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Sequential Solid-State and Submerged Fermentation to Increase *Yarrowia lipolytica* Lipase Production from Palm Oil Production Chain By-Products

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Abstract: This study investigates the potential of sequential solid-state and submerged fermentation (SeqF) to enhance lipase production by *Yarrowia lipolytica* using by-products from the palm oil production chain. Palm fiber and palm oil deodorizer distillate (PODD) were utilized as substrates in both fermentation stages. Solid-state fermentation (SSF) yielded significant lipase activity when palm fiber was used alone (1.55 U/g in 48 h), while submerged fermentation (SmF) showed improved enzymatic production with the combination of fiber and PODD (1171 U/L in 72 h). The integration of SSF and SmF in SeqF achieved superior lipase activities, reaching 4464.5 U/L, an 8.3-fold increase compared to SmF alone, in Erlenmeyer flasks. SeqF-lyophilized biocatalysts from Erlenmeyer experiments showed better hydrolytic activity (131 U/g) when the best conditions were reproduced in a 4 L bioreactor (33 U/g). The SeqF-lyophilized biocatalyst was employed in esterification reactions to synthesize mono- and diacylglycerols, achieving a 24.3% conversion rate. The study highlights SeqF as a promising and sustainable approach for valorizing agro-industrial residues, contributing to biocatalyst production and advancing circular bioeconomy initiatives.

Keywords: enzyme production; agro-industrial by-products; sequential fermentation; *Yarrowia lipolytica*

Academic Editors: Hongbo Liu and Hongxiao Guo

Received: 2 December 2024
Revised: 18 December 2024
Accepted: 19 December 2024
Published: 24 December 2024

Citation: Souza, C.P.L.; Pereira, A.d.S.; Aguieiras, É.C.G.; Amaral, P.F.F. Sequential Solid-State and Submerged Fermentation to Increase *Yarrowia lipolytica* Lipase Production from Palm Oil Production Chain By-Products. *Fermentation* **2025**, *11*, 3. https://doi.org/10.3390/fermentation11010003

1. Introduction

Palm plant (*Elaeis guineensis*) belongs to the Arecaceae family and is a humid tropical crop, growing in areas where temperatures range from 22 °C to 33 °C [1]. Palm oil is derived from the plant's fruit, which generates two types of oils: crude palm oil (CPO), from the mesocarp of the fruit, and palm kernel oil, from the seed in the fruit. CPO, which is red in color, is mostly used for food products, whilst palm kernel oil (light yellow), is largely utilized in nonedible products, such as plastics, cosmetics, detergents, and other industrial and agricultural chemicals [2]. Indonesia and Malaysia produced, in 2018, 60 million tons of crude oil and are the two top producers in the world [3].

Palm oil deodorizer distillate (PODD) is a by-product from the CPO refining process and is composed mainly of free fatty acids (FFAs) (>85%). Other non-triacylglycerides (TAG) components and unsaponifiable compounds are also detected in PODD (1.61%), such as higher aliphatic alcohols, sterols, pigments, hydrocarbons, and squalene, which

can promote undesirable flavor, odor, and appearance in edible oils [4,5]. No commercial value is attributed to PODD; however, it could be considered a promising feedstock to synthesize commercially valuable products, such as biodiesel, biolubricants, emulsifiers, and enzymes [6–8]. PODD was used to induce lipase production via submerged fermentation by *Yarrowia lypolytica*, resulting in approximately 2300 U/L of enzyme activity after a 48 h process using 1000 mL Erlenmeyer flasks with 200 mL of the production medium and 2.5 g/L of PODD at 28 °C and 250 rpm. This result shows the potential of this feedstock since a higher value was obtained in 48 h in comparison to other oily wastes [9].

Another co-product of the palm-processing chain is the fiber, which is the result of the milling and extraction of palm oil. Palm fiber is largely used to feed the industries' boilers, in animal food enrichment, and as an organic fertilizer [10]. Palm fiber is mainly constituted by a lignocellulosic material, with lignin representing the most abundant compound (41.5%), followed by cellulose (33.8%) and hemicellulose (12.4%). Its water content is about 4.3%, and its lipid and protein compositions are, respectively, 12.9% and 4.9% [8]. According to a Brazilian palm oil industry, in 2021, around 68% of palm oil production resulted in low-value residues, and fiber represented 30% of this amount. Although palm fiber has a high calorific value, enabling its use for energy generation in industries and as organic fertilizer, it holds limited economic value in the international market. Additionally, during CPO production, 3–10% of palm oil decanter distillate (PODD) is generated. However, its market price is significantly lower compared to refined oil, at USD 397.54/tonne versus USD 493.90/tonne, as reported by the Malaysian palm oil board (MPOB) in 2019 (MPOB, Overview of the Malaysian oil palm industry 2019). As agri-food wastes still lack efficient management, palm fiber shows an interesting potential as an alternative source to obtain high-value products [10]. Palm fiber has been used as solid support for lipase production by solid-state fermentation (SSF). With *Rhizomucor miehei*, a filamentous fungus, an enzyme activity of 30 U/g was obtained after a 72 h process [8]. A similar result was also reported by Ávila et al. [11] when SSF was performed using babassu cake and palm fiber with *R. miehei*, resulting in 28 U/g of lipase activity. Palm fiber and palm alkaline soapstock were also used to produce lipase via SSF using *Aspergillus niger*, reaching 77 U/g of enzyme activity [12]. However, there are no reports in the literature regarding the use of palm fiber to produce lipases from *Yarrowia lipolytica*.

Y. lipolytica is a strict aerobic yeast that can be isolated from dairy products and other lipid-rich environments, such as sewers, polluted by oils and marine or hypersaline ecosystems [13]. The microorganism is the most studied non-conventional yeast [14], considered non-pathogenic, and has received GRAS (generally regarded as safe) status by the Food and Drug Administration (FDA) [15]. *Y. lipolytica* is recognized as one of the most studied unconventional yeasts due to its metabolic versatility and applications in industrial biotechnology [16]. Unlike *Saccharomyces cerevisiae*, traditionally used in alcoholic fermentation, *Y. lipolytica* stands out for its high capacity to metabolize hydrophobic substrates [13]. In addition, *Y. lipolytica* also assimilates and ferments different hydrophilic carbon sources, such as glucose, glycerol, alcohols, and acetate, producing several important metabolites [17]. *Y. lipolytica* produces high amounts of lipase, a triacylglycerol hydrolase enzyme that catalyzes the hydrolysis of ester bonds of tri-, di-, and monoglycerides in free fatty acids and mono- and diacylglycerols. In the absence of moisture or in low moisture content, these enzymes can catalyze interesterification, transesterification, and esterification reactions [18]. *Y. lipolytica* can produce intracellular (Lip 1, Lip 3, and Lip 6), extracellular (Lip 2), and membrane-bound lipases (Lip 7 and Lip 8) [15,19].

