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Optimised Protocol for Drying Aqueous Enzyme Solutions in Organic Solvents – Comparison of Free and Immobilised *Candida antarctica* Lipase B

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Here, we propose an optimised protocol for controlling the initial water activity (a_w) in organic reaction mixtures with soluble lipase to ensure reproducible and consistent enzyme activity measurements. Pre-equilibration above saturated salt solutions was used to set a_w of the reaction media, where a_w was tracked with a hygrometer. Consistent stirring of the suspension and volume correction to compensate for solvent evaporation were found to be critical. The protocol was tested on *Candida antarctica* lipase B (CALB) in soluble and immobilised forms in three organic solvents (acetonitrile, methyl *tert*-butyl ether, and hexane) at four different water activities ranging from 0.12 to 0.97. Soluble and immobilised CALB had similar a_w-profiles and showed the highest enzyme activity in hexane. The optimised protocol expands the possibility to study enzyme kinetics from immobilised enzymes to soluble enzymes.

Introduction

Increased use of lipases (EC 3.1.1.3) is seen across a broad range of industries including the paper, food, biodiesel, pharmaceutical, textile, and detergent sectors.[1–5] The use of lipases is advantageous because of their high specificity and often also high chemo-, regio-, and enantioselectivity in hydrolysis, esterification, transesterification, and aminolysis reactions.[6] In recent years, the use of lipases has also been expanded to perhydrolysis, catalysing synthesis of peracids that can be used in Baeyer-Villiger oxidations.[7,8] Naturally, lipases catalyse the hydrolysis of triglycerides into free fatty acids and glycerol in the presence of water. Substituting water with an organic solvent shifts the thermodynamic equilibrium from hydrolysis towards esterification, making it possible to synthesise esters.[9,10] Additional advantages of carrying out lipase reactions in organic solvent include elimination of microbial contamination, limitation of undesired side reactions, higher solubility of substrates and products, and increased thermostability of the lipase.[11]

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The first lipase catalysed reaction in organic media was discovered in the 1930s.[12–15] However, the field did not take off before the 1980s with Klibanov and Zaks' studies on porcine pancreatic lipase catalysed reactions in hexane.[16,17] Before that, it was believed that enzymes required an aqueous reaction medium to function, as the hydrogen bonds between the protein and the water molecules ensured the protein's proper fold. Since Klibanov and Zaks' work, multiple enzymes with catalytic activity in organic solvents have been identified; a large portion of these is lipases.[6] This further opens up for non-aqueous enzymatic cascade synthesis reactions, as exemplified very recently using lipase-tyrosinase co-immobilisation.[18]

Lipase activity in organic solvent is greatly affected by the amount of water present in the reaction medium.[19–23] Water molecules act as lubricants for proteins, giving them the required flexibility to catalyse reactions. Therefore, exhaustive drying often leads to inactive enzymes.[24] On the other hand, high water content also halts the esterification reaction, pushing the thermodynamic equilibrium instead towards hydrolysis. In addition, it is hypothesised that water molecules form clusters on the lipase's surface that sterically hinder the entrance of substrate molecules into the active site.[25] Hence, controlling the water activity is of great importance.

In the 1990s, it became good practice to report the amount of water in the reaction medium by the thermodynamic water activity (a_w), rather than the total amount of water.[26,27] The reaction medium is composed of at least two phases; an organic phase with the organic solvent(s) and an aqueous phase with the enzyme.[19] Even though the aqueous phase is small, it is still present, and reporting the total amount of water will not adequately describe the lipase's micro-environment. At equilibrium, the water activity is identical in all phases. Additionally, water activity is not affected by the organic solvent's characteristics, such as log(P), which is not the case for the total amount of water. An additional advantage of reporting the

water activity is that it is measurable from the vapour phase with a hygrometer.[28]

At least two approaches for controlling the water activity have been proposed to date; in the first approach, the initial water activity is set prior to the catalytic reaction, while in the second approach, the water activity is controlled throughout the duration of the reaction.[29] If the final yield of the reaction is of importance, it is often necessary to control water activity during the reaction, as an esterification (condensation) reaction produces water. However, if the initial reaction rate is the essential variable, an initial pre-equilibration of the water activity may prove adequate.[30]

