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Bio-Based Materials versus Synthetic Polymers as a Support in Lipase Immobilization: Impact on Versatile Enzyme Activity

Karina Jasińska [1,2], Bartłomiej Zieniuk [2], Urszula Jankiewicz [3] and Agata Fabiszewska [2,*]

[1] Department of Food Engineering and Process Management, Institute of Food Sciences, Warsaw University of Life Sciences—SGGW (WULS—SGGW), 159c Nowoursynowska St., 02-776 Warsaw, Poland
[2] Department of Chemistry, Institute of Food Sciences, Warsaw University of Life Sciences—SGGW (WULS—SGGW), 159c Nowoursynowska St., 02-776 Warsaw, Poland
[3] Department of Biochemistry and Microbiology, Institute of Biology, Warsaw University of Life Sciences—SGGW (WULS—SGGW), 159 Nowoursynowska Street, 02-776 Warsaw, Poland
* Correspondence: agata_fabiszewska@sggw.edu.pl

Abstract: To improve enzyme stability, the immobilization process is often applied. The choice of a support on which the enzymes are adsorbed plays a major role in enhancing biocatalysts' properties. In this study, bio-based (i.e., chitosan, coffee grounds) and synthetic (i.e., Lewatit VP OC 1600) supports were used in the immobilization of lipases of various microbial origins (yeast (*Yarrowia lipolytica*) and mold (*Aspergillus oryzae*)). The results confirmed that the enzyme proteins had been adsorbed on the surface of the selected carriers, but not all of them revealed comparably high catalytic activity. Immobilized CALB (Novozym 435) was used as a commercial reference biocatalyst. The best hydrolytic activity (higher than that of CALB) was observed for Novozym 51032 (lipase solution of *A. oryzae*) immobilized on Lewatit VP OC 1600. In terms of synthetic activity, there were only slight differences between the applied carriers for *A. oryzae* lipase, and the highest measures were obtained for coffee grounds. All of the biocatalysts had significantly lower activity in the synthesis reactions than the reference catalyst.

Keywords: immobilization; lipase; synthetic activity; Lewatit; chitosan; coffee grounds

Citation: Jasińska, K.; Zieniuk, B.; Jankiewicz, U.; Fabiszewska, A. Bio-Based Materials versus Synthetic Polymers as a Support in Lipase Immobilization: Impact on Versatile Enzyme Activity. *Catalysts* **2023**, *13*, 395. https://doi.org/10.3390/catal13020395

Academic Editors: Priscilla Amaral and Ivaldo Itabaiana Junior

Received: 13 January 2023
Revised: 7 February 2023
Accepted: 8 February 2023
Published: 11 February 2023

1. Introduction

Some of the most widely industrially applied enzymes are lipases (triacylglycerol hydrolases, EC 3.1.1.3). These enzymes can be sourced from microorganisms, animals, and plants. Microbial lipases are of great interest due to their high stability in catalytic performance and broad tolerance of environmental factors (e.g., pH, temperature, and organic solvents). They can catalyze various reactions, such as hydrolysis, esterification, and transesterification, in both hydrophilic and hydrophobic media. A particular advantage of these biocatalysts is their specificity (stereo- and enantioselectivity), which is key to both the classification of lipases and their application. These special features allow lipases to be used in almost every field of biotechnology, including food chemistry, biodiesel production, and synthesis of biopolymers and pharmaceuticals [1–5].

Most lipase preparations are solutions with various degrees of concentration and purification, or preparations in solid form. In many studies, researchers apply commercial lipases such as CALB (immobilized lipase B from *Candida antarctica*), Lipozyme RMIM (from *Rhizomucor miehei*), and Lipozyme TLIM (from *Thermomyces lanuginosus)* [6].

To improve the stability of enzymes, the immobilization technique is often used. There are two main ways in which biocatalysts can be linked to special supports: the first is based on physical techniques such as adsorption, entrapment, and encapsulation, while the second includes chemical bonding processes such as covalent bonding and crosslinking [1]. Due to its reversibility and simplicity, the most favored method is physical adsorption. Its advantage is that there are no significant changes in the enzymes' native conformation and

it does not cause a loss of catalytic activity. In this single-step process, biocatalysts can be adsorbed at both porous and non-porous supports [7].

Carriers for enzyme immobilization are classified into four groups: inorganic materials, polymers, MOFs (metal–organic frameworks), and DNA origami. The most popular inorganic matrices are mica, silica, zeolites, activated carbon and, for polymers, agarose, chitin, chitosan, cellulose, lignin, starch, or gelatin (natural polymers), and polyethylene glycol, polyacrylamide, polyaniline, nylon, and epoxy-activated polymers (synthetic polymers) [2,8–12].

In recent years, research efforts have been focused on boosting the catalytic reaction to achieve more ecological and economical processes. The search for more biodegradable, biocompatible, and non-toxic supports is still necessary. The present study examined various supports for enzyme immobilization, including the synthetic, commercial matrix Lewatit VP OC 1600 as a reference, the natural biopolymer chitosan, and spent coffee grounds. The aim of this study was to compare the activity of lipase preparations immobilized on different supports—namely, lipases obtained in a wild-type strain of *Yarrowia lipolytica* yeast culture, and commercial lipase solution Novozym 51032 from *Aspergillus oryzae*. The investigations focused on the usability of spent coffee grounds in lipase immobilization in comparison to other carriers. This paper focuses on the examination of the hydrolytic and synthetic activity of the obtained preparations.

