Received: 11 November 2022 | Revised: 17 June 2023 | Accepted: 3 July 2023

DOI: 10.1002/ejlt.202200196

European Journal of Lipid Science and Technology

RESEARCH ARTICLE

Lipase-mediated plant oil hydrolysis—Toward a quantitative glycerol recovery for the synthesis of pure allyl alcohol and acrylonitrile

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Funding information
German Federal Ministry of Education and Research, Grant/Award Number: 03SF0577A

Abstract

Plant based triacylglycerides are a sustainable feedstock for the chemical, bioenergy, food and cosmetic sector. While fatty acids conversion has been intensively studied, processes for glycerol valorization have been scarce. In contrast to chemical hydrolysis of plant triacylglycerides enzymatic hydrolysis methods provide a cleaner glycerol stream. This study focuses on the selection of a commercial lipase capable of quantitative hydrolysis of rapeseed- and high oleic sunflower oils. Enzymatic process using only water as the reaction medium allows simplified recovery of pure glycerol. Among the six commercial lipase preparations tested, *Candida rugosa* lipase was identified as the most effective biocatalyst. Catalytic behavior in buffer and pure water was equivalent. Glycerol generated using a washed lipase was recovered by just three purification steps. The FTIR spectrum of the recovered glycerol was equivalent to pure glycerol standard. Over the entire recovery process, 82%–88% of the theoretical glycerol yield could be obtained. Purified glycerol was further didehydroxylated to allyl alcohol by a formic acid mediated open distillation process. The enriched allyl alcohol had a quality, which allows to use it for the synthesis of bio-based acrylonitrile.

KEYWORDS
enzymatic hydrolysis, glycerol, high oleic sunflower oil, lipase, rapeseed oil, acrylonitrile, ally alcohol

1 | INTRODUCTION

Triacylglyceride's (TAG) derivatives, such as diacylglycerides (DAG), monoacylglycerides (MAG), fatty acids (FA), fatty acid esters, and glycerol, are important platform chemicals with various applications in food, feed, detergent, cosmetic, organic synthesis, and pharmaceutical industries. While vegetable oils and animal fats are natural sources of TAG, chemical glycerolysis, hydrolysis, and transesterification of TAG represent the current industrial routes for the production of their respective derivatives.[1]

Oleochemicals, including fatty acids, fatty acid esters, hydroxy fatty acids, fatty alcohols, fatty amine, and glycerol, cover a global market

Abbreviations: 10-HSA, 10-hydroxy stearic acid; BCL, Lipase from *Burkholderia cepacia*; CalA, Lipase A from *Candida antartica*; CalB, Lipase B from *Candida antarctica*; CRL, Lipase from *Candida rugosa*; DAG, diacylglyceride; FFA, free fatty acid; HOSO, high oleic sunflower oil; MAG, monoacylglyceride; PFL, Lipase from *Pseudomonas fluorescens*; TAG, triacylglyceride; TLL, Lipase from *Thermomyces lanuginosus*.

"Fats and Oils as Renewable Feedstock for the Chemical Industry".

size of about USD 19.46 billion in 2020 offering various application possibilities and are to date only produced via TAG hydrolysis.[2]

For instance, oleic acid can be converted chemically or enzymatically by oleate hydratases into 10-hydroxy stearic acid (10-HSA). 10-HSA is a drop-in alternative to the 12-HSA, conventionally sourced from castor oil and commonly used in food or pharmaceutical applications as well as in lubricant additives.[3–5] Hydroxy fatty acids like 10-HSA can be applied for estolide production, which are i.a., a constituent in bio-based lubricant formulations.[6] Further oleic acid can be converted to several other commercially desirable products such as structured lipids or by ozone splitting to polymer intermediates like azelaic acid.[7]

In this regard, glycerol, representing a side product in FA production, has a major role in food, personal care and pharmaceutical formulation as well as additive for fuel, polymers and resins.[8,9] Moreover, glycerol can serve as a starting substance for a sequential conversion process into acrylonitrile, which is subsequently polymerized to polyacrylonitrile (PAN), which is serving as universal precursors for the production of industrial, high-performance carbon fibers.[10] Conventionally, acrylonitrile is produced via the SOHIO process, which utilizes fossil-based propylene for the ammoxidation step.[11] Alternatively, the ammoxidation of bio-derived allyl alcohol derived from glycerol represents a promising process route for the production of renewable acrylonitrile (Figure 1).[12]

Crude glycerol as it accrues in biodiesel production usually exhibits 30 wt% impurities like residual alcohols, fatty acids, or salts of an alkaline catalyst.[13] However, highly purified glycerol is mandatory for the production of high-value products, such as acrylonitrile. Therefore, different methods are being developed for the purification of glycerol waste streams from biodiesel production.[14,15]

The enzymatic hydrolysis of lipids using lipases offers the possibility to recover highly pure glycerol for the production of high-value products. Alongside with the direct applications in the food, pharma, and cosmetics sector, the obtained highly pure glycerol can be utilized by the chemical industry, for the production of, for example, propane-1,3-diol,[16] propane-1,2-diol,[17] acrolein,[18] and allyl alcohol.[19] In this study, allyl alcohol is discussed as a potential intermediate on the catalytic surface during the ammoxidation of propylene to acrylonitrile.[20] Hence, allyl alcohol can also serve as a substrate for the production of bio-derived acrylonitrile[12] and/or acrylic acid[21] (Figure 1). Allyl alcohol can be derived directly from glycerol in continuous gas phase reactions over FeO_x[19,22,23] based catalysts, by deoxydehydration in H_2 atmosphere over, for example, CoFe[24] or Re/CeO_x[25] based catalysts or by didehydroxylation mediated by formic acid.[22,26,27] However, typically these studies utilize purified glycerol as feedstock to obtain high yields in allyl alcohol and suppress potential side reactions. In terms of technical implementation of such process routes for upgrading glycerol, it is therefore important to establish an efficient supply for clean glycerol as feed stream.

While there is a lot of literature regarding the enzymatic methanolysis of TAG for biodiesel production, there is a lack of information on the enzymatic hydrolysis of TAG for the production of FFA and glycerol as sustainable starting material for the chemical industry. Specifically, the biocatalytic liberation and recovery of glycerol, which is of increasing interest as a feedstock for biogenic materials, such as green carbon fibers, is not well documented in the literature. The aim of this study is to investigate the lipase-catalyzed hydrolysis of plant oils with a particular focus on the quantitative liberation of glycerol and its scalable recovery in pure form. In the first step, different commercial lipases were screened for a high conversion of fats in FFA and glycerol. In the next step, the reaction was optimized toward fewer impurities for eas-

Practical Applications

Enzymatic plant oil hydrolysis with lipases enables a biogenic route to obtain highly pure glycerol and FFA. FFA is a renewable feedstock for production of biodiesel or hydrocarbons as well as high-value compounds, such as hydroxy fatty acids or polymer intermediates like azelaic acid. The glycerol side stream from enzymatic hydrolysis with the washed CRL can be recovered quantitatively in high purity in just three steps. All steps comprising centrifugation, filtration, and distillation can be applied in an industrial scale. The water used in the process can be recycled and reused after the glycerol purification. Further, the purified glycerol serves as a raw material for the production of bio-based allyl alcohol, which can be thermo-catalytically converted to acrylic acid and also acrylonitrile, an important precursor for carbon fibers.