Protocols for drying immobilised and freeze-dried lipases have previously been presented, where molecular sieves or saturated salt solutions are used to set the water activity.[22,25,31,32] However, catalytic activity depends on the lipase formulation and the type of support used for the immobilisation.[22,31,33] Interestingly, soluble lipases can be employed to avoid the effects of different support materials on the catalytic activity. However, to the best of our knowledge, no protocol that yields consistent and reproducible results for drying of soluble lipases has been presented. In this paper, an optimised general drying method of lipases in organic media is presented. *Candida antarctica* Lipase B (CALB) in soluble (CALB L) and immobilised (Novozym® 435, NZ435) forms were used to test the method. NZ435 was included in this study because of its high stability and activity, and additionally it is one of the most studied lipases in the literature.[34] NZ435 consists of a macroporous acrylic polymer resin (Lewatit VP OC 1600) made of poly(methyl methacrylate) crosslinked with divinylbenzene, which gives the resin both hydrophobic and hydrophilic properties. The CALB enzyme absorbs to the resin through interfacial attraction; therefore, no covalent bonds are present between the carrier and the lipase. The lipases were investigated in acetonitrile (ACN), hexane, and methyl *tert*-butyl ether (MTBE), which were chosen because of their different functional groups and log(P)-values. In addition, these solvents are often reported in the literature and used in the industry for e.g. lipase-catalysed transformations and acetylation reactions.[35–37] The synthesis of ethyl butyrate from butyric acid and ethanol was used as a simple model as CALB is known to catalyse the reaction effectively.[25,30,38,39]

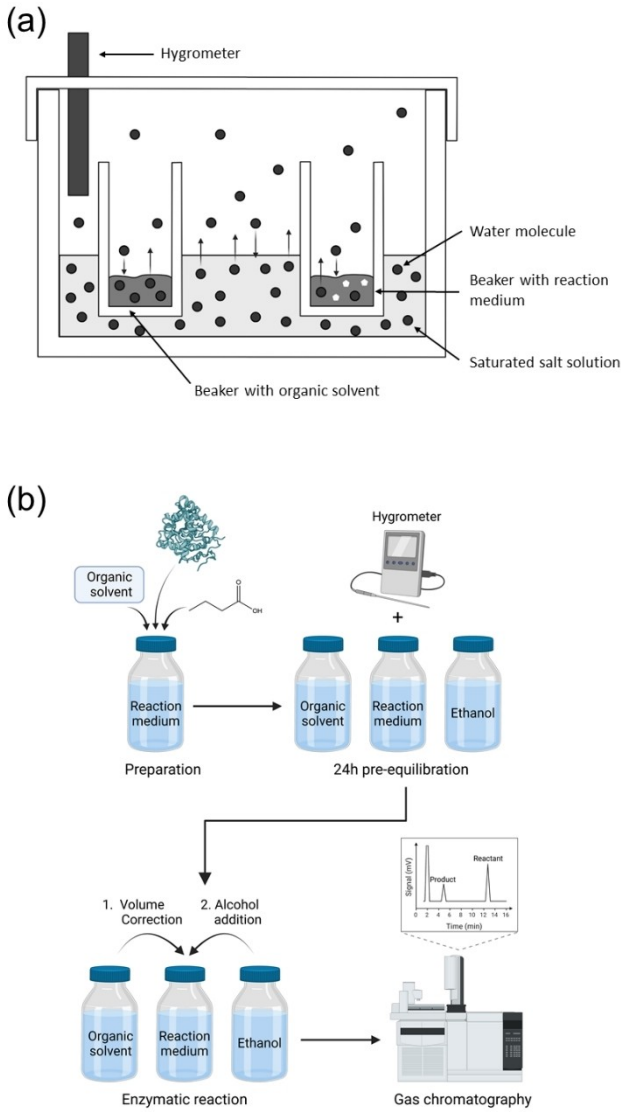

Figure 1. (a) Schematic representation of the pre-equilibration of the reaction medium and pure organic solvent. The reaction medium consists of butyric acid, lipase, and organic solvent. The water activity is measured with a hygrometer. The second substrate (ethanol) was pre-equilibrated in a separate container to ensure that the reaction did not start untimely. (b) Overview of the steps in the drying protocol and subsequent assay of lipase activity.

