatic procedures able to process large sets of cells, such as those described here, are therefore desirable to evaluate viability with the best possible statistics.

Viability measurement was used to verify cell survival rates at several different important technological steps. First, the viability of immobilized cells at different induction times was compared. Here, no significant difference between the 3 h ($95 \pm 2\%$) and 20 h ($96 \pm 1\%$) periods was found. Secondly, the cell state was compared using different immobilization technologies (Figure 5). For all the techniques used, a similar viability of $\sim 80 \pm 5\%$ was observed.

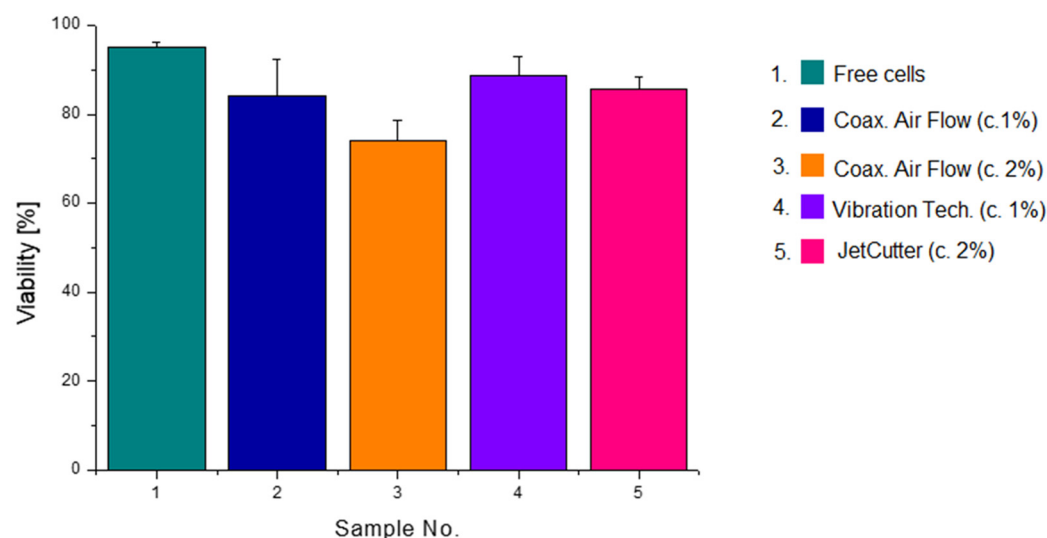


Figure 5. Cell viability compared for different immobilization techniques (see Section 3.3 for details of the immobilization protocols).

Finally, viability during subsequent cycles of the biotransformation process was evaluated (Figure 6A). In each system observed, i.e., free cells and cells encapsulated in beads of both sizes, relatively stable viability values of >80% were observed with slight diminishment towards the progression of later biotransformation cycles. On the other hand, while viability was mostly unchanged, we observed decreasing substrate conversion in all three cases (Figure 6B). We hypothesize that this effect could be linked to several mechanisms, including decreased metabolic activity, loss of redox balance, enzyme inhibition, or transport limitations, with negligible impact on cell viability. Whether these, or even other effects, took place remains to be answered. However, interestingly, the signal of cell autofluorescence (Figure 6C) partially correlates with the previous graph, showing a decreasing tendency with the increased cycle count. Therefore, we investigated the autofluorescence signal as a possible metabolic reporter in more detail, which is described in Section 2.5.

