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Concatenating microorganisms, chemical catalysts, and enzymes for the synthesis of ethyl formate from renewables

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Alternative chemical synthesis routes and the (re)utilization of CO₂ are crucial for the development of a circular bioeconomy. To establish sustainable production methods, concatenating various catalysts allows for leveraging the unique advantages of each catalyst type. During process development, however, the reaction conditions must be harmonized, the catalyst compatibility must be examined, bottlenecks must be identified, and thus the operating window for the best process mode must be found. Here, we present ethyl formate synthesis from glucose, which is achieved by concatenating three catalysts: the yeast *Saccharomyces cerevisiae*, a ruthenium catalyst, and a lipase in an immobilized enzyme formulation. This study identifies the parameters that require investigation when combining different catalyst types and integrating CO₂ as an alternative raw material. Parameters such as product stability across different reaction systems, product distribution in a two-phase system using different organic solvents, and catalyst compatibility were studied. *Candida antarctica* lipase B was identified as a suitable biocatalyst for the enzymatic esterification of formic acid and ethanol. The catalyst compatibility is shown. However, the aqueous phase, necessary for yeasts, conflicts with product stability and catalyst activity. The desired one-pot, one-step process could not be realized. As a consequence, the process can only be efficiently performed in two steps, because in a one-pot, one-step reaction, the product yield was low. The concept of a concatenated ruthenium-catalyzed hydrogenation and lipase reaction can also be adapted to other formate esters starting from CO₂.

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1. We firmly believe that concatenating different types of catalysts, such as living microorganisms, purified enzymes, and chemical catalysts, has enormous potential for developing sustainable processes. This is particularly true of the conversion of non-fossil substrates, such as renewable sugars, and the integration of CO₂.
2. The synthesis of ethyl formate with integrated CO₂ utilization was used as an example to identify the parameter space for a sustainable process involving various catalysts. The limitations and advantages of various reaction modes were identified and compared. In the coupled process consisting of a ruthenium catalyst with *Candida antarctica* lipase B, we produced 20 mM ethyl formate in the green solvent cyclopentyl methyl ether.
3. Greener processes require wider availability of green solvents, more case studies of concatenated catalytic processes, and more stable catalysts (e.g., identified via AI methods) across systems.

Introduction

Alternative carbon sources are necessary in times of defossilization, as many processes in the chemical sector rely on

carbon feedstocks. Only by integrating alternative carbon sources, it is possible to develop a circular, sustainable (bio-) economy to meet the global challenge of climate change. CO₂ and biomass are promising alternatives. As the concentration

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of CO₂ in the atmosphere is low, it is challenging to harness it.^{1,2} Nevertheless, with actual emissions of over 40 billion tons, CO₂ is one of the most abundant carbon sources.³ CO₂ can directly be converted into other, more reactive, C1 molecules such as formic acid,⁴ which has been well researched, for example, by applying (electro)chemical catalysts.⁵ Further CO₂ fixation technologies are currently under investigation, such as the use of chemocatalysis,^{6,7} whole cells,⁸ and enzymes such as formate dehydrogenases or carboxylases.^{9–11} In addition to capturing CO₂ from the atmosphere, it can also be incorporated into a reaction under controlled conditions. Concentrated CO₂ is often used for this purpose. Alternatively, CO₂ can be produced directly within the reaction system. For example, it can be produced as a byproduct of fermentation, which is the focus of this study. Apart from CO₂, other C1 molecules, such as CO, formate, methanol, and methane, serve as substrates for different types of catalysts, including (living) microorganisms, enzymes, and chemical catalysts. Each type of catalyst has its own advantages. Living microorganisms can operate as self-multiplying biofactories that catalyze multiple reaction steps simultaneously. Isolated enzymes can be highly stereoselective biocatalysts that can be applied flexibly under aqueous reaction conditions, as well as in unconventional reaction media. Chemical catalysts are well-established and often have high turnover numbers (TONs). They frequently operate under non-physiological reaction conditions.¹²

The primary goal of sustainable process development is to create the most efficient, economical, and ecological processes. Various process modes can be employed by combining or concatenating steps with different types of catalysts. Ideally, a sustainable carbon source would be converted into the desired product in a one-pot, one-step process to minimize subsequent downstream processing and reduce the process time.¹²

However, the range of adjustable parameters to define reaction conditions is limited, and all catalyst types must be compatible. This is often not the case, as each catalyst type requires specific reaction conditions. Otherwise, inhibition, instability, or toxicity of the substrates, products, or catalysts may occur. Another option is to separate the reaction steps temporally or spatially. This can be done in a single-pot multi-step process or in different pots. While this provides greater flexibility in reaction parameters, it also increases the number of downstream processing steps and the overall process time.

Guntermann *et al.* published an example of a concatenated catalytic process that uses alternative carbon sources. In this process, *Saccharomyces cerevisiae* and a ruthenium catalyst (Ru-catalyst) were employed to directly hydrogenate the microbially produced CO₂ to synthesize formate in one pot.¹³

In the present study, we extended this idea to produce the valuable compound ethyl formate (Fig. 1), which has various applications. For example, it can be used as a flavoring agent, a solvent for nitrocellulose synthesis, and as a pesticide for strawberry¹⁴ and lettuce farming.¹⁵ It has a generally recognized as safe (GRAS) status.¹⁶

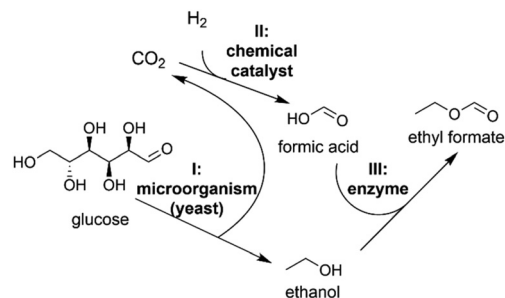


Fig. 1 Reaction scheme of the concatenated process including three catalyst types.