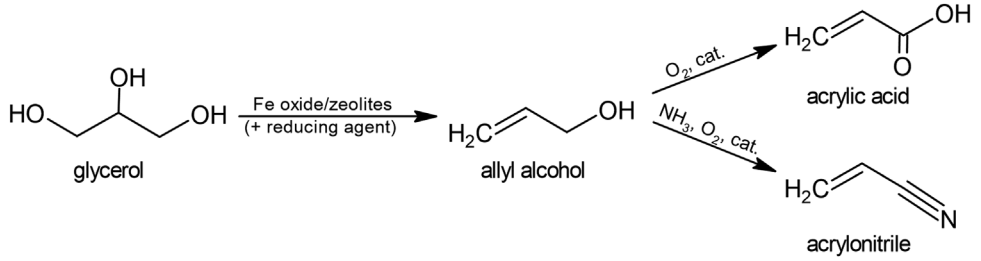

FIGURE 1 Synthesis route from glycerol via allyl alcohol as key intermediate product to acrylic acid and acrylonitrile. Glycerol conversion involves dehydration and reduction, typically carried out over Fe oxide catalyst on zeolite material and under presence of a reducing agent, such as formic acid. Allyl alcohol can be themocatalytically oxidized by oxygen to allylic acid or converted via catalytic ammoxidation (reaction with ammonia (NH_3) and oxygen) to acrylonitrile.

ier glycerol recovery and the purity of the recovered glycerol analyzed by FTIR and HPLC. Finally, the purified glycerol was converted to allyl alcohol in an open distillation process and it was shown in a proof-of-concept experiment that further ammoxidation of commercial allyl alcohol to acrylonitrile works with good yield.

2 | MATERIALS AND METHODS

2.1 | Lipase

The tested lipases from *Candida rugosa* (CRL), from *Pseudomonas fluorescens* (PFL), from *Thermomyces lanuginosus* (TLL), Lipase A from *Candida antartica* (CalA), Lipase B from *Candida antarctica* (CalB), and from *Burkholderia cepacia* (BCL) were purchased in soluble form from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH). For better comparability, all tested lipases were used with an activity of 1000 U mL^{-1} in the water phase.

2.2 | Enzymatic hydrolysis

Lipases were dissolved in buffer/water as appropriate for direct use.

For hydrolysis, 5 mL of each lipase solution and 5 mL of rapeseed or high oleic sunflower oil were mixed in 15 mL centrifugal tubes. The tubes were incubated in a rotary shaker (New Brunswick 44i, Eppendorf SE) at 120 rpm and the temperature was set according to the supplier's information (Table S1).

Samples were taken every 4 h by dissolving 10 µL of reaction mixture in 1 mL hexane.

All experiments were carried out in triplicates.

2.3 | Thin layer chromatography (TLC)

For the analysis, 5 µL of each sample were spotted on silica gel coated aluminum TLC plates with fluorescent indicator F_{254} (Merck Millipore). A mixture of hexane:diethyl ether:acetic acid (70:30:1) was used as mobile phase to develop the TLC plates.[28] The FFA and TAG were identified using a standard of stearic, palmitic, and oleic acid for FFA and triolein for TAG. After developing and drying, the TLC plates were stained with a Rhodamin B solution 0.5 g L^{-1} in ethanol.[29]

For quantification of single spots, pictures of the stained TLC plates were taken using an Imager CHEMI Premium (VWR International GmbH) with green light irradiation. The images were analyzed using the gel analysis tool from the ImageJ software.[30]

2.4 | Washing of lipase from *Candida rugosa*

The 10 kDa centrifugal filter units (Amicon Ultra-15, PLGC Ultracel-PL Membrane, 10 kDa Merck Millipore) were washed 3 times with pure water to remove the glycerol, which is used for storage prior to any filtration of the lipase solution. For washing, the lipase was dissolved in water (1 g L^{-1}) and filtered by a centrifugal filter unit at 5000 rcf at 4°C. Afterward, the volume was set to the original volume with pure water. This step was repeated 3 times. Then, the lipase solution was directly used for the enzymatic hydrolysis.

2.5 | Glycerol recovery from the water phase

After the enzymatic hydrolysis, the water phase was separated by centrifugation for 5 min at 14 000 rcf and transferred to centrifugal filter units (Amicon Ultra-15, PLGC Ultracel-PL Membrane, 10 kDa Merck Millipore). To remove the lipase from the water phase, the centrifugal filter units were spinned at 4°C and 5000 rcf.

To remove residual lipids, the flow through was washed 3 times with 1/3 of the volume of the water phase with n-hexane. Afterward, the water was completely removed.

2.6 | Upsacling of the enzymatic hydrolysis

For process scale up, the enzymatic hydrolysis was performed in a DASbox mini bioreactor system (Eppendorf SE). Therefore, the CRL (1 g L^{-1}) solution was filtered three times using a Minimate TFF system (Pall Filtersystems GmbH) equipped with an Minimate TFF Capsule with a Omega 10K membrane (Pall Filtersystems GmbH). The system was set to the original volume (600 mL) with pure water in three intervals. The resulting washed solution was directly used for the hydrolysis experiments.

For the enzymatic hydrolysis, 100 mL of the washed CRL solution were mixed with 100 mL of oil in the DASbox vessel. The hydrolysis was performed at 37°C and a stirrer speed of 600 rpm for 24 h. After the enzymatic hydrolysis, the water phase was separated in a separation funnel. To remove the lipase, the water phase was filtered again with the Minimate TFF system. The water was removed by vacuum distillation using a Rotavapor R-300 (BÜCHI Labortechnik AG).

2.7 | FTIR measurement of glycerol

IR spectra were recorded using a Nicolet iS50R FT-IR spectrometer from Thermo Fisher Scientific (Thermo Fisher Scientific) equipped with an iS50 ATR (attenuated total reflection) multirange diamond sampling station. For each sample, IR spectra were obtained in a range from 400 to 4.000 cm^{-1}. Samples were measured in triplicates.

2.8 | HPLC analysis of glycerol

Note that 50–60 mg of the purified glycerol were weight in a 5 mL volumetric flask and diluted with pure water resulting in a solution of about 10 g L^{-1}. The solution was filtered with an 0.22 µm filter and mixed with a 0.5 M EDTA solution (sample:EDTA 197:3). The samples were ana-

lyzed in a Shimadzu HPLC according to Jurkowski et al.[31] with 25 min runtime per sample.