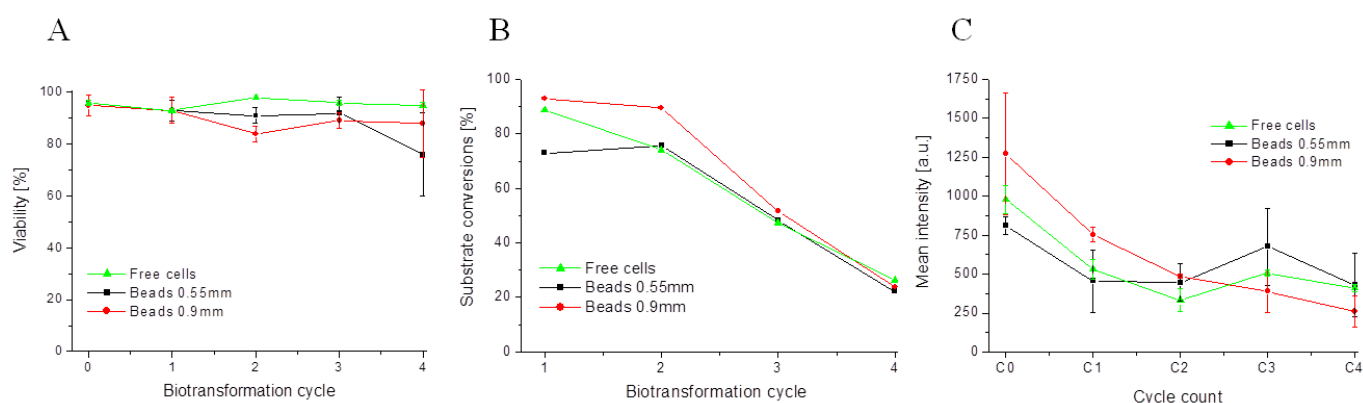


Figure 6. Various cell parameters measured over repeated cycles of a model cascade reaction converting 2-cyclohexen-1-ol to ϵ -caprolactone in free cells and cells entrapped in beads of different sizes (0.55 mm and 0.9 mm). (A) Cell viability, (B) substrate conversion efficiency, and (C) mean integral intensity of *E. coli* cell autofluorescence. 100% substrate conversion is defined as complete consumption of the substrate as determined by GC.

Additionally, preparation of barium–calcium alginate beads is considerably less demanding in terms of materials, cost, and labor compared, for example, with polyelectrolyte complex beads [11]. Simplicity, robustness, and scalability of the immobilization system based on barium–calcium alginate beads, together with slightly higher cumulative product

formation in both immobilized preparatives (Figure 3B), render a technological advantage to cell immobilization.

2.5. Advanced Imaging for Assessing Metabolic Activity of Immobilized Cells

After excitation with blue light, normally functioning bacterial cells produce a green fluorescence signal due to flavin autofluorescence ($\lambda_{\text{ex}} = 450\text{--}470\text{ nm}$, $\lambda_{\text{em}} = 525\text{ nm}$), which can be used for monitoring their metabolic state [19]. In our experiments, spatially and spectrally resolved fluorescence images within the 493–675 nm spectral range with a 23 nm step (Figure 7) were detected. From these images, the spectral profile can be reconstructed for each individual pixel.

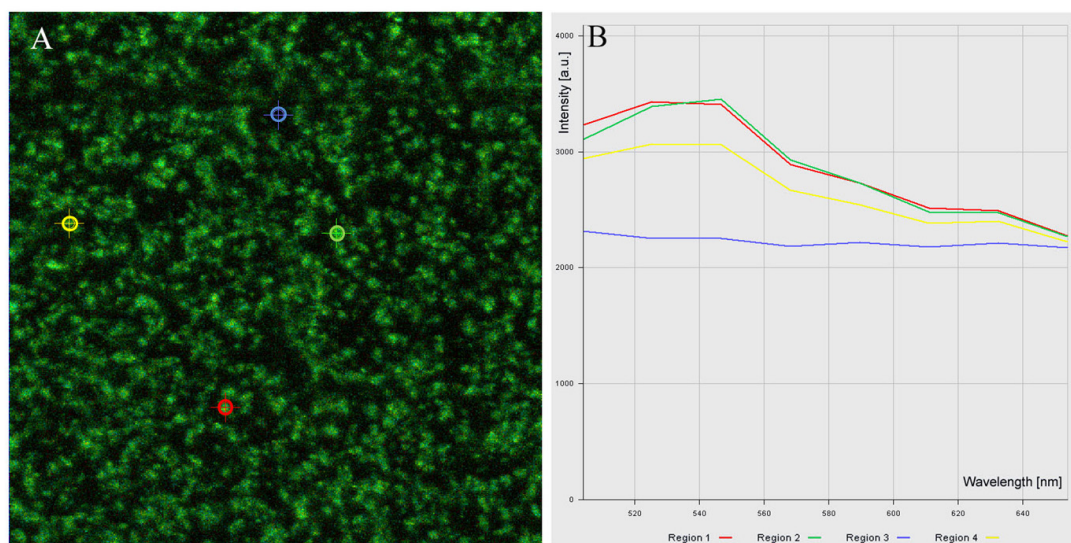


Figure 7. (A) Representative image showing a multispectral microphotograph of free *E. coli* cells. Each point in the image is represented by 8 intensity values, corresponding to the wavelength channel with a 23 nm wide bandpass. Four distinct regions of interests (ROI) are labeled with circles. (B) Example of the emission spectra from selected ROIs.