Esterification can be carried out biocatalytically using various enzymes. The most prominent ones are esterases and lipases,¹⁷ but carboxylic acid reductases have also been shown to catalyze this type of reaction.¹⁸ Carboxylic acid reductases (EC 1.2.1.30) catalyze the esterification under aqueous reaction conditions. Lipases (EC 3.1.1.3) from bacteria and fungi catalyze esterification in organic solvents and ester hydrolysis in aqueous media. They typically hydrolyze carboxylic esters of long-chain acylglycerol.¹⁸ The fungal lipase B from *Candida antarctica* (CAL-B) has been extensively studied and is used in industry for hydrolysis reactions in soap production, transesterification in the production of pharmaceutical building blocks, and in the energy sector for producing biodiesel.¹⁹ Reactions with CAL-B in various reaction solvents have been investigated.^{20,21} The substrate spectrum also comprises shorter carboxylic acids and alcohols for the esterification reaction to produce compounds such as octyl formate.²² CAL-B is commercially available as an immobilized enzyme, which increases the enzyme stability and reusability.

This investigation of process development illustrates the parameters that must be examined when integrating or using alternative carbon sources, such as CO₂, as starting materials.

Additionally, adjustable parameters are considered when concatenating various catalyst types to meet their requirements. The most favorable process mode is also evaluated: one-pot, one-step, or separation of the reaction steps.

Experimental

Chemicals and materials

All reactions performed at ambient pressure were carried out in 1.6 mL glass vials with closed caps (caps with septa) to reduce evaporation of volatile compounds, unless otherwise stated. Commercially available immobilized *Candida antarctica* lipase B (CAL-B) was used for all enzymatic reactions (Novozyme 435, specific activity of >5,000 U g⁻¹). *Saccharomyces cerevisiae* Ethanol Red (Leaf, Lesaffre, Marcq-en-Baroeul, France) was applied as the whole-cell microorganism. The ruthenium catalyst [RuCl₂(dppm)₂], carrying two bidentate bis(diphenylphosphino)methane residues, was used.

Product stability under aqueous and organic conditions

Twenty millimolar (20 mM) ethyl formate (800 μ L from a 40 mM stock solution) was incubated under aqueous (100 mM HEPES buffer, pH 8.5) and organic (dimethyl sulfoxide (DMSO)) reaction conditions. First, 53 mM formic acid (180 μ L from a 500 mM stock solution) and then 112 mM ethanol (180 μ L from a 1 M stock solution) were added. The mixture was incubated at 40 °C and 750 rpm for 24 hours, and the samples were taken at 0, 1, 3, 6, and 24 hours. The samples were diluted to 1 : 8 in acetonitrile.

Lipase-catalyzed esterification

We used immobilized CAL-B (24 mg, >120 U) for all biocatalytic reactions in the one-phase system and 12 mg (>60 U) in the two-phase system. In a two-phase system, the enzyme mass is related to the organic phase (800 μ L). Under completely organic conditions, a stock solution was prepared with 2 M ethanol and 250 mM formic acid in the corresponding organic solvent. Then, 200 μ L of the solution was added to the reaction vial ($V = 1.6$ mL) to achieve the desired working concentrations of 50 mM formic acid and 250 mM ethanol. Cyclopentyl methyl ether (CPME) or dioctyl ether was used as an organic solvent. The reaction was incubated for 24 h at 30 °C and 750 rpm.

In an aqueous–organic two-phase system (1 : 1 ratio), ethanol (2 M stock solution) was added to the aqueous phase, consisting of 100 mM HEPES or KPi buffer at pH 7 (24 °C). Formic acid (400 mM stock solution) was added to the organic phase, resulting in a final solution containing 50 mM formic acid and 250 mM ethanol. The reaction was then incubated at 30 °C and 750 rpm.

During the optimization of the enzymatic esterification under completely organic conditions in CPME, the following substrate concentrations were used: 2 M formic acid (120 μ L) and 10 M ethanol (840 μ L).

The negative control did not include any enzyme. This reaction was incubated for 24 h at 40 °C and 750 rpm.

Neat solvent system

Immobilized CAL-B (24 mg, >120 U) was weighed into glass vials, mixed with 2 M formic acid (120 μ L) in ethanol, and incubated at 40 °C and 1,000 rpm. During optimization, the substrate concentration was increased to 800 μ L formic acid with 800 μ L ethanol. The negative control did not contain any lipase. For water removal, 100 mg of 3 Å molecular sieves was added to the reaction solution consisting of 800 μ L formic acid and 800 μ L ethanol. Furthermore, in this reaction, water-free ethanol was used, and formic acid was dried over MgSO_4 for 24 h.

Influence of the ruthenium catalyst on CAL-B activity

To test the enzyme activity in the presence of the Ru-catalyst in a two-phase system, 2 μ mol of the Ru-catalyst $[\text{RuCl}_2(\text{dppm})_2]$ was added to 600 μ L of the organic phase (dioctyl ether). As the aqueous phase, 600 μ L of 100 mM HEPES buffer, pH 7,

were used. Immobilized CAL-B (12 mg, >60 U) was weighed into vials. As a negative control, CAL-B was incubated in a two-phase system (1 : 1 ratio) without the Ru-catalyst. The two-phase system was incubated for 0 h, 24 h, 48 h, and 72 h. Afterward, 200 μ L of each substrate stock solution (400 mM formic acid in dioctyl ether and 2 M ethanol in 100 mM HEPES buffer, pH 7) was added to the corresponding phase to achieve a final concentration of 50 mM formic acid and 250 mM ethanol. All reactions were incubated at 30 °C and 750 rpm.

Product distribution in a two-phase system with various solvents

Ethyl formate was dissolved in the selected solvents (CPME, dihexyl ether, dioctyl ether, dodecane, 2-undecanone) to a concentration of 40 mM. 800 μ L of the solution were added to 800 μ L of 100 mM KPi-buffer, pH 7. The phases were not fully mixed before incubation at 30 °C and 750 rpm for 24 h. Samples of the organic phase were taken at the beginning and after 24 h.