2. Results and Discussion

2.1. Morphology of Native Matrices and Immobilized Preparations

Lewatit VP OC 1600 is a synthetic matrix based on a macroporous resin of poly(methyl methacrylate) that is widely used for the physical immobilization of *Candida antarctica* lipase B (CALB) and commercially available as Novozym® 435 [13,14]. In this study, the synthetic support was applied for the immobilization of a crude enzyme solution of *Y. lipolytica* extracellular lipases and the liquid lipase Novozym 51032. The carrier was in the form of beads, as also shown in Figure 1. It was noticeable that the native Lewatit had a regular and smooth surface in comparison with the immobilized preparations (Figure 1A,B). Scanning electron microphotographs confirmed that both used enzymes were adsorbed on the surface of the support. Significant differences were observed. The carrier with lipase Novozym 51032 had a greater number of visibly adsorbed enzymes on the surface, which merged in some places to form agglomerates (Figure 1E,F). Otherwise, it looked for support with crude lipase solution from *Y. lipolytica* (Figure 1C,D). It was noticeable that the shape of both immobilized enzymes varied. Presumably, these differences resulted from the purification of the adsorbed lipases. The supernatant was separated from the yeast biomass by centrifugation and, despite the extracellular lipases, could contain some salts, sugars, other proteins or products of the yeast metabolism, and culture medium ingredients [15]. Novozym 51032, as a commercial biocatalyst, included only purified lipase proteins.

Chitosan is a natural polysaccharide that can be sourced from the walls of the shells of shellfish (mainly from shrimps, crabs, or lobsters). This derivative of chitin is characterized by biocompatibility, environmental friendliness, non-toxicity, and high stability [7,16]. In this research, the abovementioned biopolymer was used as a matrix for the immobilization of Novozym 51032. The form of the support was a powder. In Figure 2, the structure of the native chitosan and immobilized enzyme on the carrier can be observed. In comparison with commercial supports such as Lewatit, the visibility of lipases on the surface of the chitosan is scarce. Due to the more complex structure of this biopolymer, enzymes could adsorb on different elements, which might be less noticeable, or the presence of the protein could depend on the type of lipases used. Foresti et al. [17] used chitosan to carry out immobilization of different microbial lipases, such as *C. rugosa* AY lipase, *Pseudomonas fluorescens* AK lipase, and native lipase B from *C. antarctica*. Scanning electron microphotographs showed that agglomerates of *C. antarctica* B were clearly seen on the supports, while with *P. fluorescens* lipase proteins were barely visible.

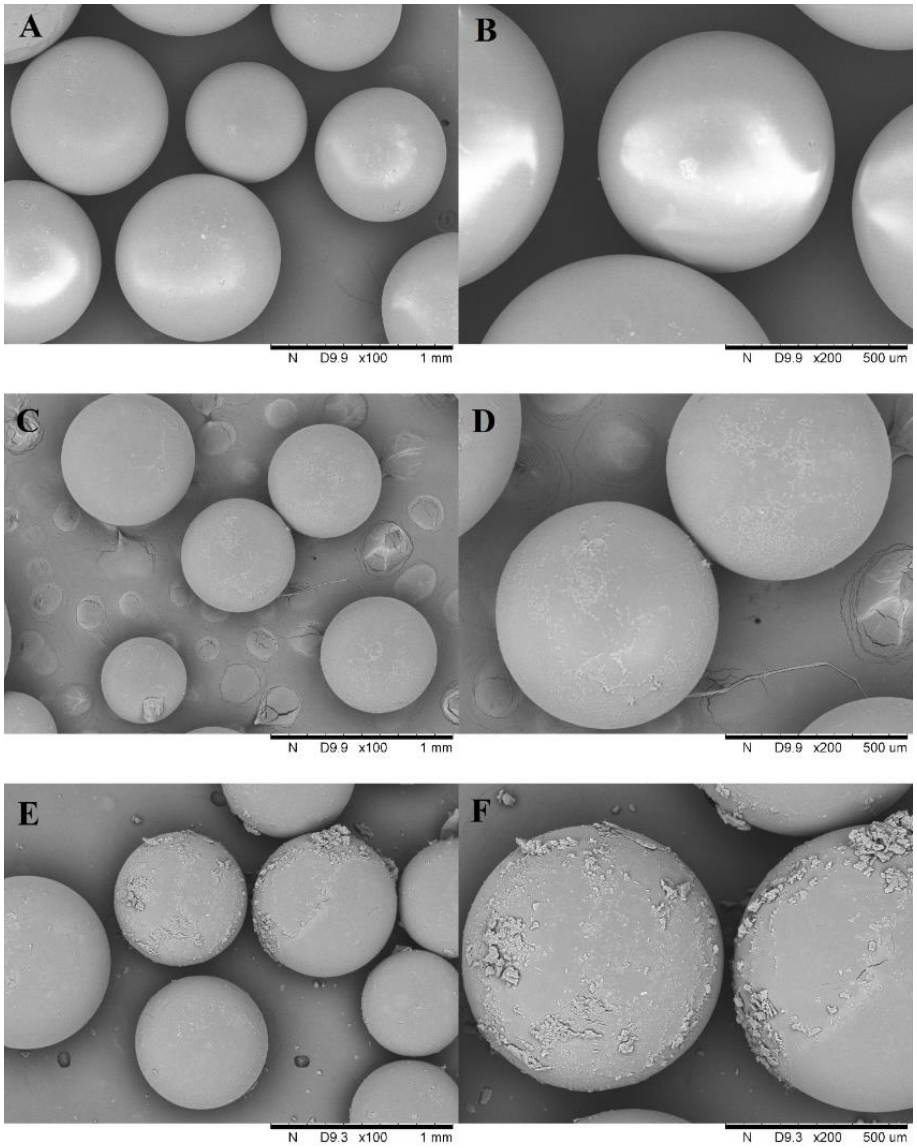

Figure 1. Scanning electron microphotographs of (**A**,**B**) beads of Lewatit VP OC 1600 (×100, ×200), (**C**,**D**) crude solution of *Y. lipolytica* enzymes immobilized on Lewatit VP OC 1600 (×100, ×200), and (**E**,**F**) liquid lipase Novozym 51032 immobilized on Lewatit VP OC 1600 (×100, ×200).