2.9 | Statistical analysis

MS Office Excel 2016 (Microsoft Co.) was used for the statistical analysis of data, namely, determination of mean and SD of the triplicates.

2.10 | Allylalcohol synthesis from glycerol

Purified glycerol was didehydroxylated to allyl alcohol using formic acid as a reducing agent, followed by a synthesis protocol as described previously.[27,32] Glycerol (15.67 g from HOSO) was placed into a 100 mL three neck round bottom flask equipped with a still bridge (15°C cooling water). Formic acid (18.88 g of 85 wt.%; purity > 99%, VWR) in water (2 mol formic acid to 1 mol glycerol) were added and the flask was quickly heated to 240°C using an oil bath. The temperatures of the swamp and the steam were monitored. Two fractions of product were taken, the initial fraction was separated at a steam temperature of around 100°C mostly containing formic acid and water. The secondary fraction was separated at a swamp temperature window of 180°C to 240°C mainly containing allyl alcohol, allyl alcohol formate and formic acid.

The second distillate was salted out and hydrolyzed to cleave the formic acid allyl alcohol ester by adding 790 mg K_2CO_3 and 290 mg NaOH in 1.59 water to 3.378 g of distillate. Then, the mixture was heated to 80°C and kept stirring for 3.5 h. After cooling on ice, two phases developed and were separated. The samples for distillate 1, distillate 2 (raw and hydrolyzed) and commercial allyl alcohol were analyzed by means of NMR spectroscopy (JNM-ECA 400 MHz spectrometer from JEOL, solvent D_2O) to determine the purity of the samples (as described in Section 3).

2.11 | Ammoxidation of allyl alcohol to acrylonitrile

Acrylonitrile was synthesized as a proof-of-concept study in a heterogeneously catalyzed reaction by ammoxidation of allyl alcohol (Sigma-Aldrich, >99%) using air and ammonia. Therefore, a pelletized (212-300 µm) commercial mixed metal (Mo, V, Te, Sb) oxide catalyst was placed into a stainless steel reactor tube equipped with a heating jacket. The reactor set-up allowed to mix controlled volumetric flows of ammonia (1.4 mol $mol_{allylalcohol}^{-1}$) and compressed air (1.4 mol_{O2} $mol_{allylalcohol}^{-1}$) with a HPLC-pump (LC-20AD, Shimadzu) controlled feed flow of a 15 wt.% allyl alcohol in water solution prior to the reactor entrance. The weight hourly space velocity (WHSV) ranged between 600 and 2000 $mg_{allylalcohol}$ g_{cat}^{-1} h^{-1}, total pressure was 1 atm and the temperature was constant at 450°C. After the reactor tube the reaction mixture was mixed with 30 wt.% acetic acid solution (1/1 (v/v) total feed to acetic acid solution) before cooling down in a condenser, where the liquid products were collected and sent to analysis for quantification. The product mixture was analyzed in a Shimadzu HPLC Prominence system equipped with a Rezex ROA-Organic Acid H+ (8%) column (300 × 7.8 mm).

3 | RESULTS

3.1 | Screening of different commercial lipases

Lipases generally show varying affinities toward the cleavage of different fatty acids as well as a certain regiospecificity toward position 1 and 3 when cleaving the FFA off the glycerol backbone. However, for a quantitative recovery of glycerol from the enzymatic hydrolysis of oils and fats, a high conversion rate of TAG into FFA and glycerol is mandatory.

Therefore, different commercially available lipases were tested for their hydrolytic activity toward rapeseed and high oleic sunflower oil (HOSO).

The conversion rate of TAG to FFA for each lipase with regard to the respective lipid type (rapeseed oil vs. high oleic sunflower oil) was plotted over time (Figure 2) to allow for detailed comparison of the enzymatic performance. Lipase B from *Candida antarctica* (CalB) did not exhibit any activity toward the conversion of TAG into FFA while lipase A from *Candida antarctica* (CalA) showed a conversion toward FFA of about 35% after 24 h (rapeseed oil: 36.3%; HOSO:33.6%). Lipase from *Thermomyces lanuginosus* (TLL) showed a slightly higher conversion rate of about 50% FFA after 24 h (48.9% vs. 51.5%). The thermostable lipases from *Burkholderia cepacia* (BCL) and *Pseudomonas fluorescens* (PFL) demonstrated a similar behavior with a maximum yield of FFA between 83% (83.0% vs. 72.2%) and 63% (63.4% vs. 65.5%), respectively. Interestingly, BCL was the only lipase to exhibit significantly different yields of FFA with the varying substrates in favor to the rapeseed oil. However, lipase type VII from *Candida rugosa* (CRL) showed the highest conversion rate with a yield of FFA of about 93% (92.9% vs. 92.6%) after 24 h independent of the applied vegetable oil type.

3.2 | Comparison of hydrolytic activity of CRL in buffer and pure water

Since CRL showed the highest conversion rates on both lipid substrates, further optimization experiments were carried out with this enzyme.

In order to improve the purity of the expected glycerol product to implement it directly in, for example, carbon fiber production workflows, the hydrolysis process parameters were optimized accordingly.

In particular, salts are a common impurity of glycerol from biodiesel production. Thus, the addition of salts or buffer components needs to be strictly limited in the process to avoid high concentrations of salts in the glycerol after recovery from the water phase. Interestingly,