Growth of *Saccharomyces cerevisiae* in the presence of different solvents

In general, *S. cerevisiae* Ethanol Red was streaked from cryopreserved glycerol stocks onto yeast-peptone-dextrose (YPD) medium agar plates and incubated at 30 °C for 24 h. A single colony was transferred to 5 mL liquid YPD medium, which was incubated in a tube at 30 °C and 200 rpm for 24 h to generate sufficient biomass. A second pre-culture was performed in 50 mL of modified Verduyn minimal salt medium for anaerobic growth (VfA), adapted from Guntermann *et al.*,¹³ in 500 mL shake flasks at 30 °C and 200 rpm (throw: 50 mm) overnight. YPD medium consisted of 10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone, and 20 g L⁻¹ glucose. VfA contained 100 g L⁻¹ glucose, 10 g L⁻¹ potassium hydrogen phthalate, 2.5 g L⁻¹ $(\text{NH}_4)_2\text{SO}_4$, 1.5 g L⁻¹ KH_2PO_4 , 0.25 g L⁻¹ $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, as well as 5 mL L⁻¹ 100 $\times$ trace elements, 0.5 mL L⁻¹ of 1000 $\times$ vitamin solution, and 0.5 mL L⁻¹ of anaerobic growth solution.

The vitamin solution contained 0.05 g L⁻¹ D-biotin, 1 g L⁻¹ calcium-D-pantothenate, 1 g L⁻¹ nicotinic acid, 25 g L⁻¹ myo-inositol, 1 g L⁻¹ thiamine hydrochloride, 1 g L⁻¹ pyridoxine hydrochloride, and 0.2 g L⁻¹ *p*-aminobenzoic acid. The trace element solution consisted of 1.5 g L⁻¹ EDTA, 0.45 g L⁻¹ $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g L⁻¹ $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.03 g L⁻¹ $\text{CoCl}_2 \cdot 7\text{H}_2\text{O}$, 0.03 g L⁻¹ $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.04 g L⁻¹ $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.45 g L⁻¹ $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 g L⁻¹ $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g L⁻¹ H_3BO_3 and 0.01 g L⁻¹ KI. The anaerobic growth solution contained ethanol and Tween80 in a 1 : 1 ratio with 15 g L⁻¹ ergosterol. In precultures for the one-pot process, 5% (v/v) of dioctyl ether was added to allow adaptation to the solvent.

The biocompatibility of solvents with *S. cerevisiae* was tested using two approaches. In a simple first screening, 800 μ L of VfA, inoculated with *S. cerevisiae* Ethanol Red, and 200 μ L of the tested solvent were incubated in a gas-tight Hungate tube at 30 °C and 200 rpm. Growth was assessed visu-

ally by detecting turbidity after 24 h of incubation. Tested solvents were CPME, dioctyl ether, and 2-undecanone.

The biocompatibility of dioctyl ether and 2-undecanone was assessed by cultivating *S. cerevisiae* Ethanol Red in their respective presence in the Transfer-Rate Online Measurement (TOM) system (Kühner Shakers, Birsfelden, Switzerland) to record the carbon transfer rates as a signal of microbial activity in shake flask cultivation. To represent the final setup in the concatenated approach, a high solvent-to-cultivation medium ratio of 9 : 1 and low power input were chosen. The total filling volume was set to 50 mL in standardized shake flasks (540–570 mL). The shake flasks were incubated at 50 rpm (50 mm throw) and 30 °C. The transfer rates were monitored at 20 min intervals.

Conditions for a simultaneous one-pot process

Immobilized CAL-B (30 mg, >150 U) was weighed into an autoclave equipped with a glass inlet. The atmosphere was exchanged by applying vacuum and refilling with argon three times. $[\text{RuCl}_2(\text{dppm})_2]$ (2 μmol) in dioctyl ether (3.5 mL) and yeast cultivation medium (VFA 0.4 mL), inoculated with *S. cerevisiae* Ethanol Red at an optical density of 10, were added. The autoclave was sealed airtight and pressurized carefully with H_2 (120 bar). The reaction was stirred (100 rpm) for 24 h at 30 °C.¹³

Coupling of the Ru-catalyst with CAL-B

Immobilized CAL-B (30 mg, >150 U) was weighed into an autoclave (inner volume = 12.5 mL) equipped with a glass inlet. The atmosphere was exchanged by applying vacuum and refilling with argon three times. Ethanol (0.12 mL, 2.00 mmol) as well as a stock solution of $[\text{RuCl}_2(\text{dppm})_2]$ (2 μmol) in CPME (2 mL) were added. The autoclave was sealed airtight and pressurized with CO_2 (30 bar) and H_2 (90 bar). The reaction was stirred (100 rpm) for 24 h at 30 °C.

Instrumental analysis

Samples were taken from the reaction using a gastight Hamilton syringe by piercing the septum to probe both phases.

Samples were diluted in acetonitrile to a volume of 200 μL in PCR tubes, centrifuged for 3 min at 2000x g, and filled into GC vials with an inlet.

GC measurements were carried out on an Agilent GC 6890N Network GC system with an Agilent HP-FFAP column (30 m $\times$ 0.530 mm; inner diameter: 1 μm). The measurement was started at 60 °C and the following temperature profile was applied: hold for 1 min, heating to 100 °C at 5 °C min^{-1} , hold for 1.5 min, heating to 210 °C at 20 °C min^{-1} , hold for 2 min. The retention times of ethyl formate and ethanol were 4.2 min and 5.3 min, respectively. Butyl formate has a retention time of 6.8 min. For product identification, commercially available standards were used.

Statistical information

All experiments were performed in technical triplicate ($n = 3$) with one batch of commercially available CAL-B. One batch of

S. cerevisiae was used for each experimental series of the concatenated reactions. The error bars represent the calculated standard deviation of the mean of the measured values.