Spent coffee grounds (SCGs) are lignocellulosic wastes obtained from the preparation of coffee. Due to the popularity of this beverage, the amount of residues around the world is continually growing. For this reason, the interest in reusing their waste is increasing, and spent coffee grounds have become a new source with potential application as a support for the immobilization of enzymes. This kind of biomaterial is characterized by biodegradability, non-toxicity, and wide availability and consists of organic compounds such as fatty acids, cellulose, hemicellulose, lignin, protein, minerals, and total sugars [18–21]. Food waste also has various interesting properties, such as high porosity—which can be observed for coffee grounds in the presence of different chemical groups—and high surface area. It is worth highlighting that using the material can have a positive effect in reducing waste disposal problems [19]. Analyzing scanning electron microphotographs (Figure 3), it was found that the immobilization process was successful and the lipase Novozym 51032 was adsorbed on the coffee grounds support. Some of the enzyme molecules settled on the surface of the carrier, while others were adsorbed inside the pores, in the channels. The structure of the coffee grounds was porous, with visible flat leaf shapes, similar to that shown by Osorio-Arias et al. [22] and Ballesteros et al. [23].

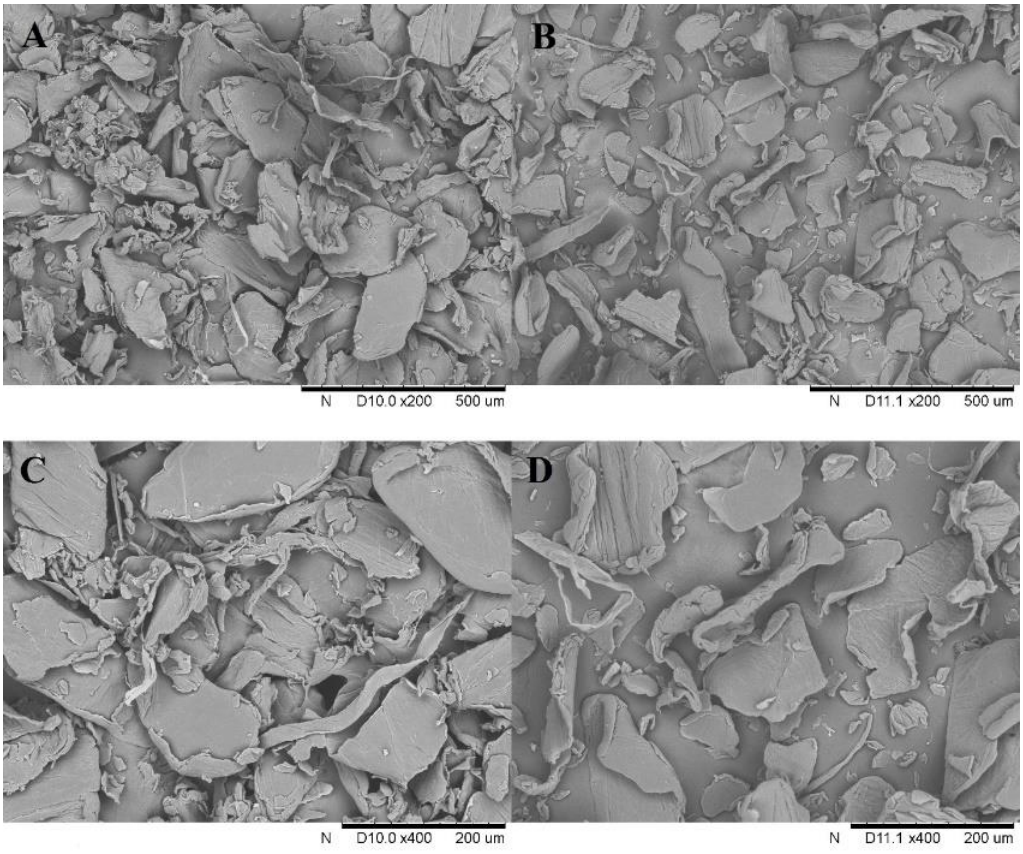

Figure 2. Scanning electron microphotographs of (**A**,**C**) liquid lipase Novozym 51032 immobilized on chitosan powder (×200, ×400) and (**B**,**D**) chitosan powder (×200, ×400).

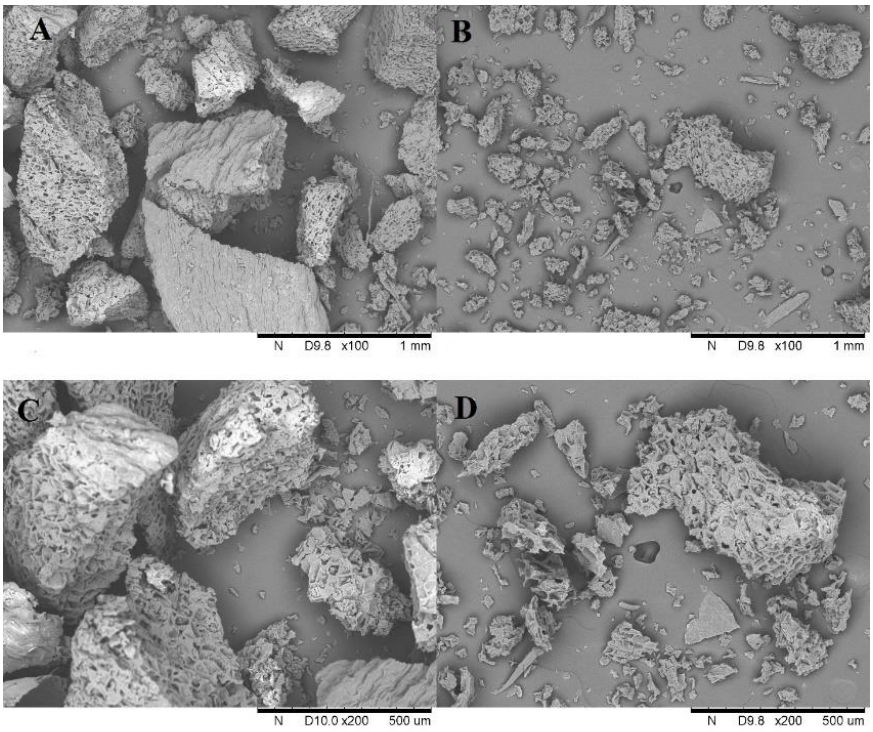

Figure 3. Scanning electron microphotographs of (**A**,**C**) liquid lipase Novozym 51032 immobilized on coffee grounds (×100, ×200) and (**B**,**D**) coffee grounds (×100, ×200).

