Electronic Supporting Information (ESI)

Automated high throughput workflow for rapid implementation of immobilized enzymes in chemical process development

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Contents

1. Materials

Enzyme carriers were purchased from Purolite and Resindion and details are given in table S1. Acetophenone starting material and (*S*)-, (*R*)- and rac-phenylethanol reference compounds were purchased from Sigma Aldrich. NADP tetrasodium salt was purchased from Carbosynth Ltd. Isopropanol and Methanol were purchased from Sigma Aldrich. Gluteraldehyde (50 wt%) was purchased from Alfa Aesar. Buffer salts and magnesium chloride were purchased from Sigma Aldrich. All chemicals were used without further purification. Milli-Q ultrapure water was used for preparing aqueous stock solutions and dilutions.

Table S1: Supplier, resin name and functional group of all resins used at the screening stage of this work.

Supplier	Resin	Linker	Pore diameter	Particle size
Purolite	ECR8304F	ethylamino	300-600 Å	150-300 µm
	ECR8309F	ethylamino	600-1200 Å	150-300 µm
	ECR8315F	ethylamino	1200-1800 Å	150-300 µm
	ECR8404F	hexamethylamino	300-600 Å	150-300 µm
	ECR8409F	hexamethylamino	600-1200 Å	150-300 µm
	ECR8415F	hexamethylamino	1200-1800 Å	150-300 µm
Resindion	HA113/S	hexamethylamino	200-500 Å	100-300 µm
	HA403/S	hexamethylamino	400-600 Å	100-300 µm
Purolite	ECR8204F	methylepoxy	300-600 Å	150-300 µm
	ECR8209F	methylepoxy	600-1200 Å	150-300 µm
	ECR8215F	methylepoxy	1200-1800 Å	150-300 µm
	MEP/C	epoxy	1000 Å	100-300 µm
Resindion	EP403/S	methylepoxy	400-600 Å	100-300 µm
	HFA403/S	amino elongated-epoxy	400-600 Å	100-300 µm

2. Analytics

The biocatalaytic reduction of acetophone (**1**) to phenylethanol (S)-**2** and (R)-**2** was analyzed on an Agilent SFC system with a 1260 DAD WR detector using a Chiralpak AD-3 (3 µm, 4.6 mm x 150 mm) column and supercritical CO_2 (A) and Methanol (B) as mobile phase. BPR pressure and temperature was 150 bar and 60 °C respectively. Column temperature was 40 °C and detector wavelength is 210 nm. Retention times of starting material and products as well as experimental relative response factor for each compound is given in table S2.

Table S2: Retention time and relative response factors (RRF) for analyzed compounds.

Compound	Retention time / min	RRF
Acetophenone	1.79	1
(*R*)-1-phenylethanol	2.22	1.054
(*S*)-1-phenylethanol	2.28	1.054

The gradient of the analytical method is given in table S3.

Table S3: Solvent gradient of analytical SFC method.

Time / min	A / %	B / %	Flow mL/min
0	98	2	2
1.75	70	30	2
3	70	30	2
3.5	98	2	2
4	98	2	2

3. High throughput methodologies

3.1. General considerations

For resin that need any form of pre-activation, either with glutaraldehyde (for covalent immobilization with amino-functionalized resins) or metal salts (for affinity immobilization) we use method A0, A1, 2 and 3. For resin that don't require pre-activation we used method B1, 2 and 3 (Figure S1). The respective deck layouts and method parameters are found in the following chapters.

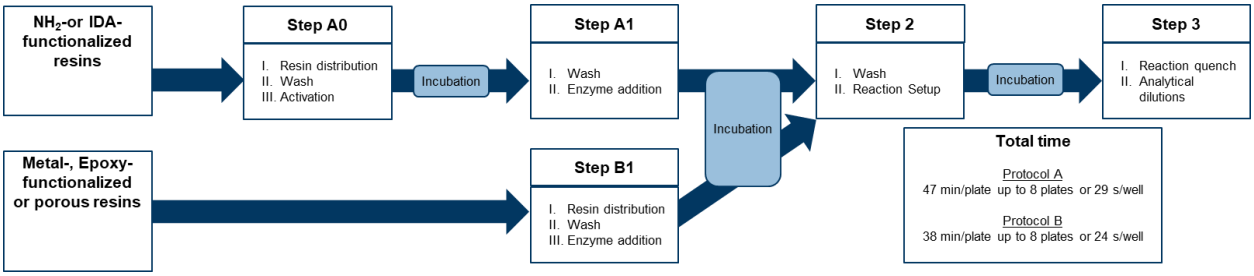

Figure S1: Order of steps for resins requiring pre-activation (e.g. amino- or iminodiacetic acid- functionalized) and requiring no pre-activation (e.g. epoxy-functionalized, metal loaded or porous).