Results and discussion

The most practical approach when concatenating different catalyst types is to perform all steps in a one-pot, one-step process. This is because no downstream processing steps are required between the different catalytic steps in this mode. However, there are few degrees of freedom when it comes to choosing the reaction conditions, since the concatenated reaction conditions must meet the requirements of each catalyst. In addition, aspects such as product and substrate stability must be compatible with the process settings. Consequently, a temporal or spatial separation of reaction steps is often required. We based our work on a process developed by Guntermann *et al.*,¹³ which we extended with an additional biocatalytic step to produce ethyl formate (see Fig. 2). First, we studied the necessary process parameters for a simultaneous one-pot, one-step process.

Process parameters for a simultaneous one-pot process with two phases

When developing the one-pot process, it was necessary to investigate various parameters, such as the stability of the product and substrate, identifying suitable catalysts, ensuring compatibility between all catalysts, and preventing toxic side effects that inactivate one catalyst in the presence of another. This study examined these factors and determined the crucial parameters for a simultaneous one-pot, one-step system.

Product stability in the different phases of the one-pot process

In the initially published system, tetradecane was used as the organic solvent, which is highly hydrophobic ($\log P = 7.2$),

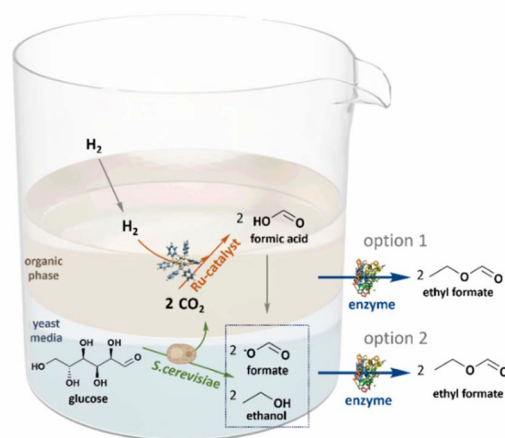


Fig. 2 Scheme of ethyl formate synthesis in a one-pot, one-step process. The enzymatic esterification can occur in the organic phase (option 1) or in the aqueous phase (option 2).

whereas formic acid ($\log P = -0.2$),²³ ethanol ($\log P = -0.1$)²⁴ and ethyl formate ($\log P = 0.5$)²⁵ are hydrophilic compounds. Consequently, the substrates and the product will be predominantly solubilized in the aqueous phase. Such systems require more suitable biocatalysts for esterification under aqueous conditions. For these systems, carboxylic acid reductases (CARs) or only the A-domains of CARs (CAR-As) are potential candidates (Fig. 2, option 2).²⁶ First, the stability of the product under aqueous conditions was investigated. Therefore, ethyl formate was incubated in the presence of ethanol and formic acid under buffered aqueous and organic conditions (Fig. 3). Dimethyl sulfoxide (DMSO) was used as the organic solvent, as it is aprotic. Under aqueous conditions, the concentration of ethyl formate decreased continuously. After 6 h, only 10% of the initial concentration remained, and after 24 h, no ester could be detected. In contrast, the ester was stable in DMSO over 24 h (Fig. 3).

Various mechanistic and kinetic studies have already been performed to explain the aqueous neutral ester hydrolysis.^{27,28} There are two parallel catalytic pathways. One mechanism is the autoionization of water, which can be initiated by an acid or a base. The other is proton-catalyzed hydrolysis. As a weak acid, formic acid is a source of protons in water ($pK_a = 3.7$ at 25 °C).

An increased amount of ester hydrolysis leads to an accumulation of protons, which accelerates hydrolysis. The same thing happens when the substrates formic acid and ethanol are initially added. Under HEPES-buffered reaction conditions, salts can play an additional role. Furthermore, esterification is an equilibrium reaction. Under aqueous reaction conditions, the equilibrium shifts towards the substrates, resulting in ester hydrolysis.

In an aprotic solvent, hydrolysis does not occur due to the absence of water and protons (see Fig. 3). However, an aqueous phase is essential for yeast growth and is therefore required in a one-pot process for the microbial conversion of glucose to ethanol. Due to its demonstrated effect on product

stability, enzymatic esterification must take place in the organic phase, and the ester must remain there to avoid hydrolysis (Fig. 2, option 1).

Lipase-catalyzed esterification in organic solvents and auto-background reactions

The next crucial step was the identification of a suitable biocatalyst to catalyze esterification in the organic phase (Fig. 2, option 1). Since lipases are generally stable in organic solvents and at aqueous–organic interfaces, and the corresponding immobilized enzymes are easy to handle,²⁹ we chose *Candida antarctica* lipase B (CAL-B) for this purpose.

Monophasic organic system. CAL-B, immobilized on acryl resin (Novozyme 435), is a promising enzyme for the studied esterification, as it is used in the synthesis of similar compounds such as hexyl formate and ethyl laurate.^{30,31} To test its enzymatic activity in the synthesis of ethyl formate, fully organic reaction conditions were first used, with cyclopentyl methyl ether (CPME) as the organic solvent.

CPME is compatible with several enzymes in the microaqueous reaction system (MARS) and is environmentally friendly, making it a promising solvent for enzymatic reactions.³³ As demonstrated in Fig. 4A, CAL-B catalyzes the synthesis of ethyl formate in CPME, which makes it suitable for the process. To determine the limits of the enzymatic reaction, the concentration of formic acid was increased to 2 M, while maintaining an excess of ethanol to shift the equilibrium toward ethyl formate. A product concentration of up to 1.1 M was observed within 6 h (Fig. 4B).