2.2. Influence of the Lipase Source and Type of Support Used in Immobilization on the Hydrolytic and Synthetic Activity of the Enzyme Preparation

Immobilized biocatalysts were employed in the hydrolysis of *p*-nitrophenyl laurate and the transesterification between vinyl acetate and *n*-butanol. The obtained results are shown in Figures 4 and 5. The highest hydrolytic activity (0.0059 U/mg) was obtained for the preparation with immobilized lipase Novozym 51032 from *Aspergillus* on Lewatit VP OC 1600 (Figure 4). The results were greater than for the reference CALB when commercially immobilized on the same support. On the other hand, in Figure 5, it can be observed that the immobilized CALB had the best outcomes for synthetic activity. These differences confirm that the selection of the perfect support for immobilization had to be specific to the used enzymes. The commercial CALB supplied by Novozym is widely known in the industry, and the support on which it was immobilized was relatively hydrophobic [24]. This combination resulted in receiving a biocatalyst with great potential in many types of reactions, as also verified by the experiments in this study. Some laboratories have tried to repeat the success of Novozym 435 and immobilized other lipases on Lewatit VP OC 1600—for example, from *Rhizopus arrhizus* [25], *R. oryzae* and *Carica papaya* [26], or *Penicillium* sp. [27]—but the conclusion was often that the achievement of combining Lewatit VP OC 1600 with CALB cannot be extrapolated to other lipases. This paper showed that in some cases, e.g., catalyzing hydrolysis reactions, our own laboratory-made biocatalysts could be better than commercially available enzymes.

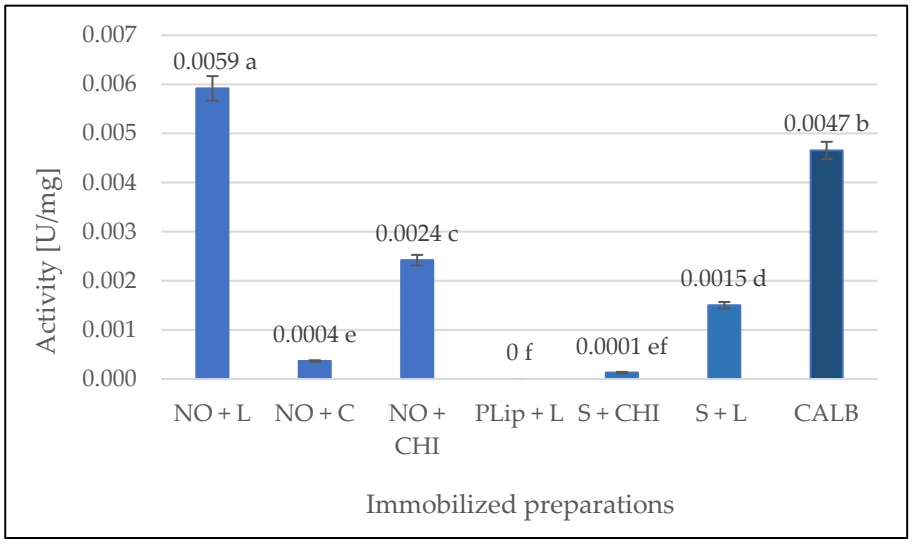

Figure 4. The hydrolytic activity of immobilized preparations: S + CHI: crude enzyme solution of *Y. lipolytica* immobilized on chitosan powder; S + L: crude enzyme solution of *Y. lipolytica* immobilized on Lewatit VP OC 1600; PLip + L: purified lipase from the supernatant of *Y. lipolytica* immobilized on Lewatit VP OC 1600; NO + L: liquid lipase Novozym 51032 immobilized on Lewatit VP OC 1600; NO + C: liquid lipase Novozym 51032 immobilized on coffee grounds; NO + CHI: liquid lipase Novozym 51032 immobilized on chitosan powder; CALB: immobilized lipase B from *Candida antarctica* as a reference. Means with the same capital letter (a, b, c, d, e, f) did not differ significantly ($\alpha = 0.05$).

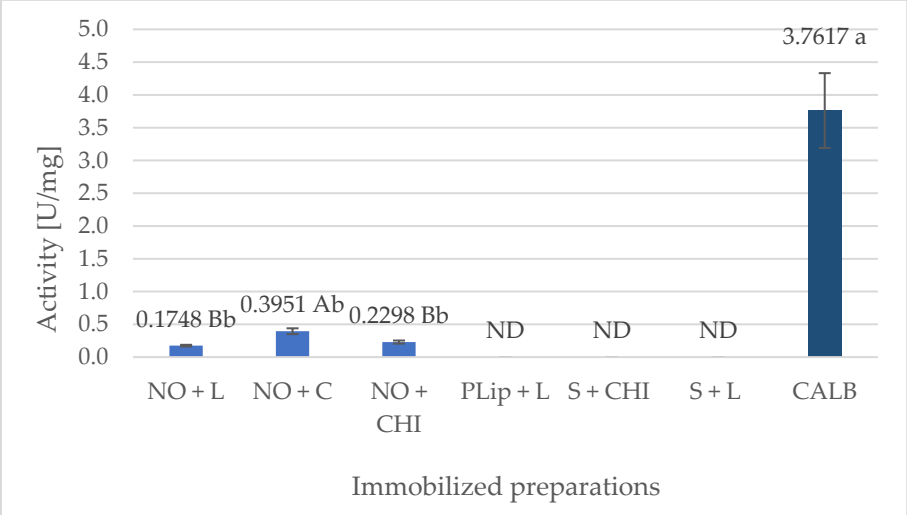

Figure 5. The synthetic activity of immobilized preparations: S + CHI: crude enzyme solution of *Y. lipolytica* immobilized chitosan powder; S + L: crude enzyme solution of *Y. lipolytica* immobilized on Lewatit VP OC 1600; PLip + L: purified lipase from *Y. lipolytica* immobilized on Lewatit VP OC 1600; NO + L: liquid lipase Novozym 51032 immobilized on Lewatit VP OC 1600; NO + C: liquid lipase Novozym 51032 immobilized on coffee grounds; NO + CHI: liquid lipase Novozym 51032 immobilized on chitosan powder; CALB: immobilized lipase B from *C. antarctica* as a reference; ND: not detected. Means with the same capital letter A or B in the case of liquid lipase Novozym 51032 immobilized on different supports (and a or b in the case of all biocatalysts) did not differ significantly ($\alpha = 0.05$).