As the intensity of AF in *E. coli* is very small, a semi-automatic segmentation procedure for identifying cells in the image and subsequent averaging of the AF signal from the whole cell-related area (see spectral data analysis for details) were again successfully utilized. After correction for background and normalization to the first channel, spectral fingerprints of the cells in various conditions were obtained. Figure 8 depicts the evolution of autofluorescence spectra in cells observed with spectrally resolved CLSM during successive cycles of a model cascade reaction converting 2-cyclohexen-1-ol to ϵ -caprolactone. After summing all the detected photons over the whole spectrum, the integral mean intensity of the cell autofluorescence can be derived (see Figure 6C). In all three cases (free cells and cells in alginate beads of two different diameters), AF intensity generally decreases with increased cycle count. The decrease was three- to fivefold, with an asymptotic trend towards higher cycle counts.

When interpreting graphs in Figure 8, it is important to say that the error bars depict natural variability of the fluorescence signal detected in cells rather than error in our measurements, which is inherently related to electronic noise in the detector electronics visible at very low signal levels and is much smaller (see, e.g., line C4 at Figure 8C).

It is also noteworthy to say that with the excitation at 450 nm we partially induce and detect the autofluorescence of NAD(P)H ($\lambda_{\text{ex}} = 330\text{--}370\text{ nm}$, $\lambda_{\text{em}} = 460 \pm 50\text{ nm}$), which may partially influence the leftmost spectral channels around 500 nm. NAD(P)H is highly fluorescent in its reduced form and loses fluorescence under oxidation, while flavins

are fluorescent in their oxidized form and their fluorescence decreases when reduced. Both act as electron carriers in oxidative phosphorylation in the mitochondria and, due to the abovementioned optical properties, they are widely used to evaluate cell oxidative metabolism conditions in cells and tissues.

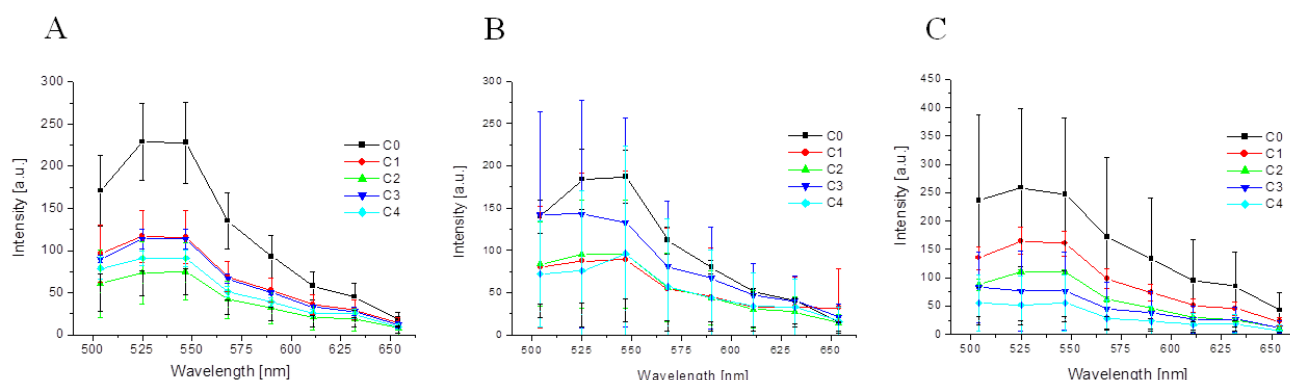


Figure 8. Autofluorescence emission spectra detected over repeated cycles of a model cascade reaction (C0–C4). (A) Free cells. (B) Cells entrapped in 0.55 mm beads, (C) Cells entrapped in 0.9 mm beads. C0–C4 are corresponding cycles of reaction, 0 being the reference state before biotransformation.

