Supplementary Information (SI) for Green Chemistry.
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Supporting Information:

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

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1. Lipase-catalyzed esterification in organic solvent and auto-background reactions

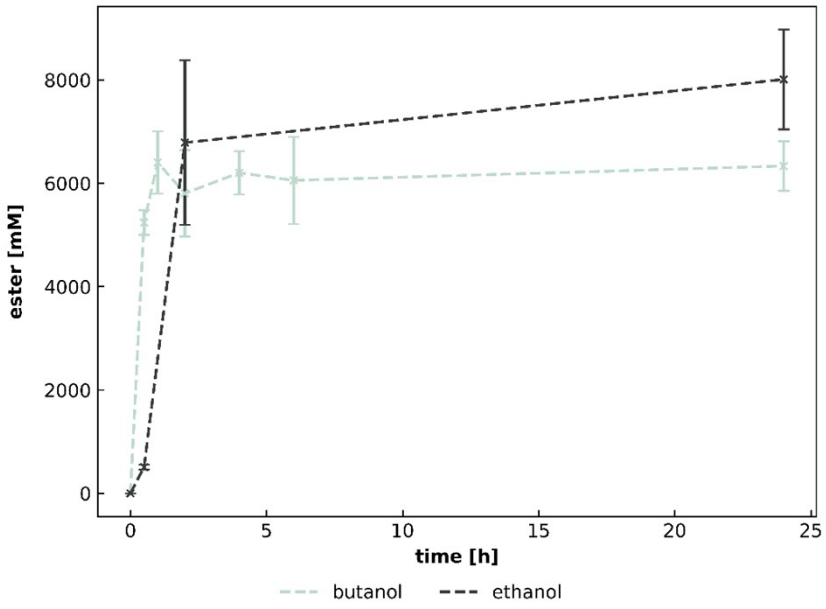

Figure S1: Comparison of the autocatalytic background reaction of formic acid with ethanol or butanol to the respective ester. 800 µL formic acid and 800 µL of the alcohol (ethanol or butanol) were incubated at 40 °C, 1000 rpm for 24 h. Samples were diluted in 100 % isopropanol for butanol and in 100 % acetonitrile for ethanol. GC analysis was performed as described in the experimental section of the main paper (n=3).

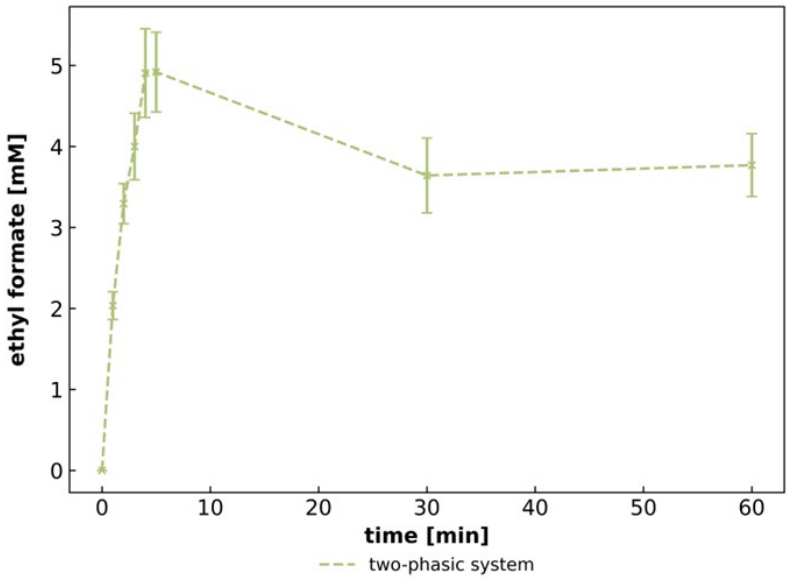

Figure S2: Time-resolved ethyl formate synthesis in the first hour. The reaction was performed in a two-phasic system. It consisted of 800 µL CPME and 800 µL 100 mM HEPES buffer, pH 7. In the reaction 12 mg (>60 U) CAL-B were used. Incubation was performed with 50 mM formic acid and 250 mM ethanol at 30 °C, and 750 rpm. GC analysis was performed as described in the experimental section of the main paper (n=3).