Neat solvent system. By testing a neat system that encompassed only liquid substrates and no additional solvent in order to increase the product concentration further, a predominant autocatalytic background reaction was identified. The esterification occurred without any catalyst when formic acid and ethanol were incubated at 40 °C, resulting in up to 5.3 M ethyl formate within 6 h (Fig. 4B).³² Adding a lipase to the system did not influence the product concentration. This background reaction also occurs with other formate esters, such as butyl formate (Fig. S1). Protons probably play a crucial role in this autocatalytic esterification, just as they do in autocatalytic hydrolysis under aqueous reaction conditions. Furthermore, water removal increased the product concentration to 10.6 M (Fig. 4B).³² This demonstrates the importance of considering background reactions and controls in process development. The studied process (Fig. 2) requires an additional organic solvent because the Ru-catalyst is not active in pure ethanol. Ethanol was diluted in a ratio of approximately 1 : 20. Adding an organic solvent, such as CPME, reduces the background reaction. Thus, to reach high product concentrations, lipase is required. With CAL-B, 1.1 M ethyl formate was synthesized, but only half of the concentration (423 mM) was obtained without the biocatalyst after 6 h (Fig. 4B). In conclusion, the enzyme catalyzes the reaction and adds the product on top of the autocatalytic background reaction in CPME.

Two-phase system. In the one-pot process, an aqueous phase is necessary for yeast growth (Fig. 2). Therefore, we

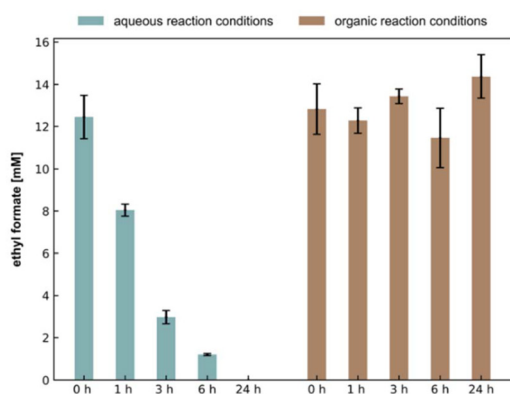


Fig. 3 Stability of ethyl formate under aqueous and organic reaction conditions. Ethyl formate (20 mM) was incubated with formic acid (53 mM) and ethanol (112 mM) in HEPES buffer, pH 8.5 (24 °C), or DMSO. Incubation was performed at 40 °C and 750 rpm ($n = 3$).

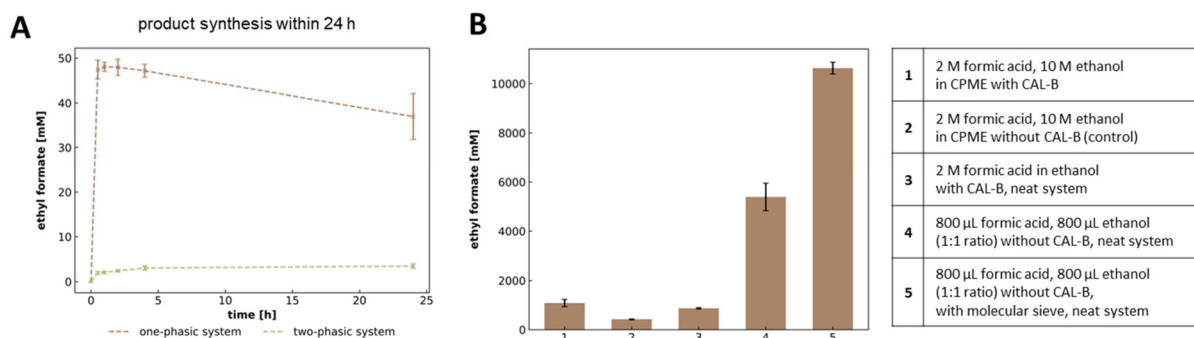


Fig. 4 CAL-B-catalyzed esterification of formic acid and ethanol. **A:** Comparison of the time-resolved ethyl formate synthesis in a one-phasic (brown) versus two-phasic system (green). The one-phasic system consists of 1.6 mL CPME, the two-phasic system 800 μ L 100 mM HEPES buffer, pH 7, and 800 μ L CPME. In the one-phasic system, 24 mg of (>120 U) CAL-B were used. In the two-phasic system, 12 mg of CAL-B (>60 U) were used, corresponding to 800 μ L CPME. The reaction was performed with 50 mM formic acid and 250 mM ethanol at 30 $^{\circ}$ C, and 750 rpm ($n = 3$). **B:** Optimization of the lipase reaction. 1: Reaction system of 2 M formic acid, 10 M ethanol, 24 mg (>120 U) CAL-B in CPME; 2: The reaction consisted of 2 M formic acid, 10 mM ethanol in CPME without CAL-B. 3: A neat system consisting of 2 M formic acid in ethanol with 24 mg of (>120 U) CAL-B. Incubation at 40 $^{\circ}$ C and 1000 rpm. 4: Neat system consisting of 800 μ L formic acid and 800 μ L ethanol without CAL-B. Incubation at 40 $^{\circ}$ C and 1000 rpm. 5: A neat system consisting of 800 μ L formic acid, 800 μ L ethanol, and 62.5 mg mL $^{-1}$ 3 $\text{\AA}$ molecular sieves without CAL-B. Incubation at 40 $^{\circ}$ C, 1000 rpm ($n = 3$). The reaction time was 6 h in all cases.

investigated a two-phase system consisting of an upper organic phase and a lower aqueous phase. In a two-phase system, a significant decrease in product concentration to 3.5 mM was observed. In comparison, a concentration of 37 mM was achieved under fully organic conditions (Fig. 4A). The highest product concentration in the two-phase system was measured after only five minutes of reaction time (Fig. S2). Subsequently, the product concentration decreased and then remained stable over time. This decrease is attributable to the differential distribution of the reaction components between the aqueous

and organic phases (Fig. 5A). No change in product concentration occurs once the equilibrium is reached. The ester partitions between both phases, while formic acid remains in the aqueous phase. Formic acid is the limiting substrate, as its addition to the organic phase resulted in a greater increase in product concentration than the addition of ethanol (Fig. S3). In the final system, the Ru-catalyst will continuously catalyze the formation of formic acid in the organic phase. Consequently, the formic acid distribution in the final process will not be limiting. The next step was to examine the effects