Solid-state fermentation (SSF) microorganisms are cultivated in the absence or near absence of free water. The presence of water in the exact quantity is essential to enable the diffusion of solutes, gas, and temperature and allow the production of the target metabolites

by the microorganism [20]. In comparison to SmF, SSF presents a greater concentration and yield of metabolites of interest produced and higher stability of the products. SSF is also advantageous in relation to media cost, which can be industrial residues or by-products, generally cheaper than usual production media compounds [21]. Castilho et al. [22] reported an economic analysis comparing Smf and SSF and found that the total capital investment needed for *Penicillium restrictum* lipase production is 78% lower for SSF than for SmF. Moreover, a unitary product from SSF costs 47% less than its selling price. On the other hand, SSF confers difficulty to scale up and is a highly heterogeneous system. Furthermore, the difficulty in controlling operational conditions, such as temperature, pH, moisture content, aeration, and nutritional conditions, is a drawback [23]. When soybean meal was used in SSF with *Y. lipolytica*, the resulting lipase activity was 107 U/g after a 14 h process, and this catalyst was used to produce wax esters [24].

Submerged fermentation (SmF) is a liquid culture containing soluble nutrients and controlled conditions of temperature, pH, pressure, and aeration. Nutrients, air, and temperature are uniformly distributed in the medium, enabling microorganisms to grow and produce metabolites homogeneously [25]. In contrast to SSF, with SmF parameters, control is easier, enabling process automation. However, in SmF, a more diluted product is obtained, and there is a higher risk of contamination [26]. Diniz et al. [27] used pomegranate seed residue in SmF for *Y. lipolytica*'s growth to produce a biocatalyst with hydrolysis and synthesis capacity. The catalyst presented 4000 U/L of enzyme activity and was able to synthesize structured lipids.

Considering that both SSF and SmF have advantages and disadvantages, the integration of these processes could enable better results. Sequential solid-state and submerged fermentation (SeqF) consists of solid-state fermentation (SSF) followed by submerged fermentation (SmF) in order to maximize the production of desired metabolites and increase the activity of the enzymes [28]. Lunprom et al. [29] used microalgae in sequential fermentation (anaerobic solid-state fermentation followed by dark fermentation) to produce bio-hydrogen, resulting in a greater yield than each isolated process. Corncob, cottonseed hull, and poplar wood were used as solid substrates in SSF for 24 h, and a nutrient medium was added in subsequent SmF to produce laccase. Higher enzyme activity (192 U/L) was detected in comparison to SSF (28 U/L) or SmF (15 U/L) alone [30]. Sequential solid-state and submerged cultivation was also carried out in Erlenmeyer flasks using sugarcane bagasse as a solid substrate, and endoglucanase production by *Aspergillus niger* was assessed. Maximum enzyme activity was found after 72 h of SeqF (1052 U/L), which is higher than the 824 U/L achieved in SmF [31].

Although studies on SeqF are scarce, this process represents a promising strategy for valorizing agro-industrial solid wastes, as it leverages the unique advantages of each fermentation type while mitigating some of their inherent constraints [32]. The aim of this study was to evaluate the production of lipase by *Yarrowia lipolytica* through sequential solid-state and submerged fermentation (SeqF) using by-products from the palm oil production chain, specifically palm fiber and palm oil deodorizer distillate (PODD), as substrates, and test the biocatalyst in synthesis reactions. The novelty of this work lies in the innovative application of SeqF with *Y. lipolytica*, combining the advantages of solid-state and submerged fermentation to enhance biocatalyst production while valorizing agro-industrial residues in a sustainable and circular bioeconomy approach.

2. Materials and Methods

2.1. Materials

Palm oil deodorizer distillate (PODD) and palm fiber originating from the palm oil refining process were kindly provided by Companhia Refinadora da Amazônia (Grupo

Agropalma, Belém, Brazil) and were kept in sealed flasks at room temperature. Figure 1 shows the image of these materials. Peptone and yeast extract were obtained from Kasvi (Paraná, Brazil) and glucose from Isofar (Rio de Janeiro, Brazil). 4-nitrophenyl laurate (4-NPL) was purchased from Sigma-Aldrich Brazil (São Paulo, Brazil), and dimethyl sulfoxide was obtained from Isofar (Rio de Janeiro, Brazil).

(**a**) (**b**)

Figure 1. Palm oil deodorizer distillate (PODD) (**a**) and palm fiber (**b**).

2.2. Pre-Inoclulum

The wild-type strain of *Yarrowia lipolytica* isolated from Baía de Guanabara, Rio de Janeiro, Brazil [33], was incubated at 4 °C on YPD-agar medium. For pre-inoculum, cells were cultivated at 28 °C in a rotary shaker at 160 rpm in 500 mL flasks containing 200 mL of YPD medium (*w*/*v*: yeast extract 1%, peptone 2%, glucose 2%) for 72 h. Cells from the pre-culture were centrifuged (2000× *g*) and used to inoculate lipase production medium in sufficient amounts to obtain 1 mg dry weight of cells·mL^{-1}.

2.3. Solid-State Fermentation (SSF)

Palm fiber, the resulting product from palm oil extraction, was used as solid support for cell growth during fermentation. Palm fiber was ground in a laboratory mill and separated in a sieve, resulting in a particle size of less than 1.18 mm. The fermentations were carried out in 500 mL Erlenmeyer flasks containing 10 g of palm fiber and 1.5% (*w*/*w*) of PODD as a lipase inducer. The flasks were sterilized at 121 °C for 20 min, and then moisture was adjusted to 55% with the YP medium (*w*/*v*: yeast extract 1%, peptone 0.64%), according to the raw material (*w*/*w*). The inoculum was performed by distributing the cells from the pre-inoculum with a pipette along the solid to achieve a concentration of 0.7 mg of dry biomass per g of palm fiber. Then, the flasks were incubated in an incubator chamber with 90% adjusted moisture at 28 °C for 72 h [8,34].