1.1 Two-phase system

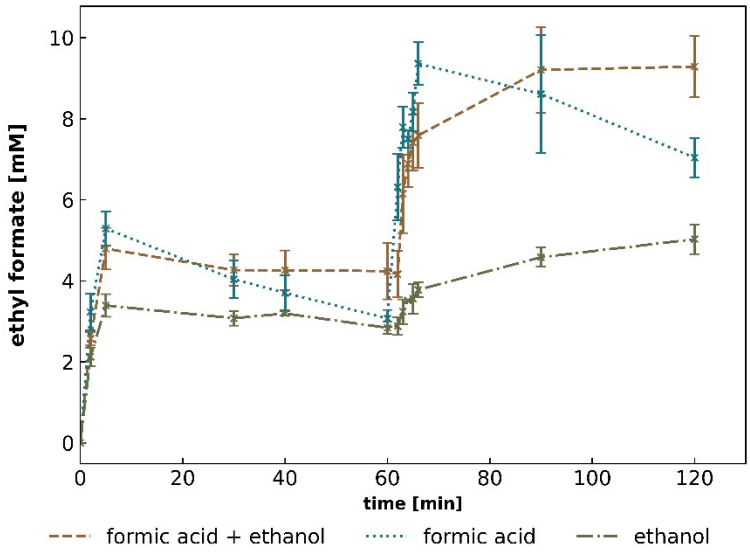

Figure S3: Feeding strategies in a two-phase system. The ratio of the aqueous phase (800 µL HEPES-buffer pH 7) to the organic phase (800 µL CPME) was 1:1 (v/v), and 12 mg (>60 U) CAL-B were used. 200 µL of the ethanol stock solution (2 M) were added to 600 µL HEPES buffer, and 200 µL of the formic acid stock solution (400 mM in CPME) were added to 600 µL CPME. Incubation was performed at 30 °C and 750 rpm. For the formic acid and ethanol feeds, 200 µL of the respective stock solutions (2 M ethanol and 400 mM formic acid) were added to the respective phase after 1 h. For the formic acid feed, 40 µL of HEPES buffer, pH 7, and 40 µL of a 2 M formic acid stock solution (in CPME) were added to the corresponding phase after 1h. For the ethanol feed, 40 µL of a 10 M ethanol stock solution (in HEPES buffer, pH 7) and 40 µL of CPME were added. Samples were taken from the organic phase after 2 min, 5 min, 30 min, 40 min, 60 min, 62 min, 63 min, 64 min, 65 min, 66 min, 90 min, and 120 min. GC analysis was performed as described in the experimental section of the main paper (n=3).