Our observations indicate that the number of fluorescent flavin molecules generally decreases during the transformation to the state of almost complete depletion of flavin resources. In this state, although the cells are not dead (see viability measurements in 2.4), they are unable to provide energy and resources for further production. While no significant changes in spectral shape were observed for free cells, the main peak (520–540 nm) in both immobilized cell groups significantly decreased compared to the reference. This could indicate different mechanisms of metabolic adaptation of the cells in entrapped particles vs. free cells to the prolonged biotransformation process. Spectral shape changes could also indicate an altered balance of NAD(P)H/FAD concentrations; however, the exact reason for such specific spectral changes requires further investigation. In conclusion, we observed that cell autofluorescence is a very sensitive indicator of internal cell metabolic balance, which—once properly deciphered and understood—could be used in the assessment of the cell catalytic properties during biotransformation.

3. Materials and Methods

3.1. Immobilization Polymers, Media, and Chemicals

High-viscosity, high-guluronic acid alginate Manugel GMB (GeniaLab, Braunschweig, Germany), calcium chloride dihydrate (VWR Chemicals, Leuven, Belgium), and barium chloride dihydrate (Sigma-Aldrich, Darmstadt, Germany) were used for immobilization. The substrate, 2-cyclohexenol (Aldrich, St. Louis, MO, USA); the analytical standards of cascade reaction intermediates, 2-cyclohexenone (Aldrich, Darmstadt, Germany) and cyclohexanone (Sigma-Aldrich, Darmstadt, Germany); and the product ϵ -caprolactone (Aldrich, St. Louis, MO, USA) were obtained from Sigma-Aldrich (Bratislava, Slovakia). Cell media for cultivations were composed of LBamp + kan agar on a Petri dish containing 10 g/L of peptone (Oxoid, Hampshire, UK), 5 g/L of yeast extract (Oxoid, Hants, UK), 10 g/L of NaCl (Slavus, Bratislava, Slovakia), 15 g/L of agar (Oxoid, Hampshire, UK), 100 μ g/mL of ampicillin (Sigma-Aldrich, Madrid, Spain), and 50 μ g/mL of kanamycin (Sigma, Jerusalem, Israel). The liquid LBamp + kan medium for cell growth had the same composition, without agar. The TBamp + kan contained 12 g/L of tryptone (Oxoid, Hampshire, UK), 24 g/L of yeast extract, 5 g/L of glycerol (Sigma, Subang Jaya, Malaysia), 16.4 g/L of $K_2HPO_4 \cdot 3H_2O$ (Carl Roth, Karlsruhe, Germany), 2.3 g/L of KH_2PO_4 (Slavus,

Bratislava, Slovakia), 100 µg/mL ampicillin, and 50 µg/mL kanamycin. Isopropyl-D-1-thiogalactopyranoside (IPTG) (0.1 mM) was from Takara Bio Inc. (Otsu, Japan). The reaction solution was composed of 6057 g/L Trizma (Sigma-Aldrich, St. Louis, MO, USA), with the pH adjusted to pH = 7 using HCl, supplemented with 1% glucose, 100 µg/mL ampicillin, and 50 µg/mL kanamycin. The pH value was maintained during the reaction using 3 M NaOH (Mikrochem, Pezinok, Slovakia). For analyzing samples using gas chromatography, we used ethyl acetate (Sigma-Aldrich, Darmstadt, Germany) with 0.5 mg/mL methyl benzoate (Sigma-Aldrich, Darmstadt, Germany) as the internal standard. All other chemicals and solvents were provided by commercial suppliers.

3.2. Cultivation of Cells

The recombinant strain *E. coli* BL21 (DE3) (pET28_CHMO_XenB and pET22b_LKADH) overexpressing the enzyme cascade of LK-ADH from *Lactobacillus kefir*, XenB from *Pseudomonas putida*, and CHMO from *Acinetobacter calcoaceticus* was kindly provided by Prof. Marko D. Mihovilović and Assoc. Prof. Florian Rudroff (TU Wien, Austria). Cells were cultivated as reported previously [11]. In brief, cells from the glycerol stock were plated onto a Petri dish containing LBamp + kan agar and incubated at 37 °C for 12 h. A single colony was then inoculated into 10 mL of LBamp + kan medium in an Erlenmeyer flask and cultivated at 37 °C for 12 h in an orbital shaker at 200 rpm. One percent (*v/v*) of the resulting culture was transferred into TBamp + kan medium and incubated at 37 °C in an orbital shaker at 200 rpm until the optical density (OD₆₀₀) reached 0.7. Subsequently, 0.1 mM IPTG was added to the TBamp + kan medium, and the culture was further induced at 25 °C for 3 h in an orbital shaker at 200 rpm. The cells were harvested with centrifugation at 15 °C and 4000 rpm for 15 min and used for cascade reactions and immobilization.