For each sample (every 24 h), 50 mL of potassium phosphate buffer, 50 mM pH 7.0, was added to the fermented solid (the content of the whole flask) and incubated in an orbital shaker at 37 °C and 200 rpm for 20 min to extract the enzyme. After that, the material was manually pressed, resulting in a crude enzyme extract, which was centrifuged (4000× *g* for 5 min) and stored at −18 °C for a few days.

2.4. Submerged Fermentation (SmF)

Lipase production by *Y. lipolytica* in SmF was performed in 200 mL of YP medium (*w*/*v*: yeast extract 1%, peptone 0.64%) with 15 g/L of PODD and/or 50 g/L of palm fiber in 1000 mL Erlenmeyer flasks. Flasks with the medium were sterilized for 20 min at 1 atm and then inoculated with cells at a 1 g dry weight of cells/mL concentration. After

the inoculation, the flasks were incubated at 28 °C in a rotary shaker at 250 rpm for 96 h. For lipase determination in a free-cell medium, samples were taken and then centrifuged (4000× *g*) for 10 min.

2.5. Sequential Solid-State and Submerged Fermentation (SeqF) in Erlenmeyer Flasks

Lipase production by *Y. lipolytica* using sequential fermentation was carried out in 500 mL Erlenmeyers flasks, beginning with SSF, exactly as described for this process. After 24, 48, or 72 h of SSF, the culture was continued in submerged fermentation (SmF) by adding 200 mL of YP* medium (yeast extract 1%, peptone 0.64%). SmF was conducted in an orbital shaker for 96 h at 28 °C and 250 rpm. Samples were taken periodically and centrifuged at 4000× *g* to determine extracellular lipase activity.

After SeqF, three lipase fractions were obtained: the solid obtained by centrifuging the SeqF content at 4000× *g* for 15 min, the supernatant from this centrifugation, and the whole content from the SeqF. All three fractions were lyophilized for 48 h, until the water content was less than 3%.

Sequential Solid-State and Submerged Fermentation in Bench Bioreactor

Lipase production by *Y. lipolytica* by SeqF was carried out in a 4 L bench bioreactor with three Rushton impellers with six vertical blades and a 4.7 cm diameter. The process was initialized with SSF, using 150 g of palm fiber inoculated with 0.7 mg of dry biomass per g of palm fiber and incubated in an incubator chamber with 90% of adjusted moisture at 28 °C. After 24 h of SSF, the culture was continued in SmF by adding 3 L of YP* medium (yeast extract 1%, peptone 0.64%), and airflow was set at 1.5 L/min. SmF was conducted in the bioreactor for 48 h at 28 °C and 600 rpm.

2.6. Determination of Lipase Activity

Determination of enzymatic activity of lipase was performed by hydrolysis of 4-NPL (4-nitrophenyl laurate). In this method, the substrate is prepared by dissolving 4-NPL in dimethyl sulfoxide (DMSO) and mixing it with 50 mM potassium-phosphate buffer (pH 7.0) to obtain 560 μM of 4-NPL. The substrate is incubated at 37 °C and then mixed with the enzyme. The production of 4-nitrophenol, which is a product of the enzymatic reaction, is monitored in a spectrophotometer at λ = 410 nm (Figure 2). One lipase unit (U) is defined as the amount of enzyme that releases 1 μmol of 4-nitrophenol per minute at pH 7.0 and 37 °C [35].

2.6.1. Enzymatic Crude Extract

For the enzymatic crude extract (extracellular lipase in the liquid phase), 0.1 mL of this extract is mixed with 1.9 mL of the substrate (560 μM of 4-NPL). The production of 4-nitrophenol is monitored by 100 s in a spectrophotometer at 410 nm.

2.6.2. Lyophilized Biocatalyst Obtained by SeqF

Biocatalysts from SeqF were lyophilized at −55 °C and 500 μHg for 48 h in a Liotop L108 freezer dryer (Liobras, São Carlos, Brazil). Determination of enzymatic activity of lipase was performed by adding 25 mL of the substrate (560 μM of 4-NPL) into 10 or 20 mg of lyophilized biocatalysts. This system was kept under magnetic stirring at 37 °C for 5 min. The absorbance at 410 nm in 30 s intervals was measured until it was completed after 5 min (Figure 2).