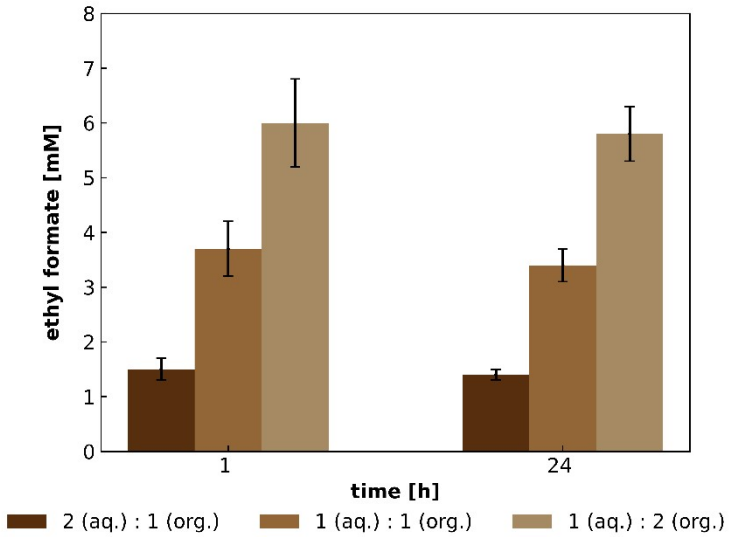

Figure S4: Influence of the ratios of the organic phase to aqueous phases on the formation of ethyl formate. Three different ratios of the aqueous to organic phases were tested: 2:1, 1:1, and 1:2. CPME was used as the organic phase, and HEPES buffer, pH 7, as the aqueous phase. The reaction was catalyzed by 12 mg (>60 U) CAL-B. In the 1:1 (v/v) ratio, 800 µL organic phase and 800 µL aqueous phase were used. 400 µL formic acid (from 400 mM stock solution in CPME) was added to the organic phase, and 400 µL ethanol (from 2 M stock solution in HEPES buffer, pH 7) to the aqueous phase. For the 2:1 ratio, 1066 µL aqueous phase and 533 µL CPME were used. The 1:1 ratio consisted of 800 µL aqueous phase and 800 µL CPME. 533 µL aqueous phase and 1066 µL CPME were used for the 1:2 ratio. Incubation was performed at 30 °C and 750 rpm. Samples were taken after 0 h, 1 h, 2 h, and 24 h. GC analysis was performed as described in the experimental section of the main paper (n=3).

2. Compatibility of catalyst types and choice of the organic solvent

2.1 Influence of the temperature

CAL-B has a temperature optimum of 40 °C. However, for *S. cerevisiae*, 30 °C is required. Therefore, the performance of CAL-B was compared between 40 °C and 30 °C. In the two-phase system, the product concentration after 24 h decreased from 4.1 mM to 3.4 mM. This demonstrates that although the activity is reduced CAL-B is still active.

2.2 Additional information on the distribution of molecules in the two-phase system as illustrated in Figure 5A

To determine the ethanol distribution 800 µL 100 mM KPi-buffer, pH 7 were added into a 1.8 mL glass vials. With a Hamilton syringe, 800 µL of a 500 mM ethanol stock solution dissolved in CPME were added. It should be noted that the complete mixing of the different phases was not performed in order to simulate the situation in the final process, where a complete phase mixing is also not the case. The approach was incubated at 40 °C, 750 rpm for 6 h. At the beginning of the reaction and after 6 h, a sample of each phase was taken with the Hamilton syringe by piercing through the septum. The samples were measured as described in the experimental section of the main paper. The formic acid distribution is not experimentally determined. Since formic acid is more hydrophilic than ethanol (-0.1) (logP = -0.2), formic acid is also found primarily in the aqueous phase. Ethyl formate is present mainly in the organic phase, as shown by the distribution in Figure 5B. In the aqueous phase, the hydrolysis reaction is predominant, whereas in the organic phase, the esterification reaction (according to Figure 1)

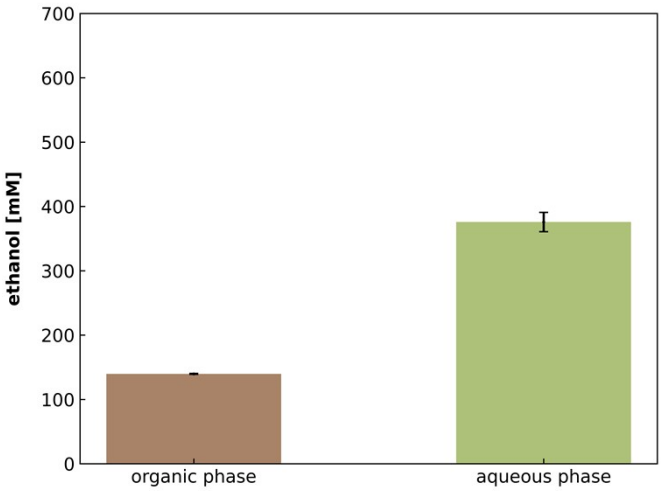

Figure S5: Ethanol distribution in the two-phase system. Ethanol is mainly in the aqueous phase. A higher ethanol concentration is observed in the aqueous phase compared to the organic phase, to which additional ethanol was initially added after six hours of incubation in a two-phasic system (Figure S5). Reaction conditions: 500 mM ethanol in a two-phasic system consisting of CPME and 100 mM KPi-buffer, pH 7.0 (1:1 (v/v) ratio). Incubation for 6 h, 40°C, 750 rpm. Concentration in the aqueous and in the organic phase were measured, therefore a sample of the aqueous phase and of the organic phase were taken with a Hamilton syringe and GC analysis was performed as described in the experimental section of the main paper (n=2).