The next carriers used in the experiments with lipase Novozym 51032 were chitosan and coffee grounds. Higher results for hydrolytic activity were achieved for samples with chitosan (0.0024 U/mg) than with coffee grounds (0.0004 U/mg), but for synthetic activity the outcomes were the opposite—a slightly higher score was obtained for coffee grounds (0.3951 U/mg). Chitosan had reactive functional groups such as amino- or hydroxyl groups, which can affect the immobilization process. Sometimes, modified functional groups in the carrier may influence the non-specific binding of the enzyme to the support, which can cause the loss of catalytic activity. There are some instances when blocking the remaining reactive groups in the carrier is necessary [7]. Due to this fact, chitosan as a support can behave in different ways in hydrolysis or synthesis because of the possibility of reacting with substrates or products of the reaction. In comparison with the literature, plenty of studies have tried to immobilize different types of lipase by physical adsorption on chitosan powder—for instance, from *A. niger* [28], C. *rugosa* [29], or *Burkholderia cepacia* [30]. Sanchez et al. [29] found that *Burkholderia cepacia* lipase immobilized on chitosan showed high activity in the *sn*-2 esterification of 1,3-dicaprin with palmitic acid. On the other hand, Kaja et al. [30] found that lipase from *C. rugosa* immobilized on chitosan had low activity values because of its lower porosity than other supports, e.g., Sephadex G-25 or Celite-545. Foresti et al. [17] obtained promising results in terms of hydrolytic and synthetic activity for chitosan-immobilized lipases from *C. rugosa*, *P. fluorescens*, and *C. antarctica* B.

Few studies are currently being conducted on coffee grounds as a carrier in the immobilization process. Lira et al. [31] made an attempt to immobilize lipase from *Thermomyces lanuginosus* (Lipozyme TL100L-Novozymes®) on spent coffee grounds. The obtained biocatalyst showed high hydrolytic activity (1715 U/g) but, surprisingly, did not present any esterification activity. Girelli et al. [20] prepared two types of biocatalyst—*Candida rugose* lipase immobilized on spent coffee grounds—by using physical adsorption and covalent methods. They obtained a preparation that could possibly be used in the hydrolysis of milk fat. In the present study, the results of enzyme activity showed that lipase Novozym 51032 immobilized on SCG can be a biocatalyst suitable for both hydrolysis and synthesis

reactions, but improvements in the values of catalytic activity are needed in future. The generally low catalytic activity of the enzyme linked to this porous support may have been due to the limitations on diffusion induced by the interaction between the biomass and the enzyme, or because of contaminants that may be present in the waste and not removed during the pretreatment [31,32].

In this study, we attempted to immobilize crude enzyme solutions of *Y. lipolytica* extracellular lipases on Lewatit VP OC 1600 and chitosan. These two supports were chosen based on the best results that were obtained for commercial lipase. Despite the fact that the crude solution of yeast lipase was a non-purified extract, it was possible to acquire an active preparation on synthetic supports that had slightly lower hydrolytic activity (0.0015 U/mg) than immobilized lipase Novozym 51032 on chitosan. Unfortunately, immobilization on chitosan was no longer as effective. It was also undertaken to carry out a laboratory experiment with self-purified lipase from lyophilized crude enzyme solution of *Y. lipolytica* extracellular lipases. However, the obtained results did not meet our expectations, and these preparations had the lowest hydrolytic activity. The process of self-purification of lipase has proven to be inefficient and needs to be refined in the future. For the measurement of synthetic activity, the method was not sensitive enough for such low activities, so the level of activity was not detected.

For preparations with detected synthetic activity, the percentage of conversion of vinyl acetate into acetaldehyde was determined, as presented in Figure 6. The highest result was obtained for commercial immobilized CALB, which Zheng et al. [33] also confirmed in their study. The best of our own laboratory-made biocatalysts was Novozym 51032 immobilized on coffee grounds. The significantly lower conversion enabled the biocatalyst to be adsorbed on chitosan.

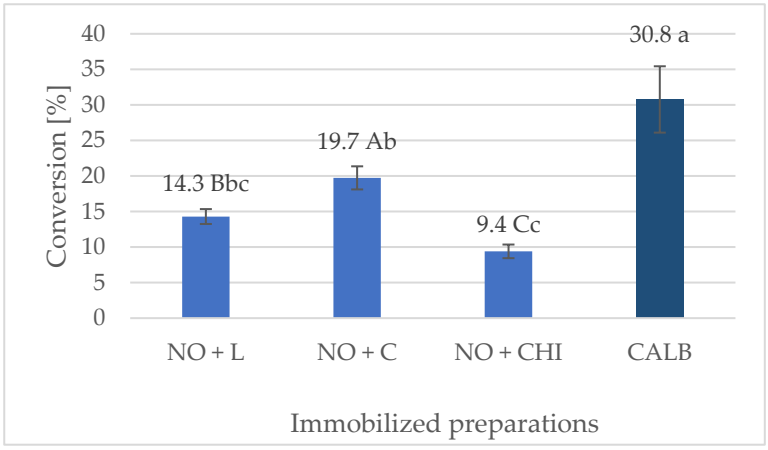

Figure 6. Conversion of vinyl acetate into acetaldehyde based on the colorimetric assay method with the immobilized preparations: NO + L: liquid lipase Novozym 51032 immobilized on Lewatit VP OC 1600; NO + C: liquid lipase Novozym 51032 immobilized on coffee grounds; NO + CHI: liquid lipase Novozym 51032 immobilized on chitosan powder; CALB: immobilized lipase B from *C. antarctica* as a reference. Means with the same capital letter A, B, or C in the case of liquid lipase Novozym 51032 immobilized on different supports, and a, b, or c in the case of all biocatalysts, did not differ significantly ($\alpha = 0.05$).