Results and Discussion

Water activity can be controlled by pre-equilibrating the lipase and organic reaction medium above saturated salt solutions (Figure 1a). Depending on the reaction mixture's initial a_w, water molecules diffuse through the gas phase from the saturated salt solution into the reaction mixture or vice versa to establish an equilibrium. When equilibrium is reached, the water activity is identical to that in the salt solution, which is also the same as that in the gas phase, and the reaction mixture. Therefore, it is possible to measure the water activity using a hygrometer.

Optimising the protocol for controlling water activity

Optimising the methods for obtaining a defined water activity by pre-equilibration over saturated salt solutions resulted in the protocol outlined in Figure 1b. During the pre-equilibration step, a considerable volume of volatile organic solvent from the reaction medium was found to evaporate. Figure 2 illustrates how solvent evaporation leads to an increase in the butyric acid concentration in the reaction medium. The volume of evaporated solvent was tracked gravimetrically, and the change in butyric acid concentration was measured with gas chromatography (GC), as described in the Experimental Section. Between 13–43 % of the original volume would evaporate independently

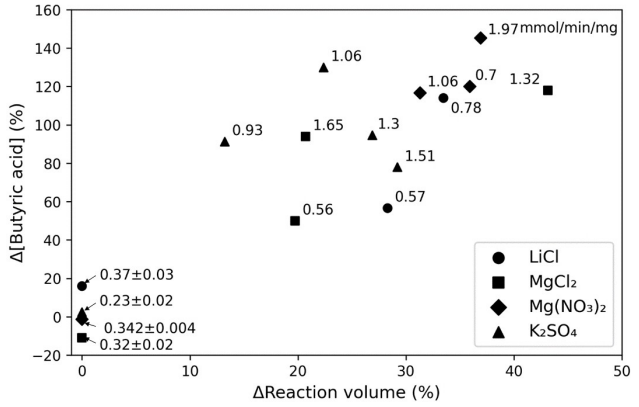

Figure 2. Correlation between the change in reaction volume and change in butyric acid concentration (Pearson correlation = 0.88). The data represents CALB L in MTBE, where the reaction medium was pre-equilibrated for 24 hours. The measured specific activity (mmol/min/mg) for each experiment is given above the individual data points.

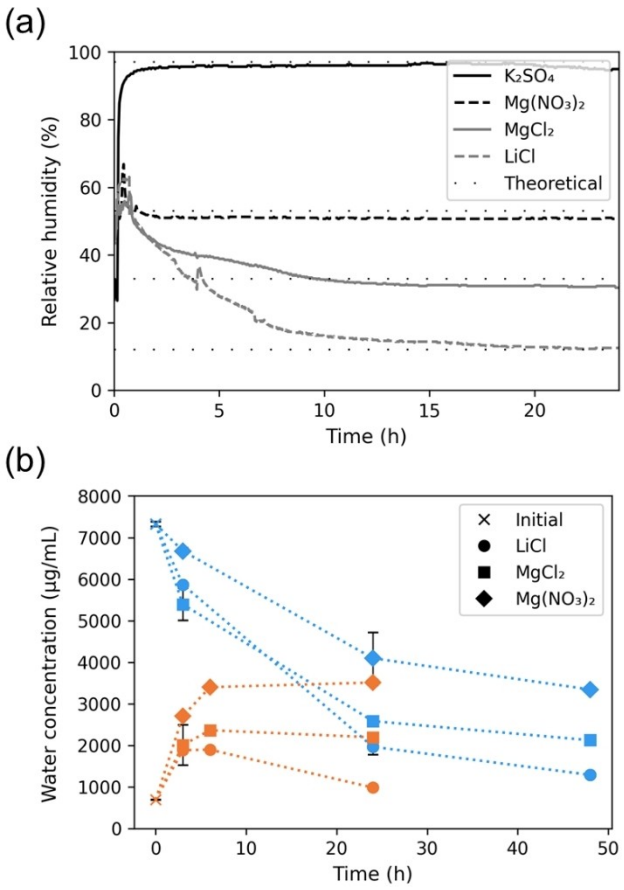

Figure 3. (a) Relative humidity of samples with CALB L in MTBE. The water activity was measured as the relative humidity by a hygrometer in the gas phase. (b) Concentration of water in samples with CALB L in MTBE during the pre-equilibration measured with Karl Fischer titration. Blue and orange colours, respectively, represent systems with CALB L and NZ435.

of the salt used in the pre-equilibration. To compensate for this, a volume of likewise pre-equilibrated organic solvent corresponding to the evaporated solvent was added to the reaction flask before initiating the reaction. The four leftmost points in Figure 2 represent experiments where the volume has been corrected. With volume correction, the change in butyric acid concentration drops to −11 to 16%; without the volume correction, the change in concentration was 50 to 145%. Figure 2 implies that increased substrate concentrations and smaller reaction volumes may yield too high specific activities compared to reactions with volume-corrected reaction media.