2.3 Compatibility of the organic solvent with *Saccharomyces cerevisiae*

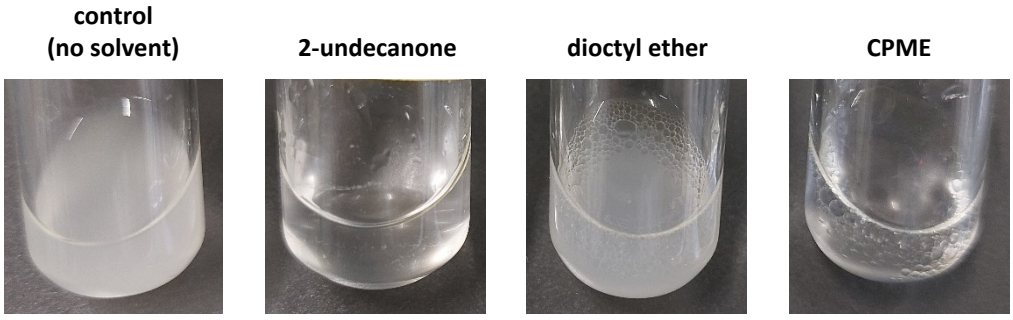

Figure S6: Qualitative biocompatibility of three solvents with *S. cerevisiae* Ethanol Red. 800 µL of modified Verduyn minimal salt medium for anaerobic growth (VfA) medium inoculated with *S. cerevisiae* Ethanol Red were incubated with 200 µL of solvent (30 °C, 200 rpm) in air-tight Hungate tubes. Pictures were taken after 24 h. Turbidity indicates growth. Dioctyl ether shows biocompatibility with the yeast, while growth was inhibited in the presence of 2-undecanone and CPME, respectively.

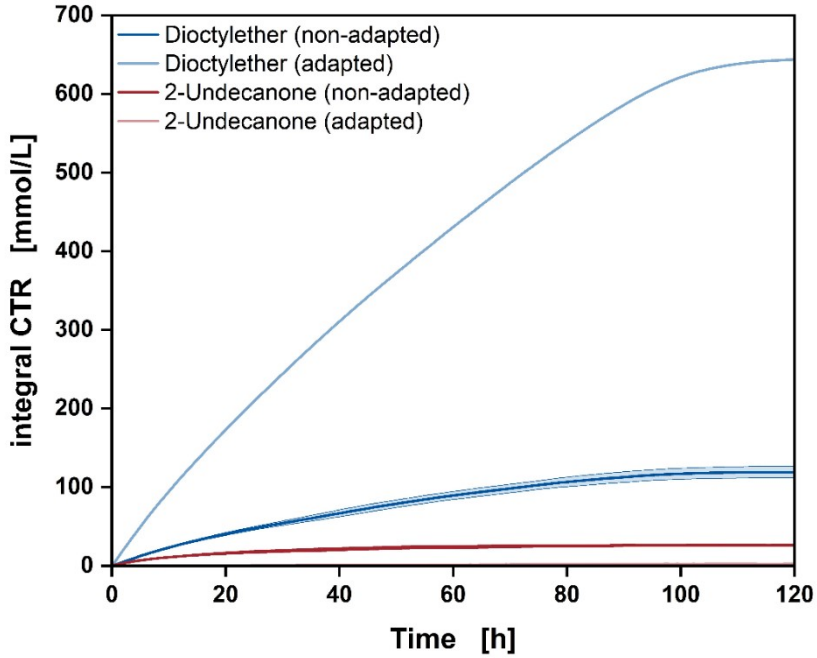

Figure S7: Quantitative biocompatibility of two solvents with *S. cerevisiae* Ethanol Red. *S. cerevisiae* Ethanol Red was cultivated in VfA medium in the presence of dioctyl ether (blue) and 2-undecanone (red) (V_L: 25 mL, ratio medium: solvent 1:9, 30 °C, 300 rpm, throw: 50 mm) in the Transfer-Rate Online Measurement (TOM) system. Non-adapted (dark) and adapted (light) yeast cells were cultivated. Adaptation was performed by adding 5% (v/v) of the respective solvent to the last pre-culture. The carbon transfer rate was recorded every 20 min. Here, the carbon dioxide transfer rate (CTR) integral over cultivation time is depicted. While activity was observed in all cultures, dioctyl ether showed a higher biocompatibility with *S. cerevisiae* Ethanol Red than 2-undecanone. Previous adaptation to dioctyl ether enhances biocompatibility.