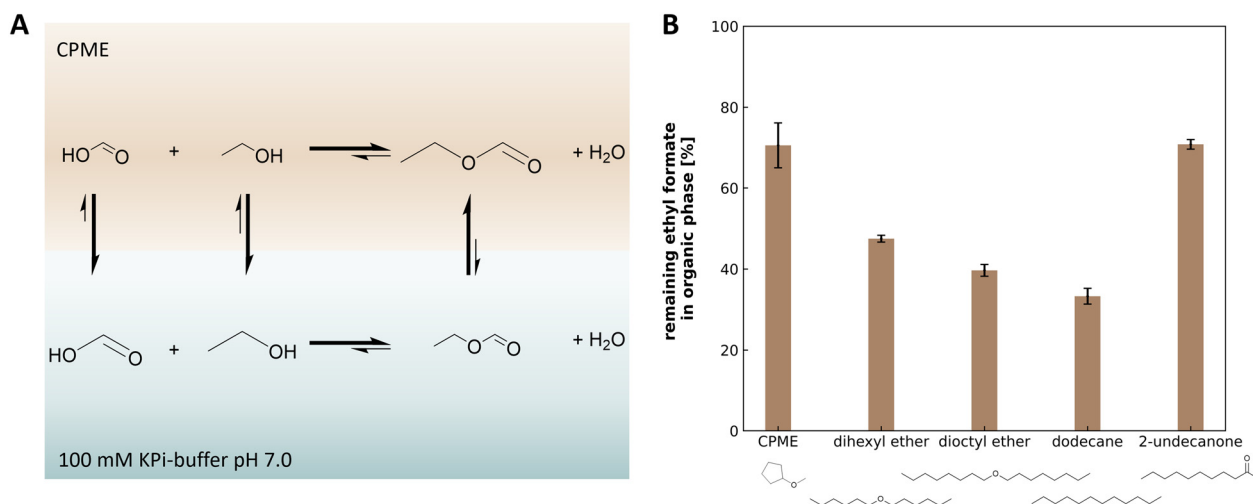


Fig. 5 Solvent candidates for the two-phase process. **(A)** Different distributions of reaction compounds in the two-phase system during the reaction. A detailed description of how the distribution was determined can be found in the SI in section 2.2 and Fig. S5. The distribution of the molecules was illustrated graphically by the size of the structural formulas and by the thickness of the arrows. **(B)** Distribution of ethyl formate after incubation in a two-phase system with different solvents. To determine ester recovery, 800 μ L of a 40 mM ethyl formate stock solution in the corresponding organic solvent was added to 800 μ L of a 100 mM KPi buffer, pH 7. The phases were not fully mixed before incubation at 30 $^{\circ}$ C and 750 rpm for 24 h. Samples were taken from the organic phase using a Hamilton syringe at the beginning and after 24 h. GC analysis was performed as described in the experimental section ($n = 3$).

of incubating CAL-B in a two-phase system prior to esterification. This is important because the formation of formic acid and ethanol requires time, and CAL-B is in contact with both phases throughout the process. When CAL-B was incubated for a longer time in the two-phasic system before the reaction was carried out, the subsequent esterification yielded significantly less ethyl formate (Table S1). As the water activity (a_w) increases, the CAL-B activity decreases.³³ One possible explanation for the negative effects of water on CAL-B is the formation of water clusters on the protein surface near the active center. This may represent a steric hindrance to substrate binding.³⁴ The esterification reaction is reversible, and CAL-B catalyzes the hydrolysis reaction as well. Under aqueous reaction conditions, an autocatalyzed hydrolysis of the ester also takes place, which can further influence the formic acid and ethanol equilibria. As aqueous and organic phases are not thoroughly mixed during the reaction, the extraction of ethyl formate into the aqueous phase should not be the limiting factor (Fig. 2).

Increasing the fraction of the organic solvent in the system increases the product concentration (Fig. S4). However, reducing the aqueous phase also reduces the medium for yeast growth. This, in turn, influences CO₂ production and ultimately affects formic acid synthesis by the Ru-catalyst and the production of the ester. Therefore, identifying the operational window, which determines the required amount of the aqueous phase in relation to the organic phase, is crucial.

In summary, we demonstrated that CAL-B is a suitable catalyst and identified the volume ratio between the aqueous and the organic phase as a possible bottleneck for a simultaneous, catalyzed, one-pot, two-phase process (Fig. S4).

Compatibility of catalyst types and choice of organic solvent

Because the different catalysts have different requirements for optimal performance (Table 1), we examined process parameters to identify a common operating window.

The reaction temperature was set to 30 °C because it is suitable for all three catalysts (SI, section 2.1).

Table 1 Basic requirements for the different catalyst types

Catalyst	Basic requirements for optimal conditions
Chemical catalyst: [RuCl ₂ (dppm) ₂]	High pressure of <i>e.g.</i> , H ₂ (120 bar) Oxygen-free atmosphere Mild temperatures (30–60 °C) Organic solvents Water tolerant
Enzyme: CAL-B	Atmospheric pressure 40 °C Organic solvents Water content as low as possible
Microorganism: <i>Saccharomyces cerevisiae</i>	Atmospheric pressure 30 °C Water required for metabolic activity & growth, the presence of (toxic) organic solvent as low as possible

Solvent selection for the two-phase system. The choice of the solvent is one crucial parameter in process development, especially in the complex multi-phasic reaction system. The solvent in the studied reaction system must fulfill different requirements. For example, it should be poorly miscible with water in order to exhibit low cross-solubility in the aqueous phase, since high concentrations of solvent are toxic to yeast. At the same time, the solvents should be as polar as possible to ensure optimal product and substrate distribution. Formic acid and ethyl formate must stay in the organic phase, while the ethanol produced in the aqueous phase must diffuse into it. There are various equilibria, which influence the distribution of substrates and product in the two-phase system (Fig. 5A). Furthermore, compatibility with the lipase CAL-B and the living microorganism *S. cerevisiae* is essential. Since yeast is more sensitive than lipase CAL-B to solvent selection, we screened different solvents for product distribution and evaluated the tolerance of yeast in their presence. After incubation, 70% of the initial ethyl formate remained in the organic phase when CPME (log *P* = 1.59) was used (Fig. 5B).