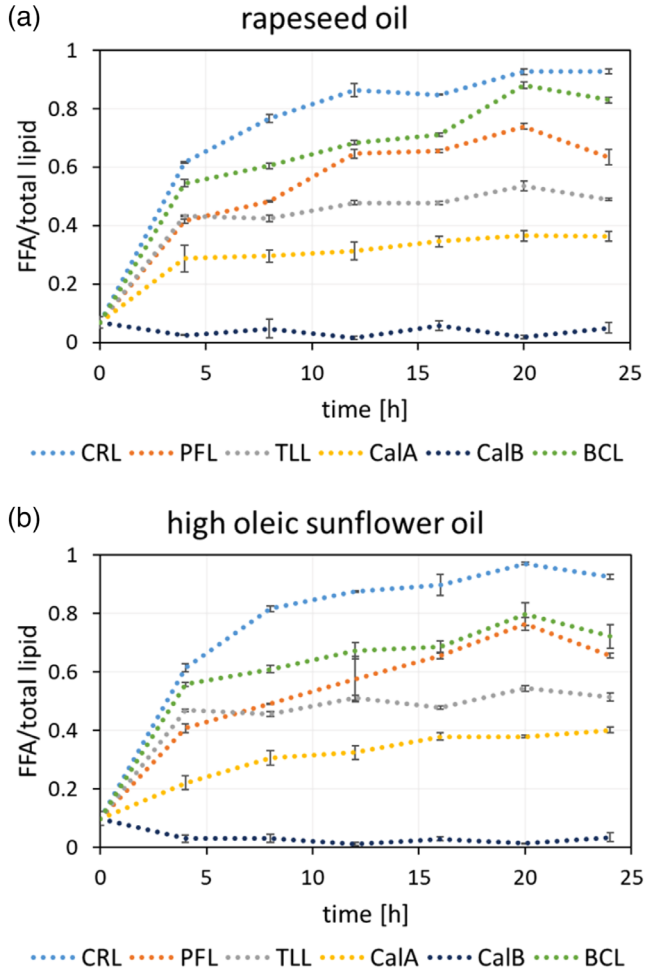

FIGURE 2 Amount of FFA developed from rapeseed oil (a) and HOSO (b) over time applying the lipase type VII from *Candida rugosa* (CRL), Amano lipase from *Pseudomonas fluorescens* (PFL), lipase from *Thermomyces lanuginosus* (TLL), lipase A *Canidia antarctica*, recombinant from *Aspergillus oryzae* (CalA), lipase B *Candida antarctica*, recombinant from *Aspergillus oryzae* (CalB), and Amano lipase PS, from *Burkholderia cepacia* (BCL). All experiments were performed in triplicates; error bars show the mean and the standard deviation of these three samples.

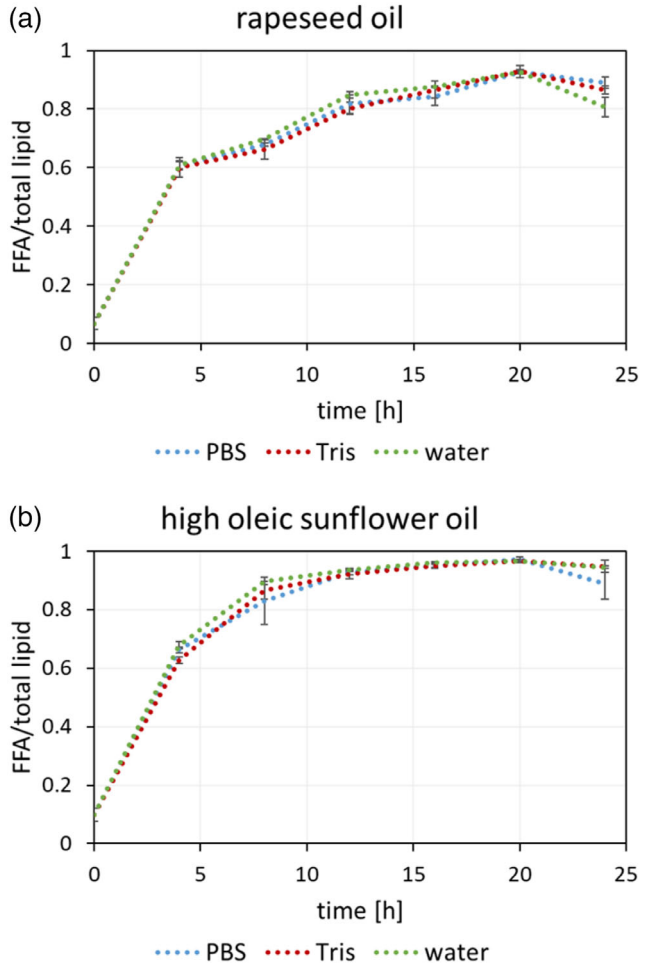

FIGURE 3 Amount of FFA developed over time during enzymatic hydrolysis of either rapeseed oil (a) or high oleic sunflower oil (b) with the lipase type VII from *Candida rugosa* in PBS pH 7.2 (PBS), 50 mM Tris/HCl buffer pH 7.2, and pure water. All experiments were performed in triplicates; error bars show the mean and the standard deviation of these three samples.

Talukder et al. showed a high activity of CRL in pure water instead of buffer on waste cooking oil.[33] Therefore, the hydrolytic activity of CRL on rapeseed oil and HOSO in two different commonly used buffers (50 mM Tris/HCl pH 7.2 and PBS pH 7.2) was compared to the activity in pure water. The experiments were performed under the same conditions as described above and the conversion rate of TAG to FFA was plotted over time (Figure 3).

The conducted experiments revealed a comparable conversion rate of TAG to FFA over time with regard to the tested buffer systems or pure water for both lipid substrates, respectively. The FFA yield reached up to 95% conversion rate for all tested conditions and is thus comparable to the conducted lipase screening experiments.

3.3 | Washing CRL for enzymatic hydrolysis

Although buffer components are a key factor for impurities in the resulting glycerol, also the enzyme itself needs to be considered. The lipase type VII from *Candida rugosa* (purchased from Sigma-Aldrich) is commercially available as a lyophilized dry matter. Thus, impurities might potentially arise from residual media components, salts, and other proteins within the lipase powder. In order to evaluate whether removing contaminants from the lipase solution by simple washing steps has an influence on the enzymatic activity, the above-mentioned procedure was developed. For a better comparison between the washed and the unwashed condition, the lipase solution for both conditions was prepared in one batch. One part was filtered, and the other one was kept at 4°C in the fridge until it was used for hydrolysis. The obtained amount of FFA was plotted over time (Figure 4).

For both lipid substrates, experiments show a decrease in the amount of FFA of about 10% for the washed compared to the

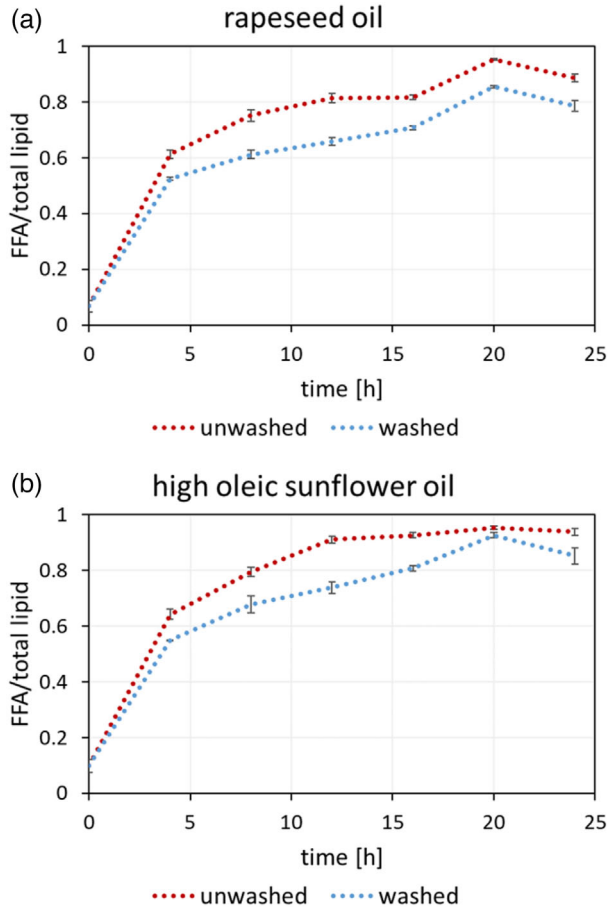

FIGURE 4 Amount of FFA observed in experiments with lipase type VII from *Candida rugosa* conducted in pure water after washing by 10 kDa filtration (washed) and without washing (unwashed). Enzymatic hydrolysis of either rapeseed oil (a) or high oleic sunflower oil (b) is shown in the course of 24 h. All experiments were performed in triplicates; error bars show the mean and the standard deviation of these three samples.

unwashed lipase solution. However, with the washed lipase solution an amount of FFA of still 79% for rapeseed oil or 85% for HOSO can be obtained, respectively.