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

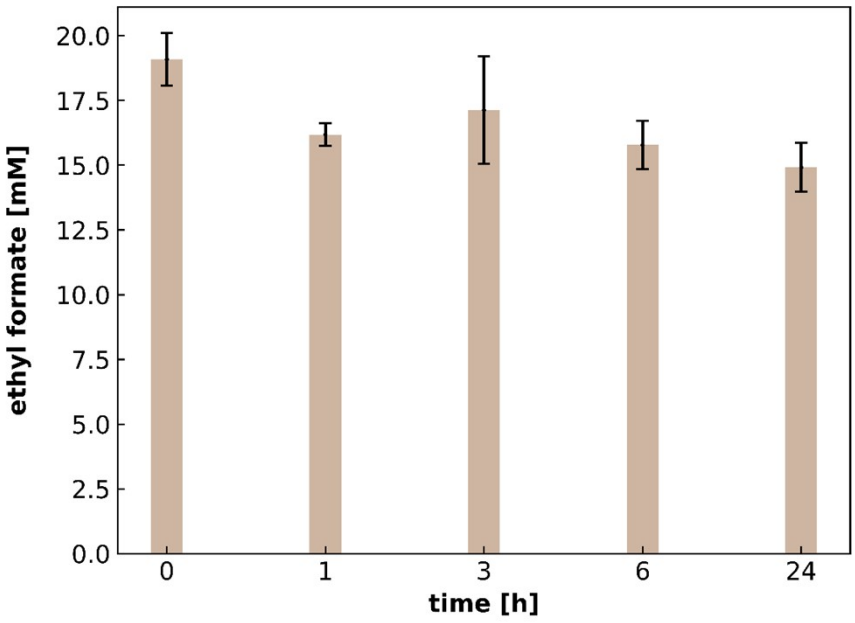

Figure S8: Stability ethyl formate in dioctyl ether. 20 mM ethyl formate were incubated in dioctyl ether with 53 mM formic acid and 112 mM ethanol (V=1.6 mL) at 40 °C, 750 rpm. Measurement using GC-analysis (see experimental section of the main paper) (n=3).

3.1 Compatibility CAL-B with Ru-catalyst

CAL-B was incubated in the presence and absence of the Ru-catalyst as described in the experimental section in the main paper. The measured ethyl formate concentrations are shown in Table S1.

Table S1: Cal-B catalyzed ethyl formate formation after incubation with and without the Ru-catalyst. 12 mg (>60) CAL-B was incubated with/without 2 µmol [$RuCl_2(dppm)_2$] in a two-phase system consisting of 800 µL aqueous phase (100 mM HEPES-buffer, pH 7) and 800 µL organic phase (dioctyl ether). Incubation was performed under ambient pressure in glass vials at 30 °C and 750 rpm for 0 h, 24 h, 48 h or 72 h. Afterwards, the reaction was performed by adding 200 µL formic acid (400 mM in dioctyl ether to the organic phase, and 200 µL ethanol (2 M ethanol 100 mM HEPES buffer, pH 7) to the aqueous phase by a Hamilton syringe. Incubation was performed at 30 C, 750 rpm for 24 h. Measurement was carried out by GC-analysis as described in the experimental section.

Incubation time [h]	ethyl formate [mM] with Ru-catalyst	ethyl formate [mM] without Ru-catalyst
0	1.25 ± 0.09	1.30 ± 0.04
24	1.12 ± 0.09	1.15 ± 0.05
48	0.75 ± 0.04	0.90 ± 0.09
72	0.60 ± 0.02	0.64 ± 0.05

As demonstrated in Tab. S1 the ethyl formate concentration is independent of the presence of the Ru-catalyst. Incubation of CAL-B in a two-phasic system alone has a significant negative impact on the ester concentration synthesized by CAL-B. This decreased continuously with longer incubation times (Table S1). After 72 h of incubation, in the following reaction, only 50 % product was measured compared to the reaction without incubation.