Evaporation of the reaction medium was not the only variable that generated inconsistent and irreproducible enzyme activity measurements. Preliminary experiments with CALB L in hexane showed that even when volume correction was incorporated into the protocol, specific activities measured at the same a_w had uncertainties in the range of 7–33%. Visual inspection of the reaction flasks revealed that the small aqueous phase with the soluble lipase was stuck at the bottom as a film. Carrying out the pre-equilibration under constant magnetic stirring (100 rpm) ensured that the two phases had better contact with each other and suppressed the film formation.

To ensure that the desired water activity was obtained during the pre-equilibration, water activity was monitored in the gas phase with a hygrometer. Goderis and co-workers have described that water activity can be measured as the relative humidity divided by 100, i.e. a relative humidity of 97% equals a water activity of 0.97.[40] As shown in Figure 3a, the relative humidity can reach a stable level after less than 15 hours; however we suggest to extend the pre-equilibration to 24 hours to add a safety margin. To further evaluate if 24 hours was sufficient, the change in the concentration of water during the pre-equilibration was measured using Karl Fischer titration. Figure 3b provides the concentration of water in the reaction mixtures containing MTBE for immobilised and soluble CALB. The results from hexane and acetonitrile are provided in Figure S1 and Figure S2. The concentration of water ranges from 20–300 µg/mL in hexane and 700–7000 µg/mL in MTBE to 3300–24000 µg/mL in acetonitrile. The amount of water increases with the decrease in the solvents' log(P)-value; hexane (log(P) = 3.9),[41] MTBE (0.94),[42] and acetonitrile (−0.34).[43] The wide range in the amount of water across the different solvents reinforces the suggestions of Valivety and co-workers that water content in such samples should be reported by the water activity and not the concentration of water if one wants to compare enzyme activity in multiple solvents.[26,27] As outlined in the Introduction, the concentration of water in the organic phase of the reaction mixture does not adequately describe the micro-environment around the lipase. In theory the water activity in the aqueous phase around the lipase should be identical even though the lipase is submerged in different organic solvents. As expected, the concentration of water reaches a similar level for CALB L and NZ435 during the pre-equilibration in MTBE and acetonitrile (Figure 3b and Figure S2), when the same salt is used for the pre-equilibration. The same trend was not seen for CALB L and NZ435 in hexane (Figure S1). Our hypothesis is that NZ435 containing the partially hydrophilic poly(methyl metacrylate) resin allow the reaction mixture

with hexane to hold a greater amount of water than the reaction mixture with hexane without NZ435. The difference between the hexane reaction media with CALB L and NZ435 is approximately 50–200 μg/mL; a difference so small that we cannot see it for the ACN and MTBE reaction mixtures where the water content is much higher. Lastly, it is evident that the saturated salt solutions with low water activities yield the lowest concentrations of water.

Enzymatic reactions

The optimised drying protocol has been tested on CALB L and NZ435 in the three solvents: acetonitrile, hexane, and MTBE. To compare activities, the esterification between butyric acid and ethanol yielding ethyl butyrate was carried out with both lipase formulations. The specific activities (in mmol/min per mg of enzyme protein) in the different solvents at four water activities are presented in Figure 4, Figure S3, and Table 1. CALB L and NZ435 showed the highest activity in hexane, with specific activities around 15–25 mmol/min/mg. CALB L's enzymatic activity was higher in acetonitrile compared to MTBE at $a_w < 0.5$. However, the opposite trend was seen for NZ435, where the lipase showed higher activity in MTBE. It may be hypothesised that the support material in NZ435 may be affected by the solvent and water activity, which can also affect the enzymatic activity. Valivety and co-workers studied five lipases on two different support materials; activity data illustrated differences between the supports at both low and high water activities.[22] This supports our hypothesis that if multiple lipases or different lipase formulations are studied in several solvents, one should aim to use soluble lipases to avoid the effects from the support material.