3.4 | Recovery of highly pure glycerol from the water phase after enzymatic hydrolysis

The conducted experiments showed that CRL can liberate the highest amount of FFA under all tested conditions. Furthermore, CRL was active in pure water without any salts or buffer components even after washing by 10 kDa filtration. Therefore, the water phases of the experiments with the washed CRL were directly used for evaluating a recovery route toward highly pure glycerol.

The remaining, highly viscous liquid after separation of enzyme remainders, washing of the reaction mixture with hexane and water removal was determined gravimetrically. The resulting weight was compared to the one of the oil used in the experiments (Table 1). A

TABLE 1 Amount of recovered glycerol after the enzymatic hydrolysis in small scale as well as the wt% compared to the oil used for the hydrolysis and the theoretical yield for rapeseed oil and high oleic sunflower oil (HOSO).

	Rapeseed oil	HOSO
Oil used for hydrolysis [g]	4.56 ± 0.14	4.47 ± 0.06
Water phase after hydrolysis [g]	4.84 ± 0.20	4.99 ± 0.16
Water phase after filtration [g]	4.10 ± 0.35	4.35 ± 0.36
Water phase after washing [g]	3.89 ± 0.42	3.58 ± 0.50
Recovered glycerol [g]	0.30 ± 0.04	0.29 ± 0.04
wt% of recovered glycerol from oil	6.55 ± 0.86	6.55 ± 0.87
Theoretical yield [%]	65.51 ± 8.62	65.46 ± 8.69

Note: The theoretical yield was calculated with an amount of glycerol of 10 wt% of the TAG. All experiments were performed in triplicates with mean and standard deviation of these three samples.

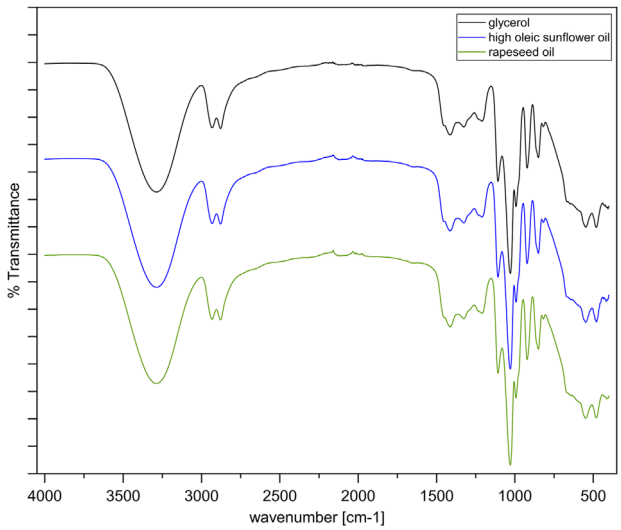

FIGURE 5 FTIR spectrum of pure glycerol (98% Ph.Eur.) recovered glycerol from the enzymatic hydrolysis of rapeseed oil and high oleic sunflower oil.

yield of about 65% of the theoretical yield of glycerol could be achieved with a high loss of glycerol-containing water phase through the steps of filtration and washing with hexane.

The recovered glycerol, for both lipid substrates tested, was analyzed by FTIR and HPLC measurements against a 98% Ph. Eur. Glycerol (Carl Roth) standard (Figure 5). The FTIR spectrum of the purified glycerol resembled the pure glycerol for rapeseed oil as well as HOSO. The small additional band at about 1650 nm wavelength, which is also present in some other IR spectra of glycerol, could be assigned to water (bending mode).[15,34]

Further HPLC analysis of the recovered glycerol fractions as well as a glycerol standard showed a single peak at 17.1 min (Figure S2). Therefore, the obtained glycerol from enzymatic hydrolysis and subsequent recovery from the water phase can be considered pure.

TABLE 2 Amount of recovered glycerol after the enzymatic hydrolysis in stirred tank bioreactors as well as the wt% compared to the oil used for the hydrolysis and the theoretical yield for rapeseed oil and high oleic sunflower oil (HOSO).

	Rapeseed oil	HOSO
Oil used for hydrolysis [g]	91.20 ± 0.08	91.27 ± 0.26
Water phase after hydrolysis [g]	99.73 ± 4.04	100.80 ± 1.10
Water phase after filtration [g]	83.50 ± 3.92	86.20 ± 2.89
Recovered glycerol [g]	7.48 ± 0.48	8.11 ± 0.28
wt% of recovered glycerol from oil	8.21 ± 0.53	8.88 ± 0.29
Theoretical yield [%]	82.12 ± 5.33	88.84 ± 2.87

Note: The theoretical yield was calculated with an amount of glycerol of 10 wt% of the TAG. All experiments were performed in triplicates with mean and standard deviation of these three samples.

3.5 | Scale up of the enzymatic TAG hydrolysis and glycerol recovery

To demonstrate the potential for an industrial application, the enzymatic hydrolysis and subsequent recovery of the glycerol were scaled up in a stirred tank bioreactor system with temperature control. The pH control was not used in these experiments to avoid intake of acid or base instead of buffer components. In addition, the washing step applying hexane was excluded to enhance process sustainability and reduce the number of process steps in order to prevent product loss during purification.

After 24 h of enzymatic hydrolysis, the lipid phase from rapeseed oil showed a composition of about 3.1 ± 0.2% TAG, 92.1 ± 0.5% FFA, 3.1 ± 0.3% 1,2-DAG, 0.8 ± 0.2% 1,3-DAG, and 1.0 ± 0.1% MAG, while the lipid phase from HOSO contained about 99.0 ± 0.2% FFA, 0.7 ± 0.1% 1,2-DAG, 0.3 ± 0.1% MAG, TAG, and 1,3-DAG were not detected.

The glycerol concentration in the water phase after the enzymatic hydrolysis was 88.6 ± 1.2 g L^{-1} (RO) and 93.8 ± 1.6 g L^{-1} (HOSO). After phase separation and water removal, the highly viscous liquid was quantified gravimetrically. The resulting weight was compared to the oil sample used in the experiments (Table 2). A yield of about 82% (RO) or 88% (HOSO) of the theoretical glycerol yield could be achieved. The glycerol was again analyzed by FTIR and HPLC. As with our lab scale results, the IR spectra of the glycerol fractions were comparable to the commercial glycerol standard with an exceptional band at about 1650 nm (Figure S3). The HPLC analysis directly after the enzymatic hydrolysis as well as after the glycerol purification showed one single peak at 17.1 min (Figure S5). From these data, the resulting glycerol can be considered as pure with residual water content of about 6.6 ± 0.7% (RO) and 5.9 ± 0.6% (HOSO), respectively.