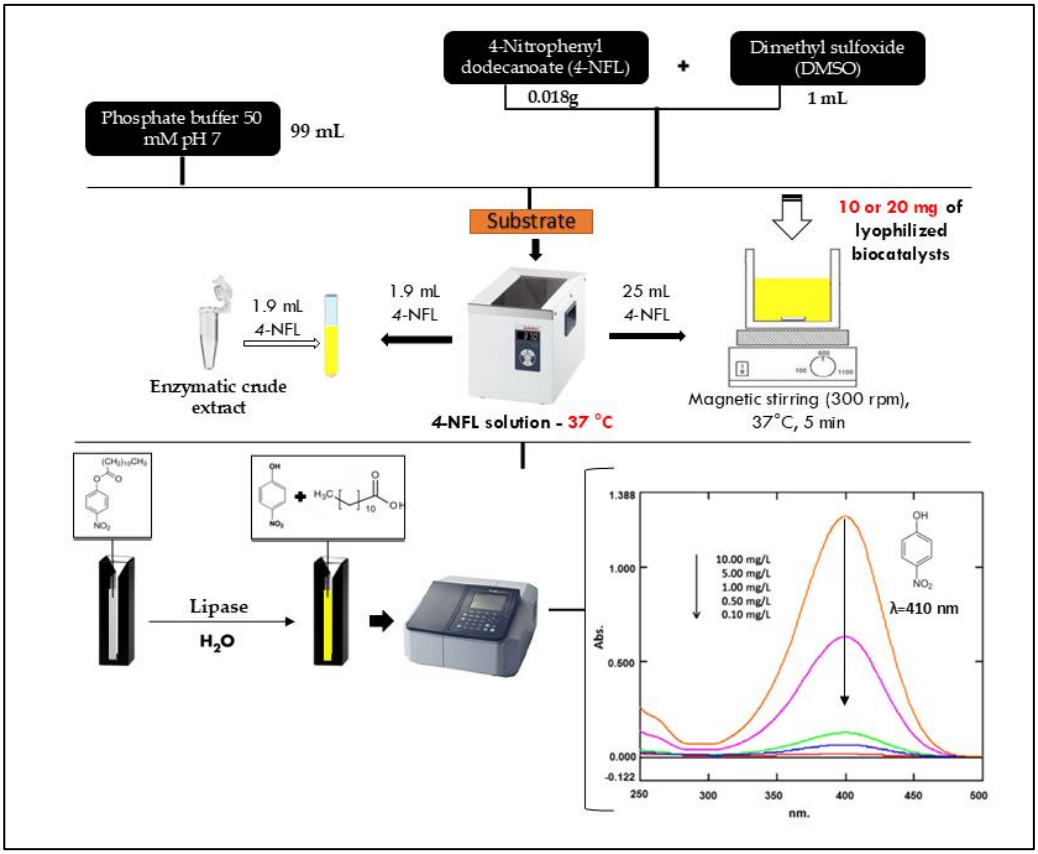


Figure 2. Schematic representation for determining the hydrolysis activity of *Yarrowia lipolytica* lipase.

2.7. Esterification Reaction

Esterification reactions were performed using oleic acid and glycerol in a 125 mL Erlenmeyer flask at 37 °C for 72 h in a water bath with reciprocal movement, as reported by Zhao et al. [36]. The molar ratio of glycerol/oleic acid was 6:1, and 10% of water (*w*/*w*) was also used. Reactions with lipase as a biocatalyst were conducted with 2.5% (*w*/*w*) of lyophilized supernatant from SeqF and lyophilized lipase from *Candida rugosa* (Sigma) as a positive control. As a negative control, oleic acid, glycerol, and water were used.

Fatty Acid Content Analysis

The residual fatty acid content of the esterification reactions was measured through a modified Lowry and Tinsley method [37,38]. In this method, 0.1 mL of the reaction medium was placed in glass tubes with 3 mL of the reaction solvent (*n*-hexane) and 1.5 mL of a 5% copper acetate-pyridine solution (pH 6–6.2). Then, the mixture was stirred for 30 s in a vortex mixer, and the supernatant was measured at 715 nm in a spectrophotometer. Each reaction was evaluated in triplicate, and the conversions were calculated as in Equation (1).

$$C\ (\%) = 100 - [(IA \times 100)/FA)] \quad (1)$$

where

C (%) = conversion.
IA = initial absorbance of sample before reaction.
FA = final absorbance of sample after reaction.

2.8. Statistical Analysis

Statistical analyses were performed using STATISTICA software version 7.1 (StatSoft, Inc., Tulsa, OK, USA). Results were considered statistically significant for *p*-values less than 0.05. Differences between means were evaluated using Tukey's test.

3. Results and Discussion

3.1. Lipase Production in Solid-State Fermentation (SSF)

SSF was carried out in Erlenmeyer flasks with palm fiber or palm fiber and palm oil deodorizer distillate (PODD), as shown in Figure 3.

(**a**) (**b**)

Figure 3. Erlenmeyer flask with palm fiber for solid-state fermentation (**a**) and palm fiber with palm oil deodorizer distillate (PODD) after sterilization (**b**).

Figure 4 illustrates that the use of palm fiber alone results in significantly higher lipase production compared to its combination with PODD, achieving approximately double the enzymatic activity. Palm fiber primarily consists of lignin, cellulose, lipids, and hemicellulose [8]. According to Collaço et al. [8], the fungus *R. miehei* can utilize the lipid content in palm kernel cake as a growth substrate, as evidenced by its reduction after fermentation.

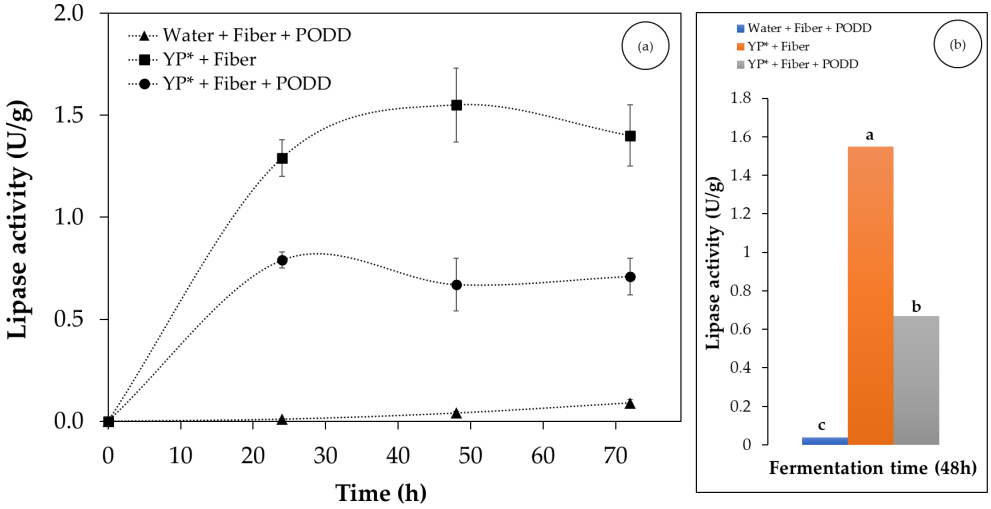


Figure 4. Lipase production by *Yarrowia lipolytica* during solid-state fermentation in 500 mL Erlenmeyer flasks containing 10 g of palm fiber with or without 1.5% (*w*/*w*) of palm oil deodorizer distillate (PODD) and moisture content adjusted with water or YP* medium. (**a**) Lipase production kinetic profile; (**b**) statistical difference observed in 48 h of fermentation—a–c, mean values with different letters differ from each other with a 95% confidence level by Turkey's test.

Similarly, in the present study, the lipid content in palm fiber was probably a good lipase inducer. The presence of PODD increased the lipid content of the medium, mainly fatty acids, which are also lipase inducers, but lower values of enzyme activity were found. This effect might be related to the waxy consistency of PODD, which could have interfered with the access of the microorganism to the fiber, and, also, it may have restricted medium aeration. Carvalho et al. [39] reported lipolytic activity in SSF of 4.36 U/g in 12 h using soybean meal and 13.84 U/g using andiroba oil cake in 24 h. A high protein content is found in soybean meal, while andiroba oil cake presents a greater oil amount.