3.3. Preparation of Barium–Calcium Alginate Beads for Cell Entrapment

A cell suspension was added to a solution of polyanion consisting of 2% (*w/v*) alginate GMB in 0.9% (*w/v*) NaCl at pH = 7 to a final concentration of 10% of wet cells. The alginate beads with the cell content were produced by using a high-performance device (JetCutter, geniaLab, Braunschweig, Germany), which broke a laminar jet of polyanion with cells into monodisperse drops using the cutting tool with wires into a stirred gelation solution of BaCl₂ + CaCl₂ in a ratio of 1:1. For the production of beads with a diameter of 0.9 mm, the flow rate of polyanion with cells was 100 mL/min with the following parameters: pressure 1.3 kPa, nozzle with inner diameter 0.4 mm, cutting tool with 100 wires, rotation of cutting tool 1200 rpm, and a distance of 1 m between the nozzle and gelation solution. The collection time was 30 s and the gelation time was 10 min. Secondly, for the production of beads with a diameter of 0.55 mm, the flow rate of polyanion with cells was 70 mL/min with the following parameters: pressure 1.3 kPa, nozzle with inner diameter 0.2 mm, cutting tool with 100 wires, rotation of cutting tool 1400 rpm, and a distance of 0.4 m between the nozzle and gelation solution. The collection time was 80 s and the gelation time was 10 min. After gelation, both of the prepared beads were separated from the gelation solution and washed and stored with 0.9% (*w/v*) NaCl at 4 °C. For comparison purposes, alginate beads containing cells were also produced using alternative immobilization technologies, particularly the tailor-made coaxial air flow technique, in which the flow rate of the polyanion–cell solution was 1 mL/min for 1% alginate and 0.7 mL/min for 2% alginate, and was operated at air pressures of 0.5 kPa and 1.3 kPa, respectively, using a nozzle with an inner diameter of 0.4 mm for both samples. In contrast, the jet break-up Encapsulator Startup (EncapBioSystems Inc., Greifensee, Switzerland) employed a significantly higher flow rate of 12 mL/min, and operated under the conditions of an air pressure of 0.5 kPa, a nozzle with an inner diameter of 0.3 mm, and a vibration frequency of 450 Hz. The

dynamic viscosity of alginate solutions was determined at 25 °C using a Brookfield DV-II+ rheometer (Brookfield, Middleboro, MA, USA).

3.4. Repeated Biotransformations in Bioreactor

Twenty grams of alginate beads with the cell content (10% *w/w*) and an equivalent amount of free *E. coli* cells were placed into Tris-HCl buffer (pH = 7) supplemented with 1% glucose, ampicillin, and kanamycin. This mixture, with a final reaction volume of 200 mL, was transferred into the 3-vessel Biostat QPlus Bioreactor system Biostat® Q plus—3 × 0.5 L UniVessel® Culture Vessels and Equipment controlled by BioPAT® DCU Tower with Touch Panel and SCADA System BioPAT® MFCS/DA 3.0 software (Sartorius Stedim Biotech GmbH, Göttingen, Germany) and, by adding the 4 mM substrate (2-cyclohexen-1-ol) in ethanol (volume ratio 1:9), we initiated the model cascade reaction. The conditions of reaction in the bioreactor were 25 °C, a speed of stirrer of 90 rpm, a flow of oxygen of 1.5 L/min, and pH = 7 maintained by automatically adding 3 M NaOH. The samples of the reaction mixture were taken every hour and extracted in a 1:1 ratio with ethyl acetate containing methyl benzoate, for analysis with gas chromatography using an Agilent technologies 7890A GC system (Agilent technologies, Santa Clara, CA, USA) as reported previously [11]. Illustrative chromatograms are depicted in Supplementary Materials as Figures S2–S4. After 8 h of reaction, immobilized cells were separated with filtration, and free cells were separated with centrifugation; both were stored at 4 °C and then washed with the substrate-free Tris-HCl buffer immediately before the next reaction cycle.