The activity of NZ435 has been investigated numerous times previously in different organic solvents and at varying water activities.[25,30,36,37] NZ435 has, therefore, been included in this study to validate the protocol. The decrease in enzymatic activity going from hexane to MTBE to acetonitrile at $a_w = 0.53$ is similar to previously published data.[25] Here, we have extended the study, and we observe that this trend is more pronounced at low water activities. In contrast, at $a_w = 0.97$, the difference between the specific activity in acetonitrile and MTBE is insignificant. In addition, soluble CALB's behaviour in different solvents and at varying water activities has been presented. As for NZ435, the differences in the specific activities for CALB L in the different solvents are more pronounced at low water activity. An interesting observation, seen in Figure 4 and Table 1, is that soluble and immobilised CALB show surprisingly similar activities. It was initially hypothesised that NZ435 would display higher activity compared to CALB L, as immobilisation has been shown in many cases to enhance enzyme stability and activity.[31,33,44] An effect of immobilization on the specific activity of the CALB enzyme in NZ435 is hence not supported by our data. The stabilizing effect may however become visible in

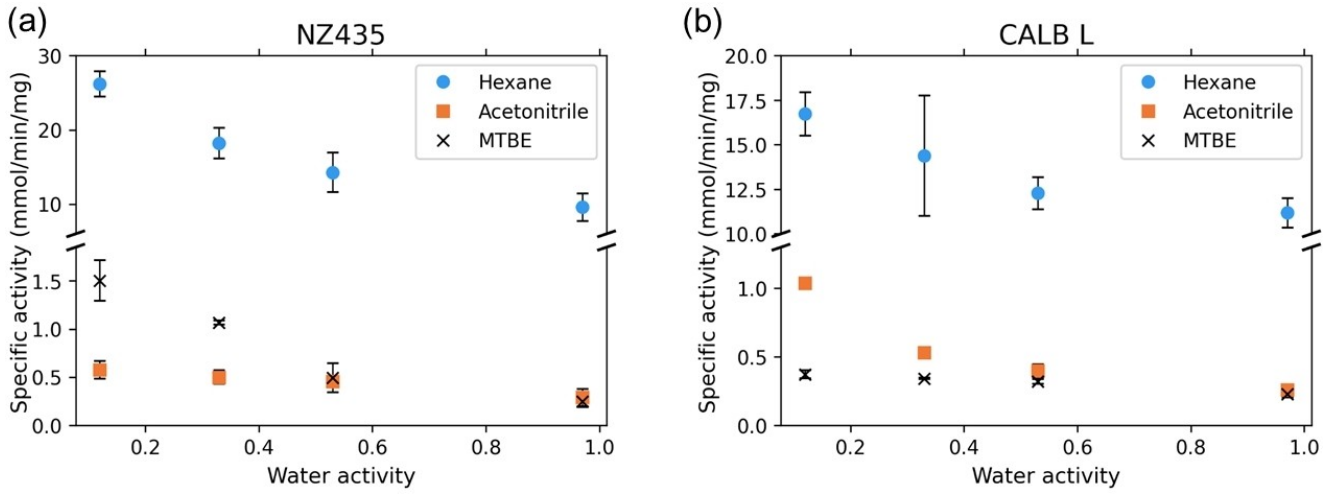

Figure 4. Specific activities of NZ435 (left) and CALB L (right) in acetonitrile, hexane, and MTBE. The specific activities were calculated from duplicate experiments. All data points have an error bar; however, it is too small to be visible for some of them.

Table 1. Specific activities of CALB L and NZ435 in acetonitrile, hexane, and MTBE. The specific activities were calculated from duplicate experiments.

Specific activity [mmol/min/mg]						
		CALB L			NZ435	
a_w	ACN	Hexane	MTBE	ACN	Hexane	MTBE
0.12	1.039 ± 0.002	17 ± 1	0.37 ± 0.03	0.58 ± 0.09	26 ± 2	1.5 ± 0.2
0.33	0.531 ± 0.001	14 ± 3	0.342 ± 0.004	0.50 ± 0.07	18 ± 2	1.08 ± 0.02
0.53	0.40 ± 0.05	12.3 ± 0.9	0.32 ± 0.02	0.46 ± 0.03	14 ± 3	0.5 ± 0.1
0.97	0.26 ± 0.03	11.1 ± 0.8	0.23 ± 0.02	0.29 ± 0.09	10 ± 2	0.25 ± 0.06

reactions with more challenging conditions (over longer time, higher temperature or destabilising reagents or solvents).