3.6 | Glycerol didehydroxylation to allyl alcohol

Formic acid-assisted didehydroxylation of glycerol to allyl alcohol was conducted by an open boiling reactive distillation method. Two main product fractions were obtained: A first one consisting of formic acid and water with a total mass of 10.97 g (Figure S6). The second fraction with a total mass of 9.76 g contained formic acid, allyl alcohol, and allyl formate (Figures S8–S9). Next to the two main distillates, a third fraction taken in between the two distillates and with unreacted left over glycerol in the swamp added up to the overall mass balance.

The ratio between allyl alcohol and allyl formate in the second fraction was determined spectroscopically by ^{1}H-NMR using the ratio of the signal areas related to protons in α position to the double bond at the C_1 carbon. The signal of the C_1 protons of the alcohol was at 3.98 ppm (*d*, *J* = 5.6 hz, 2H) and the C_1 protons of the ester were downshifted to 4.60 ppm (*d*, *J* = 5.2 Hz, 2H) (Figure S9). The final molar ratio of alcohol to ester in the distillate was 1:0.27. The total molar yield of allyl alcohol and allyl formate with respect to glycerol was 74 mol-% (calculation detailed in Supporting Information; Figure S9).

By adding K_2CO_3 to this mixture, strong bubbling indicated the neutralization of residual formic acid, resulting in separation of two phases. After the alkaline hydrolysis, the organic phase mainly contained allyl alcohol (Figures S10 and S11). The molar yield of the second purification step was 65 mol-% resulting in an overall allyl alcohol yield with respect to glycerol of 48 mol-%.

3.7 | Ammoxidation of allyl alcohol

Allyl alcohol was successfully converted into acrylonitrile showing a yield between 34 and 45 mol-% at full allyl alcohol conversion. The condensed phase contained no further products. Another product was CO_2, which formed by overoxidizing the allyl alcohol.

In order to avoid the formation of orange, solid side products potentially formed by in situ oligomerization of the acrylonitrile formed in the process, acetic acid addition to the reaction mixture was indispensable. For this proof-of-concept study, the overall production rate of acrylonitrile had to be increased. Therefore, the WHSV of allyl alcohol, defined as $WHSV = \frac{\dot{m}_{allyl\ alcohol}}{m_{cat}}$, was manipulated by changing the volumetric flow of the feed. As a result, the acrylonitrile production rate, defined as $prod.\ rate = \frac{\dot{m}_{acrylonitrile}}{m_{cat}}$, increased with increasing WHSV at almost constant yield (Figure 6).

4 | DISCUSSION

The hydrolysis of plant oils and fats enables the utilization of FFA and glycerol as renewable and sustainable precursors for the chemical industry as well as biodiesel or hydrocarbon production from the FFA fraction. To date, the hydrolysis of oils and fats for FFA production is performed on the industrial scale using the Colgate-Emery process.[35,36] This is a continuous hydrolysis process for the separation of oil into FFA with a conversion of up to 99% , resulting in a glycerol-containing water phase.[36] While this process meets some principles of green chemistry and sustainability, it is very energy consuming because temperatures higher than 250°C and a pressure of about 5 MPa are required.[36,37] To find a more sustainable and less

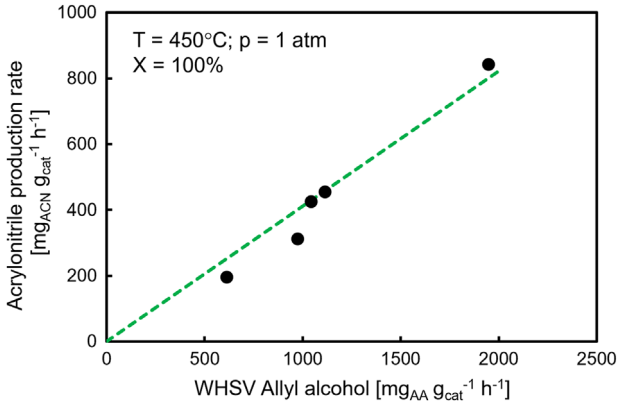

FIGURE 6 Acrylonitrile production rate at varying weight hourly space velocity. WHSV was increased by higher volumetric flow of allyl alcohol and the amount of catalyst loaded. For this proof-of-concept study, oxygen (as air) and ammonia were kept constant at a 1.4 molar ratio with respect to allyl alcohol; the reaction temperature was 450°C and an overall pressure of 1 atm was applied. The line represents the 45 mol-% yield.

energy-consuming alternative, commercial lipases were evaluated in this study for their hydrolytic activity on rapeseed oil and HOSO. Most of the biodiesel produced in Germany is derived from rapeseed oil,[38] while HOSO with an amount of oleic acid higher than 90% also has industrial applications in the chemical industry, for example, lubricant production.[39]

While all tested lipases were used with the same activity, they showed high differences in the FFA yields after the enzymatic hydrolysis under the experimental conditions applied in this study. The hydrolytic activity of the lipases is depended on the experimental conditions like temperature, pH value, buffer system, water-to-oil ratio, and the lipid substrate itself. From the supplier's information, the specific activity was determined with tributyrin for CalB, TLL, and PFL; for CalA, triolein was used; and for CRL olive oil and for the BCL, there was no information on how the specific activity was determined. The temperature as well as initial pH values of the buffers used for the screening experiments was according to the supplier's information. However, during the enzymatic hydrolysis, FFA is released, which can affect the pH value during the reaction, even in the presence of a buffer system. These slight changes in the pH value can also affect the activity of the lipases.

The hydrolytic activity of the tested TLL on rapeseed oil and HOSO was just about 50% and lower than for the tested PFL, BCL, and CRL. To this end, with lipase from *Thermomyces lanuginosus* described as a highly stable, but regiospecific (positions 1 and 3 of the TAG) lipase, only a maximum yield of about 67% of FFA without acyl transfer can be achieved.[40,41] In this context, the regiospecificity (e.g., at positions 1 and 3 of TAG) can potentially explain an incomplete hydrolysis of the lipid substrate.

In addition, the hydrolytic activity of the tested PFL was between that of TLL and BCL. However, one lipase from *Pseudomonas fluorescens* HU380 showed a higher activity on positions 1 and 3 but was also active on position 2.[42] Another lipase from *Pseudomonas fluorescens* AK102 showed no regiospecificity at all.[43] Both studies showed conversions of about 100% from olive oil as lipid substrate into FFA.[42,43] The maximum yield of FFA with PFL on rapeseed oil and HOSO was about 65% and lower than that on olive oil as lipid substrate. The different substrate and the experimental conditions might explain this difference.