3.5. Microscopy

To analyze the formed alginate beads, we used an LM6-3 microscope (Kvant, Bratislava, Slovakia) at 40× magnification with an Olympus digital camera (Olympus Optical Co. Ltd., Seoul, Republic of Korea). The dimensional analysis (size and shape) of the beads was performed using the Impor 5.0 program, and photographs of the beads were created using the Camera Control program Cam2Com Version 2.0.0.22 software.

3.6. Mechanical Stability of Beads

The mechanical stability of the alginate beads was determined using the Texture Analyser TA-XT2i (Stable Micro Systems, Godalming, UK), which evaluates the strength of the beads with piston compression of the bead against a base. Before measuring the mechanical strength, microscopic dimensional analysis was used to evaluate the average bead size in each sample to adjust the texture analyzer piston from the base. To determine the mechanical strength of the bead, the force required to compress the bead by 60% [21] was monitored using the Exponent Connect program Version 8.1.14.0. The samples were measured at a laboratory temperature of 25 °C. The obtained values were then processed in Excel.

3.7. Cell Preparation for Physiological Imaging

Cells were suspended in a physiological solution (0.9% NaCl) or PBS buffer. The particles were first mechanically crushed (by hand, using a frosted glass cup and handle), and then centrifuged at low speed (500 rpm) to separate larger polymer remains from free cells. For the experiments, we used supernatant fluid. Condensed free cells were diluted to approx. 20 uL to 1 mL of the final solution to match the cell concentration of the entrapped samples. In each case, 35–50 µL of the sample was transferred to a coverslip glass for imaging, and covered with a Petri dish to avoid evaporation. Coverslip glass was modified by applying 0.2% polyethyleneimine (Sigma-Aldrich, PEI) prior to each experiment, to stabilize the motile cells on the glass surface according to [30]. For viability measurements,

the cells on the coverslip were further incubated for 5–10 min with propidium iodide (PI) at $\sim 1 \mu\text{M}$ concentration and subsequently observed.

3.8. Spectrally Resolved Confocal Imaging

Confocal fluorescence images were taken with an Axiovert 200M microscope equipped with an LSM 510 NLO Meta laser scanning confocal head (both Zeiss, Jena, Germany) and a C-Apochromat $40\times/1.2$ NA water immersion lens (Zeiss, Jena, Germany) was utilized in our experiments. For both autofluorescence and PI excitation, we used a 450 nm diode laser (Kvant, Bratislava, Slovakia). For detection, we used either standard detectors—transmission photodiode and photomultipliers (PMTs) in a confocal fluorescence setup—or a META detector (32-channel photomultiplier array) for confocal spectral measurements. Spectrally resolved images were gathered over a 493–675 nm range with a 23 nm step size. The autofluorescence and shape of the bacteria were detected using a bandpass BP 480–520 nm filter (confocal AF detection) and simultaneous transmission imaging. A two-channel detector configuration with bandpass BP 500–550 nm (AF) and BP 620–680 nm (PI) filters was used for viability measurements. The field of view of most measurements was $122.5 \times 112.5 \mu\text{m}$ with a physical resolution of $0.22 \mu\text{m}/\text{px}$.

3.9. Cell Viability

Cell viability was measured using the intake of propidium iodide (PI, Merck/Sigma-Aldrich 81845) into necrotic cells with simultaneous recording of autofluorescence as a reference indicator of living cells [18]. Cell viability was evaluated as a ratio $(N_{\text{all}} - N_{\text{PI}})/N_{\text{all}}$, where N_{all} is the total number of bacterial cells and N_{PI} is the number of necrotic cells identified with PI fluorescence. More specifically, images taken on two channels corresponding to the living cells (green AF) and necrotic cells (red PI) were processed as follows. Pixel-wise segmentation was performed for both channels independently using Otsu's thresholding algorithm [31], which classified pixels into two classes: one representing pixels with cells, and the second corresponding to the background. Following this binary segmentation, overall viability was quantified by calculating the abovementioned ratio from the number of pixels associated with living cells in the green channel vs. necrotic cell-associated pixels in the red channel (see Section 2.4 and Figure 5 for more details). Viability values evaluated from at least three independently measured images were determined and averaged with the respective standard deviation.