Conclusion

An optimised protocol for drying soluble lipases that yields reproducible and consistent results has been developed. The protocol elaborates on well-known procedures with equilibration over saturated salt solutions, resulting in a careful drying to reach a desired water activity. Solvent volume compensation and continuous stirring were found to be important. The pre-equilibration process was evaluated using both a hygrometer (measuring a_w) and Karl Fischer titrations (measuring total water in the system). The protocol was applied on soluble and immobilised CALB in acetonitrile, hexane, and MTBE at four water activities. The results showed that CALB L and NZ435 have very similar specific activities (measured per mg enzyme protein), as well as similar a_w-profiles, with the highest enzyme activity seen at low water activities. Both soluble and immobilised CALB showed higher specific activity in hexane compared to acetonitrile and MTBE; the enzyme activity was similar in the latter two solvents. Our results show that the optimised protocol expands enzyme kinetics studies to soluble lipases and likely also other enzyme classes.

Experimental Section

Chemicals

Candida antarctica Lipase B in soluble (Lipozyme CALB L) and immobilised (Novozym® 435) forms were provided by Novozymes A/S (Bagsværd, Denmark). Ethyl butyrate, butyric acid, ethanol absolute, HCl, 1-hexanol, acetonitrile (≥99%), hexane (≥98%), methyl *tert*-butyl ether (≥99%), lithium chloride (LiCl), magnesium chloride ($MgCl_2$), magnesium nitrate hexahydrate ($Mg(NO_3)_2 \cdot 6\ H_2O$), and potassium sulfate (K_2SO_4) were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Chemicals, where the purity is not given in parenthesis, were of analytical grade.

Methods

Buffer exchange and concentration

Lipozyme CALB L was filtered through a 25 mm GD/X syringe filter with pore size 0.45 μm (GE Healthcare, USA). Afterwards, the lipase was transferred to a phosphate buffer (10 mM NaH_2PO_4, 140 mM NaCl, 27 mM KCl, pH 7.0) and concentrated using a 10 kDa spin filter (Vivaspin Turbo 15, Sartorius, UK). After the concentration was adjusted to 35 mg/mL, the lipase was aliquoted and stored at −20 °C. The concentration was measured in triplicates using UV absorbance at 280 nm on an ND-1000 NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA), where the theoretical extinction coefficient was calculated from the protein sequence (41285 $M^{-1}\,cm^{-1}$) using the ExPASy server.[45]

Preparation of reaction medium

The reaction medium consisted of organic solvent, lipase, and 50 mM butyric acid. The volume of organic solvent and amount of lipase varied depending on the experiment to achieve a suitable reaction rate. The different setups are summarised in Table 2. It was assumed that NZ435 has an enzyme content of 10% (w/w).

Pre-equilibration of water activity

The water activities of the reaction media were set using saturated salt solutions with known water activities: LiCl ($a_w = 0.12$), $MgCl_2$ (0.33), $Mg(NO_3)_2 \cdot 6\ H_2O$ (0.53), and K_2SO_4 (0.97).[46] LiCl and $MgCl_2$ react exothermically with water, and preliminary experiments showed that starting the pre-equilibration before the salt solutions had cooled to room temperature caused significant evaporation of the reaction medium. The reaction medium was pre-equilibrated together with pure organic solvent to ensure that the solvent for the volume correction had the proper water activity. The second substrate (ethanol) was pre-equilibrated in a separate container to ensure that the reaction did not start untimely. The pre-equilibration was carried out under magnetic stirring (100 rpm) at room temperature for 24 hours, where the water activity was tracked with a hygrometer (Tinytag Plus 2 – TGP-4505, Gemini Data Loggers, UK).