BCL showed no regiospecificity as well as high hydrolytic activity regardless of the chain length of the FA.[44] In the conversion of TAG to FFA, there was just a small difference (83% on rapeseed oil and 72% on HOSO) in the conversion between the tested plant oils. In a study on biodiesel production, BCL also showed a high hydrolytic activity on vegetable oils, that is, virgin olive oil, rapeseed oil, and sunflower oil compared to waste cooking oil, hence exhibiting a high substrate promiscuity when compared to the other lipases.[45]

However, lipase from *Candida rugosa* type VII (CRL) showed the highest conversion in the conducted screening experiments with a yield of FFA of about 93% with regard to the total lipid concentration and was identified as the most promising candidate for the enzymatic hydrolysis of vegetable oils and fats. Moreover, CRL is also reported as a high-fidelity enzyme for the enzymatic hydrolysis of lipids.[46–48] Similar to the conversion of rapeseed oil and HOSO in this study, the CRL showed a high hydrolytic activity on a wide range of different plant oils like canola oil, olive oil, and sunflower oil.[49]

Taking into account the amount of lipase used for the experiments combined with FFA yield, the lipases showed considerable differences in the turnover number (TON[50]). The highest TON of 168 105 $mol_{product}/mol_{catalyst}$ was obtained with CRL, followed by TLL (5079 $mol_{product}/mol_{catalyst}$), BCL (2544 $mol_{product}/mol_{catalyst}$), PFL (1961 $mol_{product}/mol_{catalyst}$), CalA (123 $mol_{product}/mol_{catalyst}$), and CalB (33 $mol_{product}/mol_{catalyst}$).

Commonly, enzymatic hydrolysis takes place in buffers to keep the pH value stable during the reaction. Furthermore, the addition of salts to the reaction mixture can help to adjust the ionic strength. Therefore, two different buffers were tested in this study, PBS with sodium chloride and TRIS/HCl without other salts, in comparison to pure water. There was almost no difference in the FFA yield after enzymatic hydrolysis with the various reaction media. A high yield of FFA using CRL in pure water was reported in literature.[33] In addition, CRL showed a high hydrolytic activity over a wide range of different pH values ranging from 4 to 8.[49,51] So, a change in the pH value during the reaction does not seem to have any influence on the FFA yield. Interestingly, there was no significant difference between the PBS-based reaction medium compared to the other tested conditions, although an inhibitory effect of sodium ions regarding the hydrolytic activity of CRL has been reported.[52] In addition to sodium, other metal ions and their effect on the hydrolytic and transesterification activity of CRL were tested in the same publication. In this context, Ca^{2+}, Cu^{2+}, Co^{2+}, and Fe^{3+} are reported to enhance the hydrolytic activity.[52] The absence of these ions might negatively influence the CRL hydrolytic activity. This could explain the decrease in the FFA amount after the hydrolysis using the washed lipase solution.

In the scale up experiments conducted in stirred tank bioreactor systems, even higher FFA yields about 92%–99% conversion, were

observed compared to the results of the small-scale laboratory experiments. This FFA yield increase might be caused by a better mixing of the two phases and a higher energy intake of the stirrer compared to uncontrolled shaking in a shaking incubator. The achieved conversion in these experiments is in the higher range of FFA yield published for enzymatic hydrolysis, even with a washed enzyme without any buffer components or salts.[33,53,54] Furthermore, this conversion is similar to the maximum FFA yield of the steam splitting process currently used for FFA production.[37] To be economically feasible, the process has to be further optimized, for example, dosing of the lipase, shortening the process run time or reduction of the water content for a more efficient downstream processing of the glycerol by keeping a high conversion. Furthermore, the use of the lipase for several hydrolysis cycles could lower the cost of each run. Another option might be the immobilization of the enzyme, this commonly increases the costs of the enzyme itself but on the other hand simplifies the downstream processing and increases the stability of the enzyme.[49,51,55]

Glycerol from biodiesel production commonly contains different impurities like salts of FFA or esters. These impurities can be detected in the crude glycerol using FTIR spectra in comparison to a pure glycerol standard. The different functional groups show characteristic bands in the IR spectrum. For example, C = O stretching from esters typically appear at about 1740 cm^{-1}, while COO^- groups coming from salts of FFA show a band at around 1580 cm^{-1} and C = C bounds present in the carbon chain of unsaturated FA appear at about 3010 cm^{-1}.[14,15,56] To remove these types of impurities, multiple cost-, energy-, and time-intensive purification steps are required. It is reported that purification of glycerol from a biodiesel process can be achieved in seven different steps, encompassing acidification, evaporation, neutralization, solvent extraction, decolorization, methanol recovery, and refining.[14] Moreover, an additional solvent extraction step may be involved to remove all FA and lipids after the acidification.[56] During this process, sulfuric acid, sodium hydroxide solution, and methanol are required raw materials to complete the purification process.[14]

In our study, the glycerol from the enzymatic hydrolysis of rapeseed oil and HOSO was recovered in just three to four steps. While an additional solvent extraction step was included after the filtration to remove residual lipids from the water phase on the small scale, this step was not exploited in the scale up experiments. The optimized purification protocol entailed phase separation, filtration, and water removal. In the scale up experiments, about 82%–88% of the theoretical glycerol yield could be achieved. While some glycerol can be found in the form of residual glycerids in the lipid fraction, there is a high loss of the glycerol-containing water phase during the filtration step. This loss of water phase is mainly caused by the dead volume of the applied filter cartridges and therefore depends on the scale of the hydrolysis as well as the filter matrices themselves. The FTIR spectra of the recovered glycerol indicated the absence of any known impurities typically identified during glycerol recovery from biodiesel production. By contrast, our data of the obtained glycerol preparation was equivalent to the pure glycerol standard. Hence, the glycerol recovery process delineated in our study requires fewer steps, cost, energy and is less time-consuming. Moreover, in the biodiesel glycerol purification process, the acidification and neutralization steps consume acid and base, resulting in salt accumulation in the glycerol product, which cannot be easily removed. By contrast, our process is carried out in water, simplifying glycerol recovery after enzymatic hydrolysis, by preventing salt accumulation.

In comparison to the steam splitting process, there is one additional step in the here presented purification procedure. While the water removal can be performed directly after the phase separation in the steam splitting process, a filtration step to remove the lipase from the water phase is required for the enzymatic hydrolysis. The glycerol concentration in the water phase after the enzymatic hydrolysis was 8.9 wt% or 9.4 wt%, which is close to the range expected from steam splitting.[36]

To test the suitability of the purified glycerol yielded in this study for later application, its catalytic conversion to acrylonitrile via allyl alcohol as an intermediate was demonstrated.