4. Process engineering for a compatible reaction mode in a two-pot system

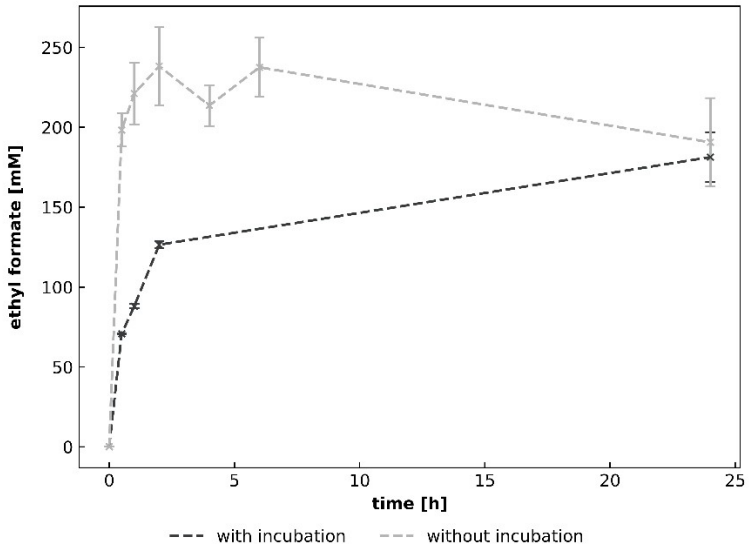

Figure S9: Comparison CAL-B-catalyzed esterification with and without previous incubation in a pressurized autoclave. Incubation of 30 mg (>150 U) CAL-B was performed at 120 bar H_2, with 2 µmol [$RuCl_2(dppm)_2$] and 2 mmol ethanol at 30 °C for 24 h in a (V=2 mL). The lipase reaction afterwards was performed at ambient pressure in a total volume of 1.6 mL. 24 mg (>120 U) of the wet CAL-B, which was previously incubated in the autoclave, was mixed with 250 mM formic acid (15 µL) and 1.25 M ethanol (116 µL) in CPME. As a control, the reaction was performed under the same conditions with 24 mg fresh CAL-B. The esterification was incubated at 40 °C, 750 rpm for 24 h. Samples were taken after 0 h, 0.5 h, 1 h, 2 h, and 24 h. GC analysis was performed as described in the experimental section of the main paper (n=3).

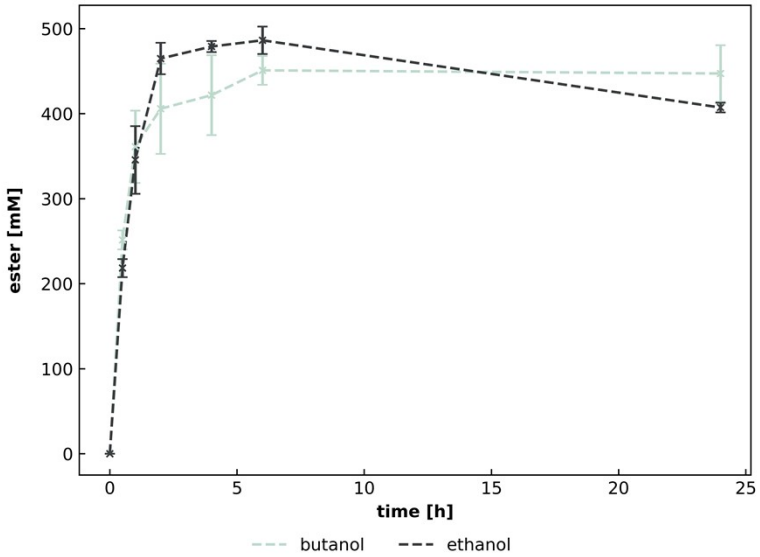

Figure S10: CAL-B catalyzed synthesis of ethyl formate and butyl formate in CPME. 500 mM formic acid (30 µL) and 2.5 mM ethanol or butanol (366 µL butanol/233 µL ethanol) were incubated in CPME with 15 mg mL^{-1} CAL-B (24 mg, >120 U) (V=1.6 mL) at 40 °C and 750 rpm. Samples for the measurement of butyl formate were appropriately diluted in 100 % isopropanol, and for ethyl formate in 100 % acetonitrile, before GC-analysis (see experimental section of the main paper) (n=3).