To evaluate if lipase was produced before 24 h of SSF, the kinetics of the process was monitored for 30 h with sampling intervals of 2 h (Figure 5).

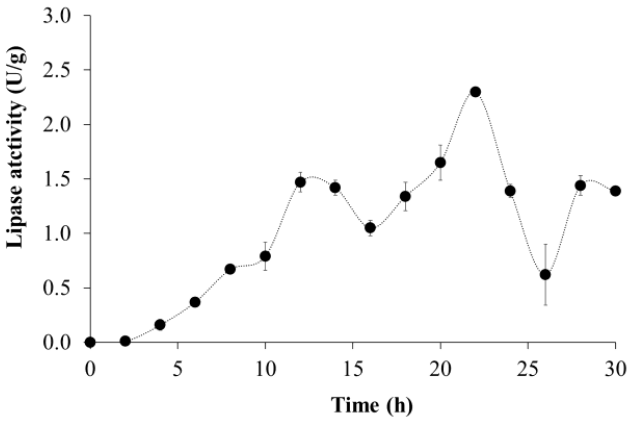


Figure 5. Lipase production by *Yarrowia lipolytica* during solid-state fermentation in 500 mL Erlenmeyer flasks containing 10 g of palm fiber and moisture content adjusted with YP* medium.

It is possible to observe that higher lipase activity occurs between 20 and 24 h, peaking at 22 h of SSF, with lipase productivity of 0.10 U/g h, although higher productivity is achieved at 12 h (0.12 U/g h). Souza et al. [34] used canola cake as a raw material to produce lipase in SSF and obtained a maximum of 91.7 U/g of enzyme activity after 28 h of fermentation. Soybean meal was also used in SSF by the authors, yet the fermentation reached 9.4 U/g of lipase activity after 10 h.

3.2. Lipase Production in Submerged Fermentation (SmF)

Lipase production during submerged fermentation was performed in a YP*-based medium, since yeast extract and peptone are usually good nitrogen sources for lipase production [35]. To test the use of palm oil chain by-products, the palm fiber, PODD, and both residues were added to the YP* medium (Figure 6).

Low values of lipase activity were detected in the control (YP* medium) (Figure 6), as expected, since no lipidic inducer was present in this medium. Diniz et al. [27] also reported similar results. Better results were obtained with the by-products of palm oil production. The best result was obtained with both residues (fiber and PODD), with a higher activity of 1171.7 U/L after 72 h. In the case of SmF, the presence of PODD did not hinder enzyme production. This can be explained by the fact that in SmF, the waxy material remains dispersed in the liquid medium, not interfering with aeration or hindering the microorganism's access to nutrients. On the contrary, this material may be gradually utilized by the yeast as a carbon source.

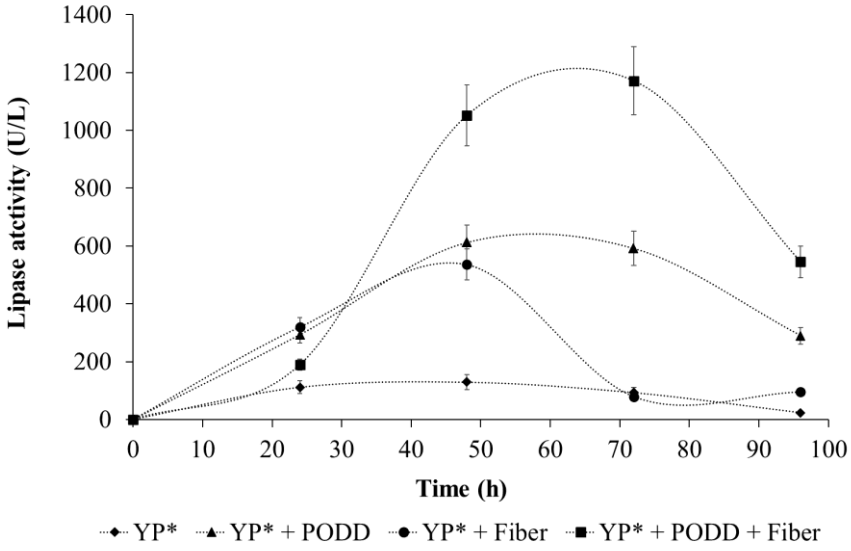


Figure 6. Lipase activity during growth of *Yarrowia lipolytica* in submerged fermentation in 1 L stirred flasks containing 200 mL of YP* medium (control) and YP* medium with PODD, YP* medium with palm fiber, and YP* medium with PODD and fiber.

3.3. Lipase Production in Sequential Solid-State and Submerged Fermentation (SeqF)

Lipase activity was evaluated in a sequential fermentation process (SeqF). The SeqF began with solid-state fermentation (SSF) conducted in 500 mL flasks containing 10 g of palm fiber and 1.5% PODD (*w*/*v*) for 72 h, with moisture content adjusted with the YP* medium. Given that PODD inhibited lipase secretion during SSF but induced the enzyme during SmF, SeqF with and without PODD in the SSF stage was investigated. Following this, 200 mL of the YP* medium was added to the flask, initiating a submerged fermentation (SmF) for an additional 72 h (Figure 7).

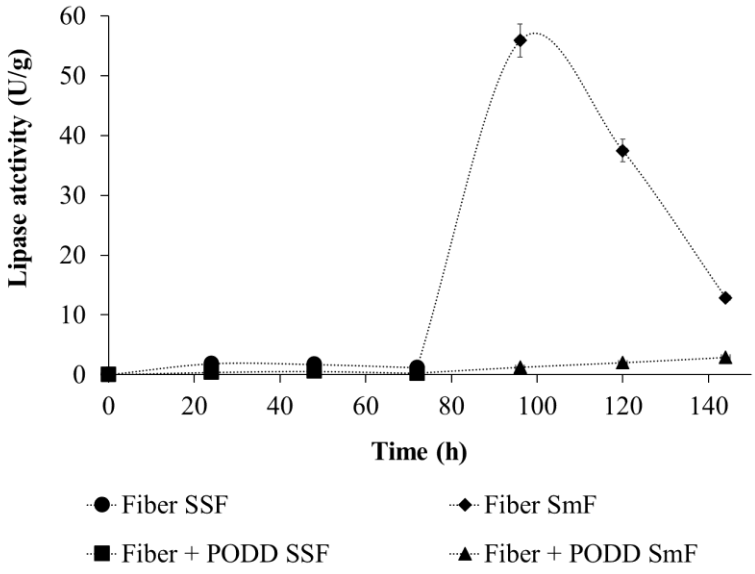


Figure 7. Lipase production by *Yarrowia lipolytica* during sequential solid-state and submerged fermentation (SeqF) in 500 mL flasks containing 10 g of palm fiber with or without 1.5% of PODD, with the addition of 200 mL of YP medium after 72 h. SSF: solid-state fermentation stage of SeqF; SmF: submerged fermentation stage of SeqF.