Conventionally, fossil-based propene is ammoxidized to acrylonitrile going through several intermediates on the catalytically active surface.[11] Allyl alcohol was previously used as a probe molecule for an allylic intermediate on the catalytic active surface during ammoxidation of propene on Bi-Mo mixed oxides.[20] Additionally, it was successfully converted to acrylonitrile on SbFe-based oxides.[12] In this study, plant oil-based purified glycerol was upgraded through its didehydroxylation to allyl alcohol and subsequent ammoxidation to acrylonitrile as versatile and value-added platform chemical. The first step of the here presented route to acrylonitrile is the conversion of glycerol to allyl alcohol using formic acid as a reducing agent. In literature several different routes from glycerol to allyl alcohol are presented, which in principle can be described as overall deoxydehydration reactions.[19,22–24,57–60] All have in common, that in a first catalytic step glycerol is potentially dehydrated to acrolein and subsequently hydrogenated to allyl alcohol either by directly utilizing H_2 or catalytically enabling an H-transfer from a reductant. These continuous, heterogeneously catalyzed reactions are carried out at elevated temperatures between 250°C and 350°C and varying total pressures of 1 atm to 20 bar. Typically, side products are formed and unintended overhydrogenation to propanol and other saturated alcohols additionally lower the selectivity and total yield of allyl alcohol at usually full conversion of glycerol. In this study we decided on an open distillation protocol utilizing formic acid as both, catalyst and reducing agent for the didehydroxylation reaction of glycerol to allyl alcohol.[26] With this method, side product formation is minimized and potential unwanted products remain in the swamp, respectively.

During the reactive distillation process the chemical transformation from glycerol to allyl alcohol starts at elevated temperatures of 230°C–240°C.[26] As a consequence, the low-boiling compounds formic acid and water are constantly removed as a first distillate fraction during the heating process. At the same time, formic acid glycerol ester is formed in the swamp. The formic acid in the first distillate fraction might be recovered and reused in another synthesis. In the second stage of the distilling process at temperatures above 230°C, the formed allylic compounds are constantly removed

($bp_{allyl\ formate}$ = 85°C, $bp_{allyl\ alcohol}$ = 97°C) and collected. Notably, the products in this distillate were purely allyl alcohol, formic acid, and allyl formate. For a final purification step, this raw product underwent an alkaline (NaOH) hydrolysis step in combination with salting out using K_2CO_3 for final purification of the allyl alcohol. According to NMR analysis, the allyl alcohol was nearly phase pure when compared to a commercial product and therefore could act as a feedstock for upgrading it to higher value products such as acrylic acid or acrylonitrile. In this study, we also demonstrate the conversion of allyl alcohol to acrylonitrile on a commercial mixed metal oxide catalyst. However, we note, that for this conversion step we used commercial allyl alcohol, as the volume of allyl alcohol synthesized from the glycerol yielded from lipase-mediated TAG hydrolysis was too small to feed the ammoxidation in a continuous reactor. Nevertheless, there is no doubt that the process would perform as well based on the glycerol from lipase-based hydrolysis, given the purity of the allyl alcohol we could synthesize from it. The end-to-end demonstration of the entire process route TAG to glycerol to allyl alcohol to acrylonitrile will be subject of a follow-up publication, which is currently in preparation.

Ammoxidation of allyl alcohol was performed in a continuous heterogeneous catalytic process at 450°C and atmospheric pressure. The maximum yield of acrylonitrile was between 34 and 45 mol-%, independent of the applied WHSV. Anyhow, since all allyl alcohol was converted, undetected side products must have formed. Most likely allyl alcohol was over oxidized to CO_2. Data also indicated that the quench flow of acetic acid stabilized acrylonitrile in the hot downstream part of the reactor and inhibited its polymerization. However, in future we will investigate and optimize the process of allyl alcohol ammoxidation to acrylonitrile on novel, industrial mixed metal oxides to gain a deeper understanding of the catalytic steps involved in the chemical transformation process.

It is also worthwhile to briefly consider the economic potentials of establishing process routes based on glycerol as side product from biodiesel production. After an all-time high in 2022, glycerol market prices have reached normal levels of the period before the war in Ukraine, that is, ranging from about 130 USD/t for low-grade glycerol up to 930 USD/t for the purified product.[61] This compares to current market prices for conventional (i.e. fossil-based) acrylontrile of about 1300–1500 USD/t.[62] In this figure, it has to be taken into account that the value of truly renewable ("green") acrylonitrile will be higher, allowing for a price premium and increasing the economic potential of its production. It has also to be considered that the lipase-mediated TAG hydrolysis and the subsequent glycerol purification, as suggested in the present study, offers the potential for efficient and high-performing glycerol extraction. Future research will show how the economic performance of this approach can translate into supply costs for glycerol, for example, if the costs will be at the lower end of the glycerol price range mentioned above or rather at the upper end. The glycerol supply costs will be decisive for the economic viability of the synthesis of compounds like acrylonitrile and acrylic acid based on glycerol.

5 | CONCLUSIONS

Lipases enable the use of a broader range of substrates for biodiesel production and offer a more sustainable alternative to the conventional, chemical production routes. In the present study, six different lipases were tested for their hydrolytic activity on rapeseed oil, a common substrate for biodiesel production in Europe, and on high oleic sunflower oil. To this end, the lipase type VII from *Candida rugosa* was identified as the most promising candidate achieving a high turnover from TAG to FFA even in pure water. The resulting FFA can be converted to biodiesel but also to other value-added products like 10-HSA, a highly valuable, sustainable viscosity modulator in the cosmetic and lubricant industry.[5] In addition, the recovered glycerol from the enzymatic hydrolysis using the washed CRL showed a high purity. Thus, it can serve, for example, as precursor for the carbon fiber production based on acrylonitrile. A promising intermediate in the synthesis of acrylonitrile from glycerol is allyl alcohol. The purified glycerol successfully underwent a formic acid-assisted didehydroxylation to yield phase-pure allyl alcohol, thus proofing, that the applied glycerol purification method in this study favorably compares to established protocols. Additionally, the yielded allyl alcohol can be further converted to acrylonitrile through an ammoxidation reaction, thereby opening a range of attractive options to valorize glycerol as side product of TAG hydrolysis and/or transesterification, such as applied in biodiesel production. In summary, the lipase-based hydrolysis of plant oils possesses a significant potential to serve as sustainable supplier of resources to be converted by the chemical industry into innovative high-performance materials.

ACKNOWLEDGMENTS

FM, FV, MR, MM, AR, and TB gratefully acknowledge funding of the GreenCarbon project by the German Federal Ministry of Education and Research (grant no.: 03SF0577A).

Open access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Melcher, F., Vogelgsang, F., Haack, M., Masri, M., Ringel, M., Roth, A., Garbe, D., & Brück, T. (2023). Lipase-mediated plant oil hydrolysis—Toward a quantitative glycerol recovery for the synthesis of pure allyl alcohol and acrylonitrile. *European Journal of Lipid Science & Technology*, *125*, e2200196. https://doi.org/10.1002/ejlt.202200196