2.3. Protein Content and Specific Activity of Selected Immobilized Preparations in Comparison to the Native Forms of the Enzymes

The protein content and the specific hydrolytic and synthetic activity were determined for free lipase and for preparations with lipase Novozym 51032 immobilized on different supports (i.e., Lewatit VP OC 1600, chitosan, coffee grounds). Results were presented in Table 1. For each prepared biocatalyst, it was confirmed that the lipase had been well adsorbed, and the percentage of protein immobilization for the samples was 93.68%, 92.75%, and 88.26%, respectively. The scanning electron microphotographs presented earlier in

Section 2.1 also confirmed the presence of enzymes on the surface of the carriers. In comparison to free lipase, all of the biocatalysts had a higher catalytic activity, proving that physical adsorption is a proper method of immobilization for improving the hydrolytic and synthetic properties of enzymes. Yielding more active and selective biocatalysts—especially when used to catalyze complex reactions such as regioselective hydrolysis or synthesis—can be possible if physical adsorption takes place on hydrophobic supports, because it results in the lipase being mainly in its "open" form [7]. The physiological role of lipase is to catalyze the hydrolysis reaction, and this study showed that the best specific hydrolytic activity was that of the biocatalyst immobilized on the synthetic support Lewatit VP OC 1600 (0.314 U/mg protein), while for specific synthetic activity better results were obtained for lipase immobilized on biomaterials. There was only a slight difference between enzymes immobilized on chitosan (24.61 U/mg protein) and coffee grounds (22.23 U/mg protein). This may have been because the enzymes were located inside the pores of the support, protecting them against medium alterations and enabling transesterification reactions to take place under hydrophobic medium conditions [1]. The low specific synthetic activity of the biocatalyst immobilized on Lewatit VP OC 1600 may have been caused by lipase's formation of dimers or aggregates, as shown in Figure 1E,F, which may have negatively affected the immobilization [7].

Table 1. The protein content, specific hydrolytic activity, and specific synthetic activity of various free and immobilized lipases: NO + L: liquid lipase Novozym 51032 immobilized on Lewatit VP OC 1600; NO + C: liquid lipase Novozym 51032 immobilized on coffee grounds; NO + CHI: liquid lipase Novozym 51032 immobilized on chitosan powder.

	Protein Content (mg/mL)			Specific Hydrolytic Activity (U/mg Protein)		Specific Synthetic Activity (U/mg Protein)	
Preparations	**Free Lipase**	**Filtrate after Immobilization Process**	**% Protein Immobilization**	**Free Lipase**	**Immobilized Preparations**	**Free Lipase**	**Immobilized Preparations**
NO + L		1.27 ± 0.80	93.68		0.314 ± 0.013		9.27 ± 0.68
NO + CHI	20.13 ± 2.84	1.46 ± 0.69	92.75	0.022 ± 0.002	0.259 ± 0.011	5.20 ± 0.14	24.61 ± 2.51
NO + C		2.36 ± 0.71	88.26		0.021 ± 0.001		22.23 ± 2.48

3. Materials and Methods

3.1. Materials and Biocatalysts

In the present study, as a support for lipase immobilization, the following materials were used: chitosan (Glentham Life Sciences, Corsham, UK), Lewatit VP OC 1600 (Lanxess, Cologne, Germany), and coffee grounds (household waste). Chemical reagents were purchased from Sigma-Aldrich (Poznań, Poland) and Avantor Performance Materials Poland S.A. (Gliwice, Poland). Immobilized lipase B from *Candida antarctica* (CALB) (Sigma-Aldrich, Poznań, Poland), liquid lipase Novozym 51032 from *Aspergillus oryzae* (Novozymes, Basgvaerd, Denmark), and supernatant from *Yarrowia lipolytica* were used as biocatalysts. The supports Lewatit VP OC 1600 and commercial lipase Novozym 51032 were kindly gifted by the companies Novozymes and Lanxess, respectively. The yeast strain *Yarrowia lipolytica* KKP 379 was purchased from the Collection of Industrial Microorganisms at the Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology State Research Institute in Warsaw, Poland. Furthermore, culture media and their components were acquired from BTL Sp. z o.o. (Łódź, Poland).

3.2. Culture Media and Yeast Cultivation

For yeast cultivation, YPO medium (2% peptone, 2% olive oil, 1% yeast extract) pH 5.0 was used, with 0.1% Tween 80 as an emulsifier. Inoculation was conducted by adding 0.1% (*v/v*) of a 24-h *Yarrowia lipolytica* KKP 379 inoculum in YPD medium (2% peptone, 2% glucose, 1% yeast extract) to 200 mL of sterile medium in flat-bottomed flasks, which

were then cultured on a rotary shaker (140 rpm) for 48 h. The obtained yeast culture was centrifuged, and the crude enzyme solution of *Y. lipolytica* extracellular lipases was separated from biomass.

3.3. Freeze-Drying and Purification of Yeast Lipase

The obtained supernatant of *Y. lipolytica* KKP 379 was divided and poured into Petri dishes and then lyophilized. The samples were frozen in an Irinox freezer (Corbanese, Italy) at −40 °C and then freeze-dried in the Christ Gamma 1-16 apparatus (Osterode am Harz, Germany). The materials were stored on shelves at a temperature of 0 °C. The lyophilized supernatant was purified by using ion-exchange chromatography (elution with a linear gradient with 0.7 M NaCl + 15 mM Tris-HCl, pH = 6.8, TRIS buffer) and molecular sieves (50 mM phosphate buffer, pH = 7.0). The obtained active fractions were concentrated in a centrifuge (4.000× *g*, 10 min) on a VIVASPIN Centrifugal Concentrator Membrane 10.000 MWCO PES (Sartorius, Göttingen, Germany).