Higher lipase activities were observed when only palm fiber was used, peaking at 96 h (55.9 U/g), corroborating the results obtained with SSF. The sequential submerged fermentation in the presence of PODD was not able to reverse the negative impact of the

presence of this waxy material during SSF, and low values of lipase activity were observed in this process (Figure 7). Without PODD, a huge increase in lipase activity (around 30 times higher) was observed in the sequential fermentation during the submerged stage in comparison to the values obtained during SSF (1.9 g/L, after 24 h). In comparison to the process conducted through SmF (Figure 6, with a maximum lipase activity of 535.9 U/L for the palm fiber), the peak observed after 96 h in the current process (55.9 U/g) represents a 5.2-fold increase, since when converted to U/L, this corresponds to 2795 U/L. Even if compared with the condition that promoted higher lipase activity during SmF (Figure 6, Fiber + PODD, maximum lipase activity of 1171.7 U/L), the sequential fermentation favored lipase production (2.4-fold increase).

Since lipase activity in SSF peaked at 22 h, sequential fermentation (SeqF) was performed with the SmF initiating at this point. SeqF began with an SSF phase conducted in 500 mL flasks containing 10 g of palm fiber with or without PODD for 24 h. Subsequently, 200 mL of YP medium was added to the flask, initiating a submerged fermentation (SmF) for an additional 72 h in an orbital shaker (Figure 8).

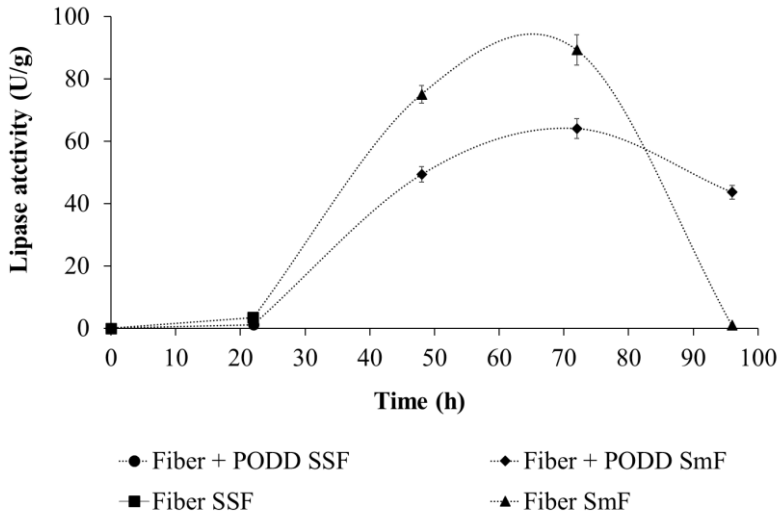


Figure 8. Lipase production by *Yarrowia lipolytica* during sequential solid-state and submerged fermentation (SeqF) in 500 mL flasks containing 10 g of palm fiber with or without 1.5% of PODD, with the addition of 200 mL of YP medium after 24 h. SSF: solid-state fermentation stage of SeqF; SmF: submerged fermentation stage of SeqF.

Combining palm fiber and PODD, lower values were achieved (3201.2 U/L after 72 h) (Figure 8), but it increased in relation to the previous SeqF (Figure 7), which might be related to the lower period in SSF. This result reinforces the hypothesis that PODD in SSF reduces the contact of the microorganism with the solid medium and the air, and, probably, after 72 h, microbial viability is significantly reduced. For the SeqF performed with only palm fiber, the highest lipase activity (89.3 U/g or 4464.5 U/L) was detected after 72 h, representing the best result and an 8.3-fold increase compared to the previously performed SmF with only palm fiber. Florêncio et al. [28] revealed that the SeqF for cultivating *A. niger* resulted in 3- and 8-fold higher xylanase and β-glucosidase activities, respectively, than the SmF process. Laccase productivity by *Pleurotus ostreatus* was also increased by SeqF, with a 2-fold and 7-fold increase in comparison to the submerged and solid-state methods, respectively [30].

Sequential solid-state and submerged fermentation has been developed mainly with the intent to produce enzymes in the SSF to hydrolyze the raw material, converting them into substrates for submerged fermentation [32]. A few works with filamentous fungi have used SeqF to boost enzyme production [28,30,31], which was explained by the maintenance

of a filamentous morphology of the fungus in SeqF during the submerged fermentation stage in contrast to the pellet formation in SmF [31]. In the present study, which is the first report in the literature of a sequential fermentation process with yeast, we believe that the beneficial effect of this approach might be related to the presence of the enzyme in the solid matrix, which is difficult to extract with a 30 min agitation in a buffer, as performed for SSF. Silva et al. [40] reported that the extraction of lipase from the solid-state fermentation with a buffer was higher after 30 min of agitation, with no increase in the enzyme activity for longer periods. However, when they added Tween 80 to the extraction buffer, a 30% increase in lipase activity was observed in the extraction solution. Carvalho et al. [39] supplemented with 0.001% (*w*/*v*) of Tween 80 the SSF of andiroba oil cake, soybean meal, and soybean oil and observed a 1.5-fold increase in lipase production by *Y. lipolytica*, resulting in 82.5 U/g. In the present work, in the SeqF, during the SmF stage, this enzyme can be released to the culture medium slowly while the microorganism continues to produce the biocatalyst, boosting the enzyme activity in a homogeneous medium.

To evaluate which biocatalyst produced during SeqF would be the best, the fermented fiber, the centrifuged fermented medium (supernatant), and the fermented fiber, together with the supernatant, were lyophilized, and their lipase activity was measured (Figure 9). Fractions obtained after 24 h (only SSF), 48 h (SSF 24 h + SmF 24 h), and 72 h (SSF 24 h + SmF 48 h) were tested.