5. Calculation of the carbon balance for the integrated CO_2

The molar quantity of CO_2 introduced into the reaction at 30 bar was calculated using the ideal gas law. 30 bar CO2 corresponds to $7.8 \cdot 10^6$ mol CO_2. 20 mM ethyl formate in 2.12 mL corresponds to $4.25 \cdot 10^5$ mol ethyl formate. Consequently, 1.6 % of the carbon from the CO_2 was integrated into ethyl formate. Currently, the value is low at 1.6%, consistent with the low product concentration. These values could be improved by further optimizing the process (e.g., by using a more effective chemical catalyst) and by increasing the product concentration.

6. Instrumental analytics

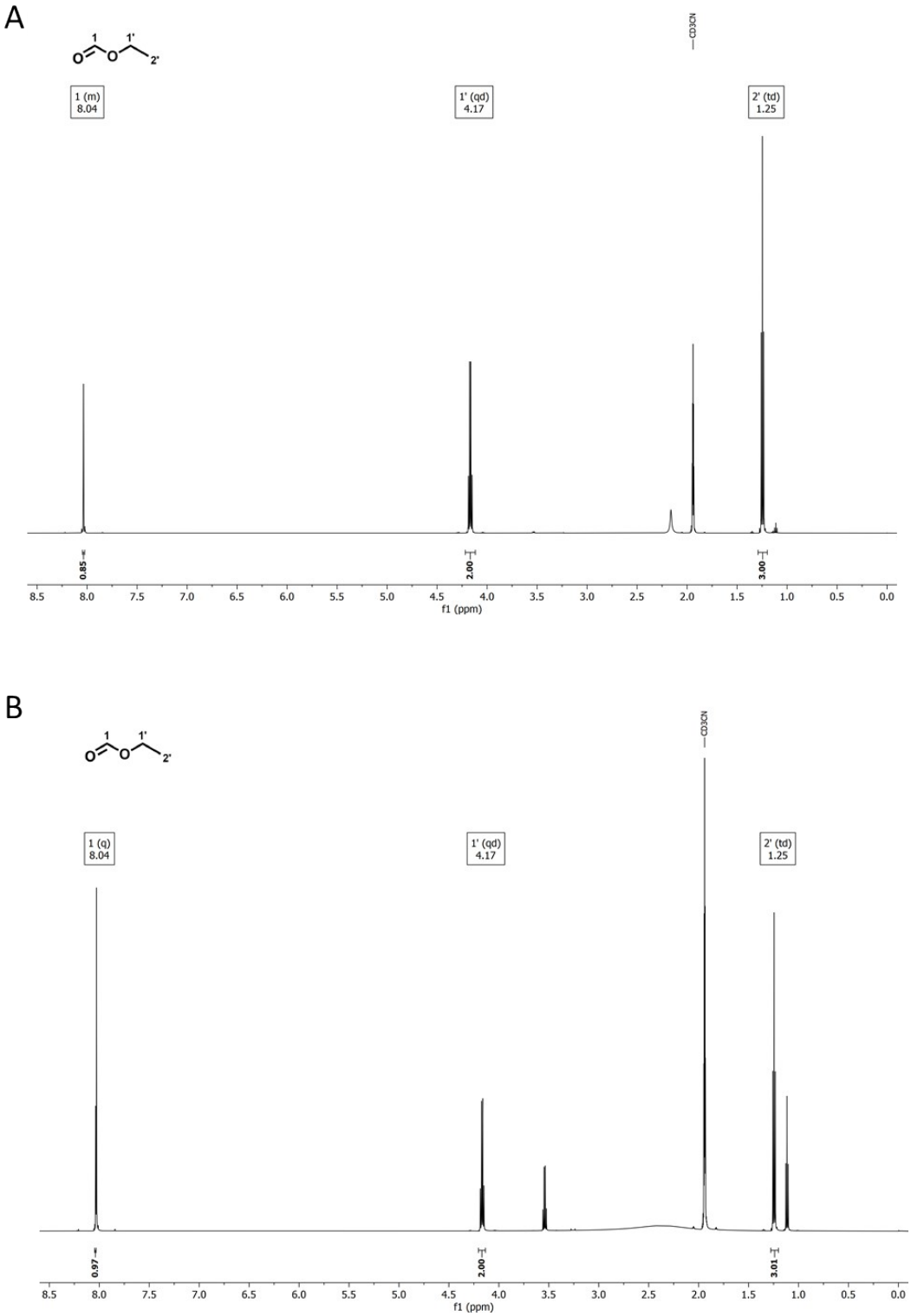

Figure S11: ^{1}H NMR spectrum (600 MHz, CD_3CN) of the ethyl formate reference (commercial analytical standard from Sigma-Aldrich) (A) and CAL-B reaction solution (B).
CAL-B reaction was performed in ethanol with 250 mM formic acid and 24 mg (>120 U) CAL-B for 24 h at 40°C and 750 rpm. The triplet at 1.12 ppm and the quartet at 3.54 ppm in B can be assigned to ethanol.

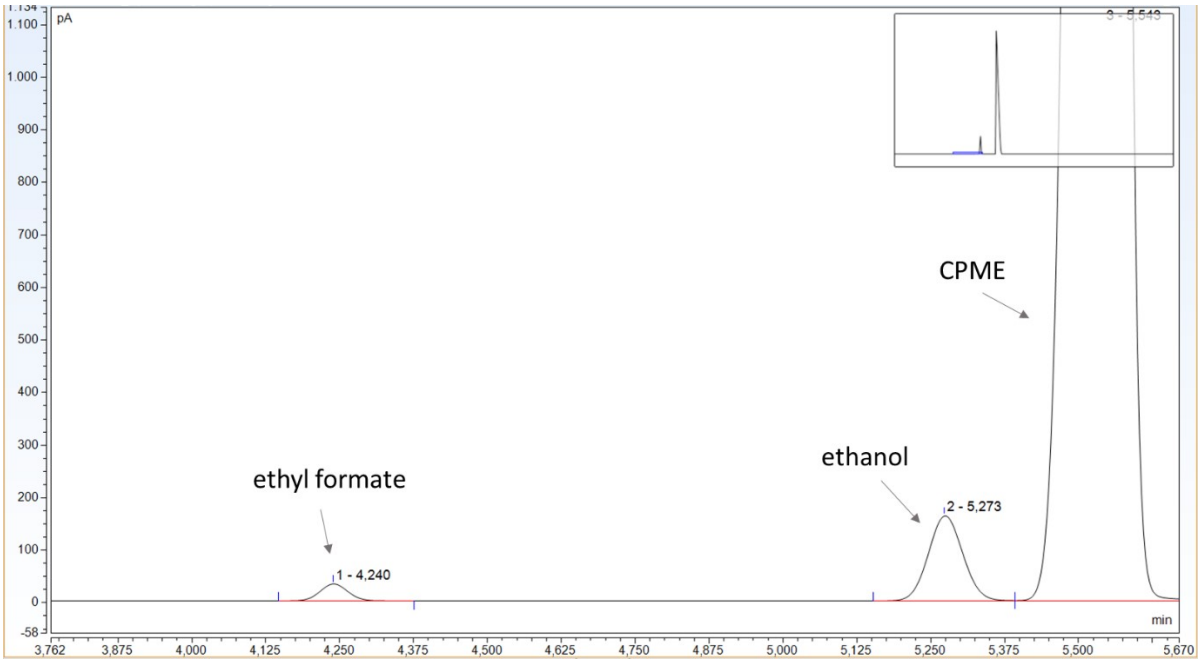

Figure S12: GC chromatogram of the CAL-B-catalyzed ethyl formate synthesis in CPME. Retention times: ethy formate 4.2 min, ethanol: 5.2 min, CPME: 5.5 min. For further details see experimental section in the main paper.