3.4. Lipase Immobilization

3.4.1. Immobilization on Lewatit VP OC 1600

Immobilization on Lewatit was carried out according to the methodology described by Barrera-Rivera and Martínez-Richa [34], with slight modifications. Firstly, the beads were activated with ethanol at a ratio of 1:10 (beads:ethanol) for 5 h, and then they were filtered with distilled water and, finally, dried under vacuum at room temperature for 24 h. After that, 1 g of beads was added to 15 mL of different lipase solutions. For immobilization, 15 mL of supernatant from *Yarrowia lipolytica*, 1 mL of purified lipase from supernatant from *Yarrowia lipolytica*, and 1 mL of lipase Novozym 51032 were used. The lipase solutions and beads were shaken in a rotatory shaker at 4 °C for 14 h. After incubation, the immobilized beads were filtered off with distilled water and dried under vacuum at room temperature for 24 h.

3.4.2. Immobilization on Chitosan

Based on the methodology described by Pereira et al. [35], with slight changes, lipase was immobilized on chitosan by physical adsorption. Before immobilization, the chitosan (2 g) was soaked in 30 mL of hexane and agitated for 1 h. After that, the hexane was removed by filtration under a vacuum, and the chitosan was washed with distilled water. The volume of lipase solution used was 20 mL of supernatant from *Yarrowia lipolytica* and 1 mL of lipase Novozym 51032 (14 mL of distilled water). The lipase solution and chitosan were mixed together for 3 h at room temperature in an Erlenmeyer flask, and then for an additional period of 18 h they were moved under static conditions at 4 °C. At the end, the immobilized preparations were filtered under vacuum, washed with distilled water, and dried at room temperature.

3.4.3. Immobilization on Coffee Grounds

Firstly, it was necessary to purify the coffee grounds before beginning the immobilization. Briefly, spent coffee grounds were subjected to extraction processes with the Soxhlet apparatus, and a 1:15 (*w*/*v*) ratio of coffee grounds to solvent was used. First of all, water was used as a solvent, and the extraction lasted about 4 h. Subsequently, *n*-hexane and then ethanol were used, where the solvents overflowed the Soxhlet chamber 12 times. The multistep extractions allowed for the removal of proteins, polyphenols, terpenes, and oils [36]. The immobilization procedure on coffee grounds was based on the methodology presented by Buntic et al. [18], with slight modifications. Lipase solution (1 mL of Novozym 51032 and 14 mL of distilled water) was agitated with 1 g of the prepared supports for 2 h. After that, the immobilized lipase was filtered under a vacuum, washed with distilled water, and dried at room temperature.

3.5. Lipase Activity Assay

3.5.1. Hydrolytic Activity

Measurement of hydrolytic activity was carried out via a spectrophotometric method based on the hydrolysis of *p*-nitrophenyl laurate. The reaction was carried out in Eppendorf test tubes. The 100 µL of free liquid lipase or 25 mg of immobilized biocatalyst in 100 µL of distilled water was stirred at 37 °C with 25 µL of 0.3 mmol *p*-nitrophenyl laurate dissolved in 2 mL of heptane. After 15 min of incubation, absorbance was measured at 410 nm with a UV–Vis spectrophotometer. The unit of lipase enzymatic activity was 1 U, i.e., the amount of enzyme that released 1 µmol *p*-nitrophenol per minute under the assay conditions.

3.5.2. Synthetic Activity

The synthetic activity of the immobilized lipase was checked using the colorimetric method developed by Zheng et al. [33], with some modifications. The measurement was based on transesterification between vinyl acetate and *n*-butanol. MBTH (3-methyl-2-benzothialinone) reacts with released acetaldehyde to produce the appropriate aldazine, which is converted to a blue-colored TAPMC (tetraaza-pentamethincyanine). The reaction was conducted in an Eppendorf tube and included 100 mM vinyl acetate and 100 mM *n*-butanol in 1 mL of hexane. To induce transesterification, 5 µL of free liquid lipase or 5 mg of immobilized lipase was added. Incubation was carried out for 5 min, at 30 °C, with agitation. The control experiment was conducted without the addition of an enzyme. After that, diluted samples (200 times, or in accordance with the requirements) for spectrophotometric measurement were prepared in test tubes. The assay began by adding 1 mL of 0.1% (*m*/*v*) MBTH solution to each sample and mixing for 10 min at 30 °C. Then, 0.4 mL of 1% (*m*/*v*) $H_4FeNO_4S_2 \cdot 12H_2O$ solution (in 0.1 M HCl) was added and agitated for another 30 min at 30 °C. The analytical wavelength was determined based on the UV–Vis spectrum for derivatives of acetaldehyde (Figure 7). Colorimetric measurements were carried out at 595 nm in a spectrophotometer. The standard curve was prepared by using different concentrations of acetaldehyde (Figure 8). The unit of lipase synthetic activity was 1 U, i.e., the amount of enzyme that converted 0.1 mmol vinyl acetate into acetaldehyde per minute under the assay conditions.

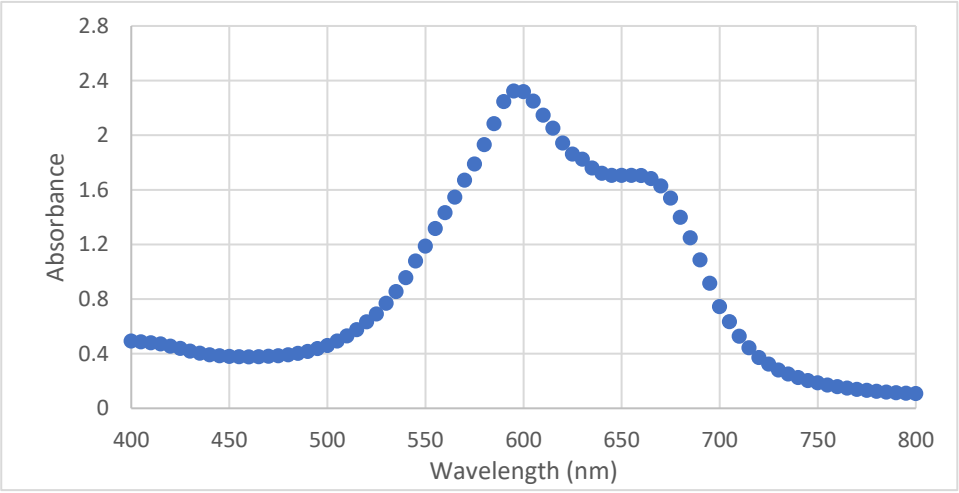

Figure 7. UV–Vis spectrum for derivatives of acetaldehyde.