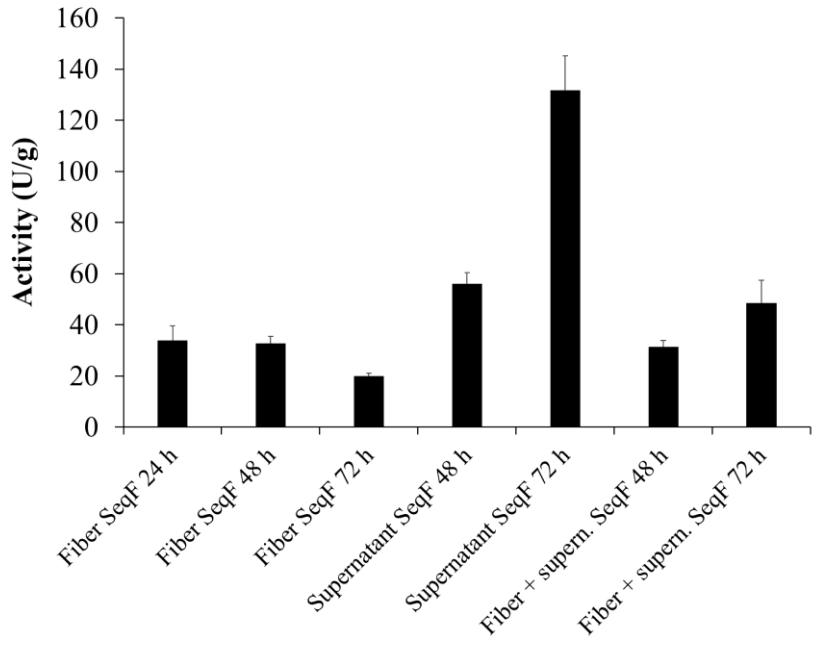


Figure 9. Lipase activity of lyophilized biocatalysts (fermented fiber (Fiber), the centrifuged fermented medium (Supernatant), and the fermented fiber + the fermented medium (Fiber + supern.)) obtained during sequential solid-state and submerged fermentation (SeqF) in 500 mL flasks containing 10 g of palm fiber, with the addition of 200 mL of YP medium after 24 h. Fractions obtained after 24 h (only SSF), 48 h (SSF 24 h + SmF 24 h), and 72 h (SSF 24 h + SmF 48 h).

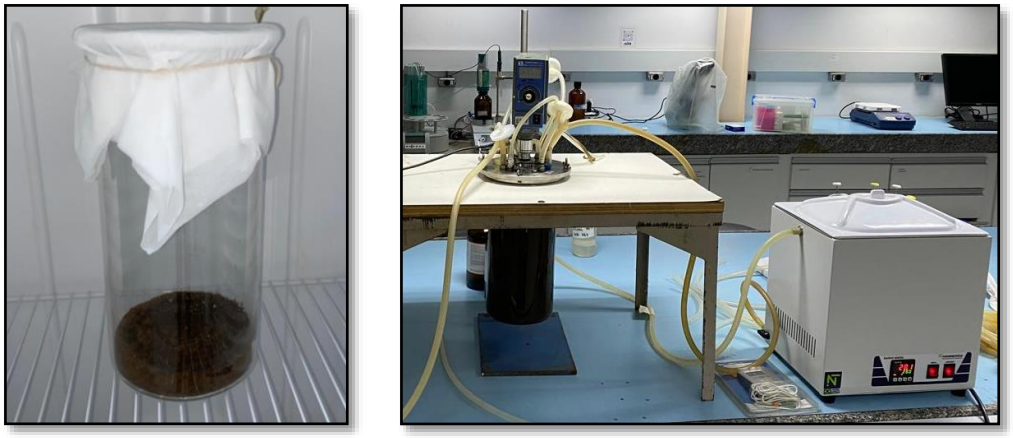
Figure 9 reinforces the hypothesis that the lipase that is adsorbed in the solid matrix (fiber) is released to the culture medium during the SmF stage of the SeqF since we detected a reduction of the enzyme activity for the lyophilized fiber and an increase in the supernatant fraction after 72 h.

The lyophilized supernatant of the 72 h SeqF showed the best lipase activity (131 U/g of catalyst) (Figure 9). Despite the lower activity observed for the fractions containing the fiber, the use of a biocatalyst immobilized on a solid support, such as the lyophilized fiber, presents a significant advantage as it facilitates enzyme reuse. In this case, the

best result was detected for the fiber with the supernatant after 72 h in SeqF (48.5 U/g). The dry fermented solid obtained from the solid-state fermentation of macaúba cake with *Rhizomucor miehei* resulted in 14.4 U/g of lipase hydrolytic activity after process optimization [41]. For the babassu cake fermented with *R. miehei*, a hydrolytic activity of 42 U/g was obtained [42].

Lipase Production by SeqF in 4 L Bioreactor

SeqF was performed in a 4 L bioreactor, beginning with an SSF phase in the 4 L bioreactor with 150 g of palm fiber for 24 h (Figure 10a). After that, the addition of 3 L of YP medium initiated the submerged fermentation (SmF) for an additional 48 h with mechanical agitation and aeration (Figure 10b).



(**a**) (**b**)

Figure 10. Sequential solid-state and submerged fermentation (SeqF) in 4 L bioreactor with 150 g of palm fiber with the addition of 3 L of YP medium after 24 h. SSF: solid-state fermentation stage of SeqF (**a**); SmF: submerged fermentation stage of SeqF (**b**).

After the supernatant was lyophilized, the maximum lipase activity obtained was 33 U/g after 72 h of fermentation (24 h of SSF and 48 h of SmF). This value was lower than the one found in fermentation using Erlenmeyer flasks, which can be attributed to the SSF stage of the fermentation, which was performed in the bioreactor vase. The bioreactor vase diameter is 137.5 mm, while the average diameter of the bottom of 500 mL Erlenmeyer flasks is 105.0 mm. Therefore, in the Erlenmeyer SSF process, the fiber density was 1.15 g/mm^2, whereas in the bioreactor, it reached 10.12 g/mm^2, nearly ten times higher. This fact may have compromised the aeration, which is an important substrate for *Y. lipolytica*. Even so, this was proof of concept, which demonstrated that the process could be carried out in a bioreactor. For further studies, the conditions of the SSF should be improved. Pomegranate seed residue was used in a bioreactor with *Y. lipolytica* for submerged fermentation with a medium supplemented with yeast extract and urea, and the dry fermented solid presented 70 U/g of enzyme activity [27]. Lipase production was also evaluated in a bioreactor in submerged fermentation using *Y. lipolytica* with mango tegument as a carbon source, and, after 38 h, an enzyme activity of 68 U/g was found when the lyophilized fermented solid was tested [43].