A similar percentage of ethyl formate remained in the organic phase when 2-undecanone, which has a log *P* of 4.09 and is less polar than CPME, was used as the organic phase. However, both solvents were toxic to *S. cerevisiae* (Fig. S6). Therefore, the hydrophobicity of the solvent candidate was increased at the expense of a higher amount of ester hydrolysis in the aqueous phase. Dioctyl ether (log *P* 6.9)³⁵ was identified as a compatible solvent for the yeast³⁵ (Fig. S7), showing also an acceptable product distribution (Fig. 5B). In dioctyl ether, the ester remains stable over time as well (Fig. S8). Finding the operational window, which is acceptable for all parameters, is essential. With dioctyl ether, we identified a suitable solvent candidate for a one-pot two-phase system.

Compatibility of different catalyst types. In a system with all three catalysts working simultaneously, it is essential that the catalyst types are compatible under the chosen reaction conditions. The Ru-catalyst's compatibility with *S. cerevisiae* has been shown previously.¹³

The Ru-catalyst was shown to be active in the presence of CAL-B, as evidenced by the detection of formic acid. Additionally, the activity of the lipase CAL-B in the presence of the Ru-catalyst was verified. CAL-B is compatible with the Ru-catalyst for at least 72 h (SI, Table S1).

Simultaneous catalyzed two-phase process in one pot. The operational window in which all catalysts were active was identified. However, given the catalysts' divergent requirements for optimal activity, this left a relatively limited parameter space (Table 1) within which the catalytic efficiency of each catalyst was expected to be far below its respective optimum. As expected, no product was detected when the reaction was performed in a two-phase system with dioctyl ether as the organic phase at 30 °C.

A ratio of 1:9 (v/v) (aqueous phase to organic phase) was used to minimize ester hydrolysis in the aqueous phase and the negative effect of water on CAL-B activity toward esterification. However, the reduced water phase limited yeast growth, which affected the yeast performance negatively. Consequently, the concentrations of CO₂ and ethanol decreased concomitantly

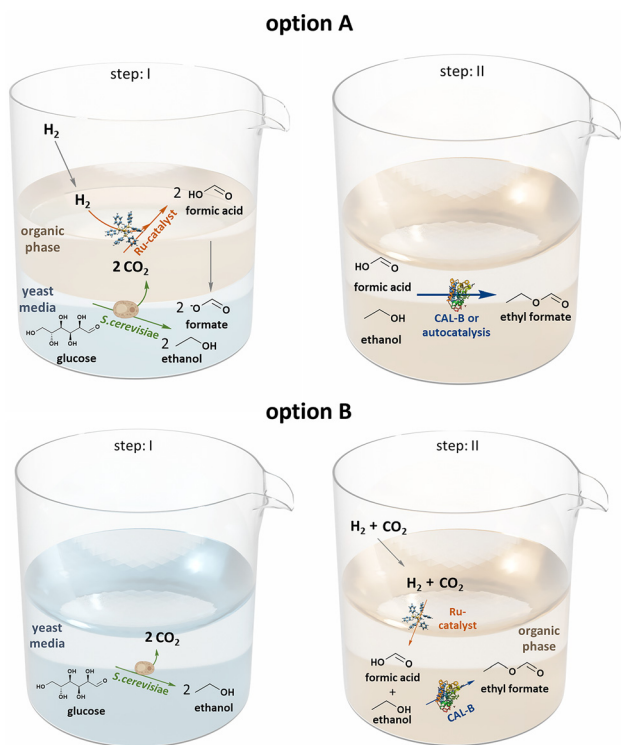


Fig. 6 Two process modes for the two-step ethyl formate synthesis from renewables. **Option A:** couples yeast fermentation to ethanol and Ru-catalyzed formic acid production in a two-phase system in a pressure autoclave (pot 1), followed by esterification with CAL-B or autocatalysis in a neat substrate system (pot 2). **Option B:** couples yeast fermentation to ethanol under aqueous conditions (pot 1), followed by Ru-catalyzed formic acid production and CAL-B-catalyzed esterification in CPME in a pressure autoclave (pot 2).

with the activity of the reactions catalyzed by the Ru-catalyst and CAL-B. Further limitations arose from the distribution of ethanol, formic acid, and ethyl formate in the two-phase system, as only the fraction of the synthesized formic acid in the organic phase was accessible by CAL-B, but not the fraction in the water phase. Furthermore, the performance of CAL-B was not optimal under the selected reaction conditions. The second phase and the lower temperature (30 °C instead of 40 °C) significantly reduced the esterification reaction. Consequently, the catalytic steps were spatially separated (see Fig. 6).

Process engineering in a two-pot system

Separating the reaction steps principally increases the degrees of freedom in the parameter space to optimize the process efficiency. There are two possible separation modes (see Fig. 6). In both cases, two pots were used. However, the reaction environments and the coupled catalysts differed.

Option A: coupled yeast fermentation to ethanol and Ru-catalyzed formic acid production in a two-phase system (pot 1) + subsequent esterification (pot 2)

The first option, as shown by Guntermann *et al.*,¹³ was to couple the Ru-catalyst with the yeast to synthesize formic acid

and ethanol in a two-phase system. Afterward, formic acid can be biocatalytically esterified under fully organic conditions in a second pot (Fig. 6, option A). At the end of the reaction in pot one, additional preparatory steps are required before esterification. The products of the first step, formate and ethanol, must be extracted into an organic phase. Ideally, the used organic solvent would be compatible with CAL-B so that the enzyme could be added directly to the organic phase. This would reduce downstream processing costs and the workload. However, before extraction, acidification would be required, as formate is insoluble in organic solvents. Afterward, CAL-B could catalyze esterification under fully organic conditions. Another option is to purify the products from the first step and then use autocatalyzed esterification of ethanol and formic acid. In this case, no catalyst is needed, and a high product concentration can be reached with 10.6 M ethyl formate (Fig. 4C). Additionally, the absence of a catalyst significantly reduces process costs.