3.10. Spectral Data Analysis

Image data were analyzed using the ZEN 5.0 SP1.1 software (Zeiss, Germany) and custom-developed procedures for image processing. Similar to the viability measurement, spectrally resolved images were processed using binary segmentation to discriminate pixels corresponding to objects from those corresponding to the background. The segmentation procedure was based on the generation of a binary mask derived from an image, which was created by summing all spectral channels into a single grayscale image. From this summed image, a binary mask was computed using Otsu's thresholding algorithm [31], which automatically determines the optimal threshold value by maximizing inter-class variance. This binary mask was subsequently utilized to extract cumulative spectral profiles separately for cell pixels and background pixels. The resulting spectral profiles were normalized by averaging across the number of pixels from which they were obtained. The background profiles were further subtracted from the signals corresponding to cells. As a final step, an average of spectral profiles computed from at least three independently measured images was determined with their respective standard deviation. For the evaluation of the spectral shape changes, selected curves were normalized to the first spectral channel.

Standard statistical procedures utilizing triplicates (where possible) were used.

4. Conclusions

An original contribution of this study is the concept of assessing the metabolic response of live recombinant cells, *E. coli* containing a cascade of three enzymes for ϵ -caprolactone production, through simultaneous measurements of viability, biocatalytic efficiency, and spectrally resolved confocal microscopy, which are collectively capable of capturing changes in cellular energetics. Moreover, based on spectral shape changes in autofluorescence emission spectra, it was possible to distinguish the metabolic response of cells in free versus immobilized states as an adaptation to repeated biotransformations. While the viability of both free and immobilized cells remained high throughout the repeated biotransformation process, the biocatalytic efficiency and energy resources of immobilized cells declined more slowly than in free cells. This suggests a possible direction to focus on in order to explain the mild stabilizing effect of cell immobilization, the deeper biological and biochemical causes of which still need to be elucidated.

The results of a unique comparative study of three different immobilization principles utilizing devices based on coaxial air-stripping, jet break-up, and jet-cutting demonstrated high cell viability following immobilization, allowing for the interchangeable use of these methods. The application of the most powerful immobilization technique—jet-cutting—led to the production of alginate particles with immobilized cells that were mechanically reinforced by known combined gelation using BaCl_2 and CaCl_2 in a 1:1 ratio. The resulting particles proved to be mechanically and chemically resistant under conditions of repeated biotransformations in parallel, three stirred, aerated, and pH-controlled bioreactors. Though four possible consecutive biotransformations may be considered as a low number, it can be justified and economically acceptable if no other solution is feasible. The hypothesis of improved accessibility of cells with enzyme cascades in particles with a smaller diameter (0.55 mm) was confirmed, as evidenced by a 22% higher ϵ -caprolactone production during repeated biotransformations compared to particles with a 0.9 mm diameter, and a 36% higher productivity compared to non-immobilized cells.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/catal16040369/s1>, Figure S1: (A) Biocatalytic activity values (U/g) at each biotransformation cycle. (B) Space-time yield of ϵ -caprolactone (mg/L·h) at each biotransformation cycle (8 h). 1 U corresponded to the formation of 1 μmol of product per minute per gram of cell dry weight under the given experimental conditions; Figure S2: GC chromatogram corresponding to the sample withdrawn at the start ($t = 0$ h) of the first run of enzyme cascade reaction catalysed by recombinant cells *E. coli* immobilized in 0.55mm barium-calcium alginate beads. Peak at the elution time of 1701 min corresponds to substrate 2-cyclohexenol. Peak at the elution time of 3299 min corresponds to internal standard methyl benzoate; Figure S3: GC chromatogram corresponding to the sample withdrawn at the reaction time $t = 1$ h of the first run of enzyme cascade reaction catalysed by recombinant cells *E. coli* immobilized in 0.55 mm barium-calcium alginate beads. Peak at the elution time of 1701 min corresponds to substrate 2-cyclohexenol; 1917 min corresponds to one of the intermediates 2-cyclohexene-1-one; 3299 min corresponds to internal standard methyl benzoate; 4037 min corresponds to product ϵ -caprolactone. Figure S4: GC chromatogram corresponding to the sample withdrawn at the reaction time $t = 8$ h of the first run of enzyme cascade reaction catalysed by recombinant cells *E. coli* immobilized in 0.55 mm barium-calcium alginate beads. Peak at the elution time of 1701 min corresponds to traces of substrate 2-cyclohexenol; 3300 min corresponds to internal standard methyl benzoate; 4035 min corresponds to product ϵ -caprolactone.

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