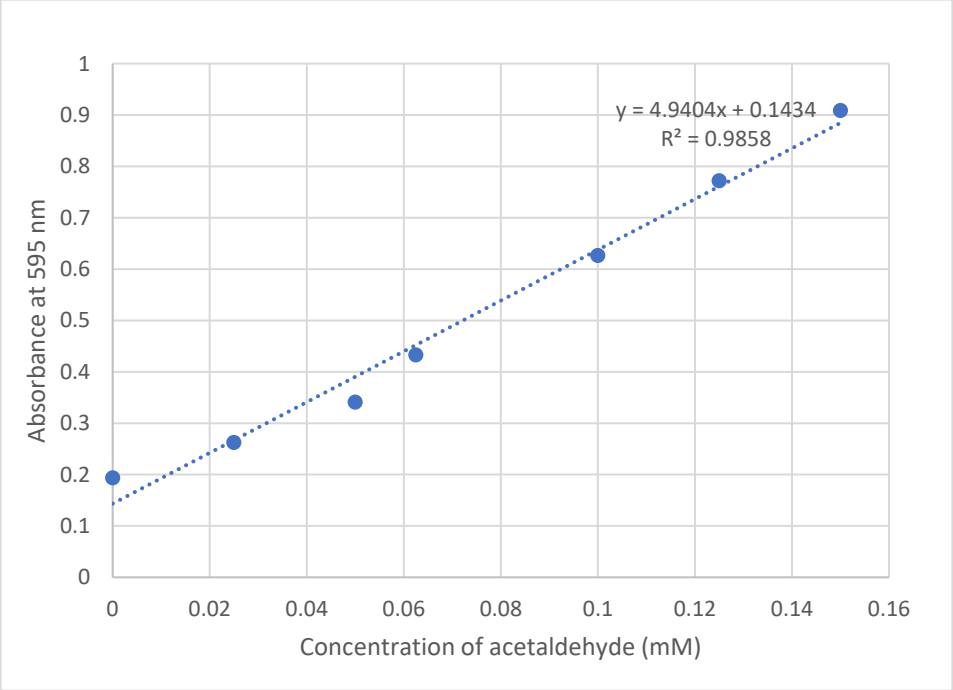

Figure 8. Standard curve of acetaldehyde by MBTH derivatization against absorbance at 595 nm.

3.6. Protein Content

The protein concentration in free lipase and in filtrates after immobilization was indicated spectrophotometrically using Lowry's method [37]. This process is based on the reaction between peptide bonds and aromatic amino acids with Folin–Ciocâlteu phenol reagent. For measurement, 1 mL of each tested solution was used. Fiftyfold dilutions of the samples were also prepared. The reaction was carried out in probes, to which 5 mL of copper reagent (2% Na_2CO_3 in 0.1 M NaOH, 1% $CuSO_4$, and 2% potassium sodium tartrate at a ratio of 100:1:1) was added. After 10 min, 0.5 mL of Folin–Ciocâlteu phenol reagent was added. The incubation lasted 30 min, and then the measurements were taken at 750 nm with a Rayleigh UV-1601 spectrophotometer (BRAIC, Beijing, China). A calibrated curve obtained with albumin as a standard was used to calculate the protein content. The amount of adsorbed protein on the particles was estimated based on differences between free lipase and the enzyme solution after immobilization.

3.7. Scanning Electron Microscopy (SEM)

The surface and morphology of the supports and immobilized preparations were studied using an electron microscope (HITACHI TM 3000, Ramsey, New Jersey, USA). Before observation, the samples were dried under a vacuum and coated with a layer of gold (Cressington Sputter Coater 108 auto, Cressington Scientific Instruments, Watford, UK). Microphotographs were taken at a magnification of 100, 200, and 400x.

3.8. Statistical Analysis

The obtained results of the conducted studies were analyzed by statistical methods, using the STATISTICA 13 program (StatSoft, Krakow, Poland). The Shapiro–Wilk test was used to verify the statistical hypothesis of normality of the distribution of the experimental data, while Levene's test and the Brown-Forsythe test were used to check the hypothesis of homogeneity of variance. The significance of the grouping variables was assessed by conducting an analysis of variance (ANOVA). Homogeneous groups for the experimental data that met the assumption of normal distribution were separated using Tukey's test. Values of $p \leq 0.05$ were considered to be statistically significant.

4. Conclusions

In this study, we investigated the process of immobilization of various microbial lipases on different supports. The obtained results proved the diverse influence of the used carriers (i.e., chitosan, coffee grounds, and synthetic Lewatit VP OC 1600) on the catalytic activity of the immobilized biocatalysts. The most promising results were achieved for coffee grounds and chitosan, which have potential in enzyme catalysis due to their biological origins, biodegradability, and susceptibility to chemical and/or physical modifications. Reusing food waste can provide more sustainable supports for enzyme immobilization. To the best of the authors' knowledge, this is one of the first papers to take into account immobilization on spent coffee grounds and chitosan liquid lipase Novozym 51032 from *Aspergillus niger*, opening new prospects for future researches. Moreover, it was found that, depending on the enzyme's biocatalytic properties, it is necessary to choose a specific support for the immobilization. Lipases are versatile catalysts with many contrasting activities, e.g., hydrolysis, esterification, transesterification, and aminolysis. It is crucial to indicate the types of reactions in which the prepared biocatalyst will be used, because of the differences in hydrolytic and synthetic activity observed in this study.

Author Contributions: Conceptualization, K.J. and A.F.; methodology, K.J., U.J. and B.Z.; formal analysis, K.J.; investigation, K.J. and U.J.; resources, A.F. and U.J.; data curation, K.J.; writing—original draft preparation, K.J.; writing—review and editing, B.Z., A.F. and U.J.; visualization, K.J.; supervision, A.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Acknowledgments: Two materials used for experiments were kindly gifted to us: the support Lewatit VP OC 1600 was donated by Lanxess, and the liquid lipase Novozym 51032 was donated by Novozymes.

Conflicts of Interest: The authors declare no conflict of interest.

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