Volume correction

The enzymatic reactions were conducted in 20 mL screwcap flasks. The flasks were first weighted empty, then with reaction medium before the pre-equilibration, and finally after the pre-equilibration. The volume of evaporated organic solvent during the pre-equilibration was determined from these weights and the organic solvent's density. A volume of the pre-equilibrated organic solvent corresponding to the evaporated volume was added to the reaction medium to ensure the substrate concentration was constant in all experiments. The needed amount of solvent was determined using Equation 1.

$$\text{Volume} = \frac{\text{Final weight (mg)} - \text{Initial weight (mg)}}{\text{Solventdensity } \left(\frac{\text{mg}}{\text{mL}}\right)} \quad (1)$$

Esterification reactions

After pre-equilibration and volume correction, the esterification reactions were initiated by adding pre-equilibrated ethanol to the reaction medium in equimolar amounts to butyric acid (50 mM). The reactions were performed at room temperature on a magnetic stirrer (800 rpm) for 25 minutes. At regular time intervals, 100 μL samples were withdrawn from the reaction medium and added to GC vials containing 175 μL organic solvent (acetonitrile, hexane or

Table 2. Summary of the organic solvent, type and amount of lipase, and reaction volume used in the enzyme kinetics experiments.

Lipase	Solvent	Enzyme amount [mg]	Reaction volume [mL]
CALB L	Acetonitrile	1.58	3
CALB L	Hexane	0.53	5
CALB L	MTBE	1.05	3
NZ435	Acetonitrile	1.0	3
NZ435	Hexane	0.5	5
NZ435	MTBE	1.0	3

MTBE), 10 μL 1 M HCl to terminate the esterification, and 15 μL 0.5 M 1-hexanol, which was used as an internal standard in the GC analysis. The enzymatic experiments were performed in duplicates.

Analyses

Gas chromatography

The samples extracted from the esterification reactions were analysed using a Perkin Elmer Clarus 500 gas chromatograph (Connecticut, USA) equipped with a flame ionisation detector. The components were separated using a Nukol™ (Sigma-Aldrich) column (15 m length, 0.31 mm i.d., 0.25 μm d_f). Helium was used as the carrier gas with a flow rate of 0.75 mL/min. The samples were injected using split injection with a ratio of 1:50, and the injector temperature was set to 200 °C. Initially, the column temperature was kept at 50 °C for 5 minutes. Afterwards, the temperature was increased to 100 °C at a rate of 25 °C/min and kept at 100 °C for 2 minutes. Lastly, the temperature was increased to 175 °C at a rate of 25 °C/min and kept at 175 °C for 2 minutes. The detector temperature was set to 260 °C. The retention times were as follows: hexane (1.31 min), MTBE (2.20 min), ethanol (2.53 min), acetonitrile (3.50 min), ethyl butyrate (4.01 min), 1-hexanol (9.30 min), and butyric acid (11.91 min). 1-Hexanol was employed as an internal standard to determine the concentrations of ethyl butyrate and butyric acid in the samples. The response factors for ethyl butyrate (0.84 ± 0.07) and butyric acid (0.5 ± 0.1) in relation to 1-hexanol were determined from standard curves. The response factors were calculated using Equation 2, using known concentrations of ethyl butyrate or butyric acid, and 1-hexanol.[47] The units for the peak areas and concentrations are μV·s and mol/L, respectively.

$$\text{Response factor} = \frac{\text{Area}_{\text{Sample}} \cdot [1 - \text{Hexanol}]}{\text{Area}_{1-\text{Hexanol}} \cdot [\text{Sample}]} \quad (2)$$

Calculation of the specific activity

The specific activities in different organic solvents were calculated from the initial reaction rates of the production of ethyl butyrate from ethanol and butyric acid. The first 10% of substrate conversion was included in the calculations, to ensure the calculations were based only on the linear region of the reaction.[48] The ester formation was plotted against the reaction time, and the slope was determined using linear regression; the unit of the slope is mM/min. The specific activity was calculated from the slope using Equation 3 (unit is mmol/min/mg).

$$\text{Specific activity} = \frac{\text{Slope} \cdot \text{Reaction volume}}{\text{Mass}_{\text{enzyme}}} \quad (3)$$

Karl Fischer titration

The concentration of water in the pre-equilibrated organic reaction mixtures were measured on a TitroLine KF trace (Schott Instruments, Germany), and Aqualine Electrolyte AD was used as anolyte. Samples were extracted during the pre-equilibration with a syringe, and the amount of solvent added to the titration chamber was determined gravimetrically.

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Conflict of Interest

The authors declare no conflict of interest

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Biocatalysis · Enzyme catalysis · Lipase · Organic solvent · Soluble enzyme · Solvent effects · Water activity

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