Option B: yeast fermentation to ethanol under aqueous conditions (pot 1) + subsequent coupled Ru-catalyzed formic acid production and CAL-B-catalyzed esterification conditions in CPME (pot 2)

Another option is to decouple the production of ethanol by fermentation from the concatenated formic acid synthesis and esterification. This approach has the advantage that bioethanol fermentation and purification are well-established processes. For example, cocoa waste is already used.^{36,37}

Consequently, we decided to separate the reaction according to **Option B**, in which yeast fermentation takes place in the first pot, and the second pot contains the Ru-catalyst and CAL-B. CPME can therefore be used as a solvent, and the aqueous phase can be omitted. The latter is the main hurdle in reaching high product concentrations. In the concatenated mode of the Ru-catalyst and CAL-B in CPME, 20 mM ethyl formate was synthesized in the second pot. As shown in Fig. 4B (1 and 2), CAL-B is necessary for the esterification reaction that occurs after formic acid is synthesized using the Ru-catalyst in CPME and ethanol. The Ru-catalyst is likely inactive in ethanol (data not shown). Furthermore, the presence of CPME resulted in a less pronounced autocatalytic background (Fig. 4B). Therefore, using CAL-B is essential for effectively catalyzing the esterification in the system.

Additionally, the product spectrum can be increased with the compatibility of both catalysts. A variety of alcohols can be used to produce a wide range of formate esters, in which formate is synthesized directly from CO₂. However, when assessing sustainable sources of various alcohols, it must be considered that synthesis is not always possible directly from glucose-containing waste streams, as with ethanol. A significant advantage is that CAL-B remained active after use in the pressure autoclave (Fig. S9), allowing for reuse and minimizing process costs, as catalysts are a substantial cost factor in processes.³⁸ The coupling method of pot 1 with pot 2 is not yet established.

Table 2 Comparison of the separation of options A and B

Option	Advantages	Disadvantages
A	CO ₂ for formic acid production is produced by yeast fermentation if purified formic acid & ethanol are used, esterification occurs autocatalytically	extraction of formic acid and ethanol from the aqueous phase in pot 1 has to be developed
B	bioethanol fermentation and downstream processing are well established, also from renewables, easier reaction system and scale up, due to monophasic systems in both pots	CO ₂ must be added from an external source, or direct transfer from fermentation must be evaluated

Comparison of Option A and Option B

In general, both option A and option B can be used for ethyl formate synthesis. In comparison, every option has its own advantages and disadvantages (Table 2).

Option A has the huge advantage of direct CO₂ integration from fermentation into the Ru-catalyzed formation of formic acid. However, **Option B** has a simpler reaction setup, making it less costly and easier to scale up. Future studies must fully implement and optimize both options of coupled processes to assess their applicability. The possible general concepts are presented herein. As part of the optimization and process intensification efforts, sustainable alternatives to the Ru-catalyst for formate synthesis should also be investigated. After process intensification, a comprehensive life-cycle analysis should be conducted to quantitatively assess the process's sustainability.

Expanding the scope of products

Lipase CAL-B can also catalyze the synthesis of other formate esters, such as butyl formate (Fig. S10). Thus, the general concept of coupling of the Ru-catalyst with CAL-B could be applied to synthesize an expanded spectrum of formate esters.

Conclusions

In this study, we propose combining different catalyst types and their specific requirements to convert renewable resources and directly reuse CO₂, using the synthesis of ethyl formate as an example. We identified and analyzed critical parameters, such as product stability, solvent selection, and catalyst compatibility. When concatenating different catalyst types in a one-pot, one-step process – which is preferred to reduce downstream processing and the processing time – determining suitable reaction conditions is essential due to the limited parameter space of adjustable variables. The aqueous phase was especially limiting and could not be omitted in a one-pot system. Since a concatenated two-phase one-pot approach was too inefficient, we separated the catalyst steps in space and time. Two separation modes were studied:

In **Option A**, the yeast fermentation occurs with the Ru-catalyzed reaction in one pot while the lipase-catalyzed esterification occurs in the other. In **Option B**, the yeast fermentation takes place in one pot, and the reactions catalyzed by the Ru-catalyst and CAL-B occur in the other pot. With **Option B**, 20 mM ethyl formate was synthesized in 2.12 mL. Further optimization, investigation, and a technoeconomic analysis are required to determine the most appropriate process mode. This demonstrates that splitting reaction modes can sometimes be more advantageous than combining them.

Our developed coupling concept of a Ru-catalyst with CAL-B can be adapted to other products, such as different formate esters. However, further process optimization is necessary to enable the development of a CO₂-integrated process that can compete with existing fossil resource-based systems. Switching from the Ru-catalyst to more sustainable alternatives would further improve the process sustainability. These alternatives include a combination of Mg-chlorophyll-a catalysts (Mg Chl-a) with formate dehydrogenases³⁹ and formate synthesis *via* electrochemical pathways.⁵

Regarding the product ethyl formate, autocatalysis of ethanol and formic acid can be used in its synthesis. This method does not require a catalyst and produces a high concentration of 10.6 M of the product under ambient reaction conditions. In addition to existing routes, further synthesis routes for the supply of ethanol and formic acid are expected to emerge in the future. Due to climate change and limited resources, the integration of alternative raw materials will play a decisive role in future processes. In this context, the synergistic interaction between chemo- and biocatalysis will be crucial for concatenating the advantages of both types of catalysts.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: <https://doi.org/10.1039/d6gc02757a>.

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