3.4. Enzyme Reactions with Biocatalyst Produced by SeqF in Bioreactor

To evaluate the potential of the biocatalysts produced during SeqF, the esterification of glycerol with oleic acid was performed in an attempt to produce monoacylglycerols and (MAGs) and diacylglycerols (DAGs), which are important emulsifiers [8]. Esterification reactions were carried out for 72 h with the lyophilized biocatalysts from SeqF performed

in the bioreactor (Figure 11). As the reactions occurred in solvent-free systems, they were performed in 125 mL open flasks to reduce water content through evaporation. Both biocatalysts produced during SeqF showed esterification activity, although low values were achieved. It seems that longer periods would be necessary to increase conversion. The best conversion was 24.3% when a lyophilized supernatant was used. The commercial enzyme, the lipase from *Candida rugosa*, showed a 59% conversion, which was expected since this enzyme is usually used for esterification reactions [44] and commercial preparation for optimized biocatalysts.

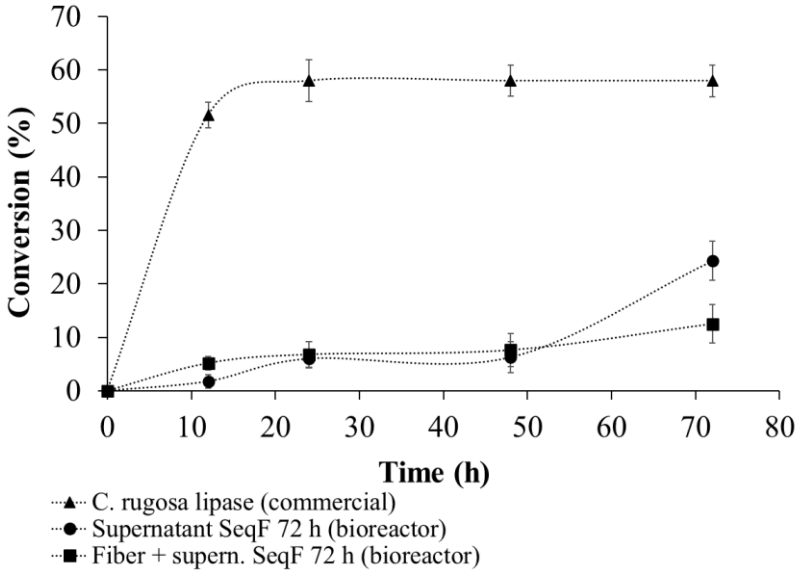


Figure 11. Conversion of glycerol and oleic acid using lyophilized biocatalysts from SeqF performed in bioreactor using *Yarrowia lipolytica* (the centrifuged fermented medium (Supernatant) and the fermented fiber + the fermented medium (Fiber + supern.) obtained after 72 h in SeqF) and a commercial *Candida rugosa* lipase in 125 mL flasks for 72 h. Molar ratio of glycerol/oleic acid was 6:1, and 10% of water (*w*/*w*) was used.

MAGs and DAGs were synthesized using a dry fermented solid from palm oil production waste in solid-state fermentation (SSF) with *R. miehei*, as reported by Collaço et al. [8]. Emulsifier production was monitored, and, after 48 h, the conversion of PODD and glycerol to MAGs and DAGs reached 80% under the following conditions: 50 °C, a molar ratio of 1:1 (glycerol:PODD), 9.6 wt% water content, and 20 wt% dried fermented solid. These results are substantially higher than those obtained in the present study. However, the lower conversion rates observed here may be attributed to increased viscosity in the reaction medium due to the lower reaction temperature, which limits substrate accessibility to the enzyme [45]. The optimization of reaction conditions could improve the performance of the biocatalyst produced herein.

4. Conclusions

The results of this study demonstrated that palm fiber and palm oil deodorizer distillate are effective substrates for inducing lipase production by *Yarrowia lipolytica* in submerged, solid-state, and sequential fermentations. Sequential fermentation (SeqF) showed the highest efficiency, with an activity of 4482 U/L (22.4 U/g of residue), surpassing the results obtained in SmF and SSF. Additionally, lyophilized biocatalysts from SeqF exhibited superior hydrolytic activity (131 U/g) and were able to synthesize mono- and diacylglycerols, achieving a 24.3% conversion with glycerol and oleic acid. These findings highlight the potential of SeqF as an efficient and sustainable strategy for valorizing agro-industrial

residues from the palm oil production chain, contributing to the production of high-value-added biocatalysts. Although promising, the study's results presented limitations, such as the reduction in hydrolytic activity during scaling up to a 4 L bioreactor, emphasizing the need for further optimization. Future research should explore more robust scaling-up conditions and expand the application of these biocatalysts to other industrial reactions. This work underscores the importance of biotechnological processes based on circular economy principles, offering a practical and economical solution for the utilization of agro-industrial residues.

Author Contributions: Conceptualization, A.d.S.P. and P.F.F.A.; methodology, C.P.L.S., P.F.F.A., A.d.S.P. and É.C.G.A.; formal analysis, C.P.L.S.; investigation, C.P.L.S.; resources, P.F.F.A.; data curation, C.P.L.S., P.F.F.A., A.d.S.P. and É.C.G.A.; writing—original draft preparation, C.P.L.S. and P.F.F.A.; writing—review and editing, C.P.L.S., P.F.F.A., A.d.S.P. and É.C.G.A.; visualization, C.P.L.S. and P.F.F.A.; supervision, P.F.F.A.; project administration, P.F.F.A.; funding acquisition, P.F.F.A. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ—grant number E-26/201.915/2020; E-26/210.821/2024), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq—Bolsa PQ: 304694/2022-3).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author due to privacy.

Acknowledgments: The authors acknowledge the Microbial Biotechnology Laboratory, Biochemical Department, Instituto de Química, Universidade Federal do Rio de Janeiro, for kindly providing the raw material PODD.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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