For the resin distribution we developed a methodology that relies on Beckmann 190 µL wide bore tips, the tips are filtered to protect the equipment from any accidental solid particles contamination. The resin can be suspended in any kind of reservoir (full reservoir, 12-row or 8-column reservoir, 96 deep well plate) using distilled water. Using the tips, the resin is pipetted from the fully settled slurry at a low speed and transferred to the reaction plate using both trailing and leading air gaps and blowout including tip touch. For small volumes the plate can be briefly centrifuged afterwards. We have validated this approach in the range of 2.5 to 35 mg of resin using between 4 and 60 µL of pipetting volume by determining the supernatant volume pipetted in addition to resin during the method (Figure S2). As the non-dried particles mostly consists of water (70-80 % according to manufacturer's analysis) we assumed a density of 1 g/mL for the resin in our consideration.

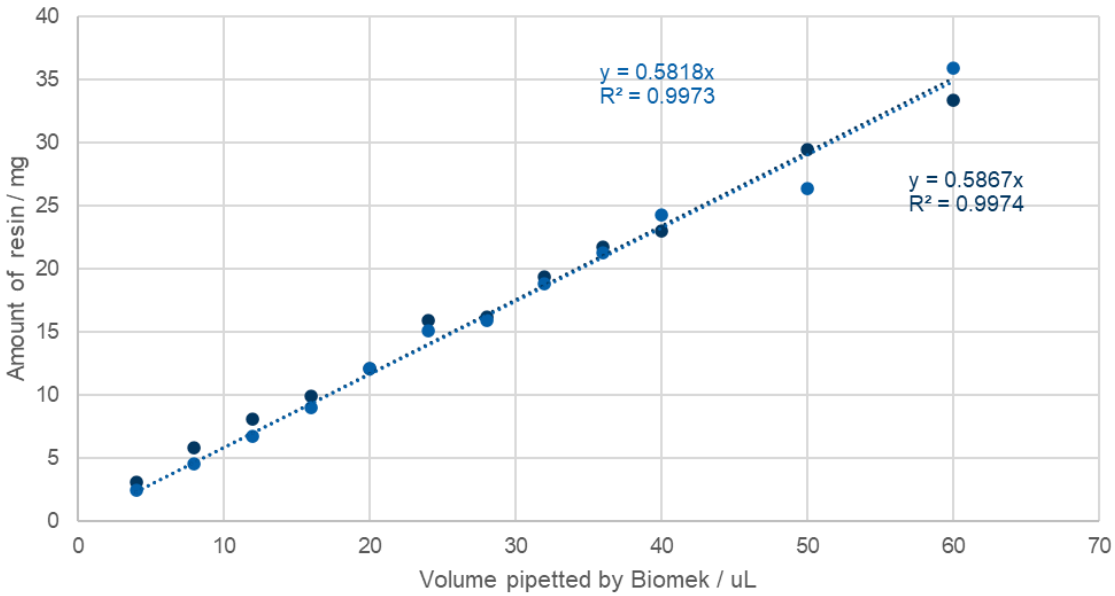

Figure S2: Validation of resin distribution method using Biomek i7. The resin was pipetted using Beckmann 190 µL wide bore tips from a fully settled resin slurry in distilled water. The input volume was pipetted by the liquid handling robot using leading and trailing airgap, blowout and tip touch. Afterwards the supernatant water pipetted by the Biomek was measured and the amount of resin was calculated with [amount of resin (mg)] = [Volume pipetted by Biomek (µL)] – [Supernatant volume (µL)] assuming a density of 1 g/mL for the resin. A range of volumes between 4 and 60 µL was pipetted twice, the results are plotted, and linear regression fitted.

The robustness and reproducibility of the HT enzyme production, immobilization and screening can be analyzed by comparing identical enzyme samples across the plates. In table S4 there are all samples of the original backbone enzyme shown on Codexis KRED plate 1 and 2 to illustrate well-to-well and plate-to-plate variation which are both very good for a HT application.

Table S4: Free and immobilized enzyme conversion of KRED parent present in Codexis KRED plate 1 and 2 taken from the data obtained in the initial panel screening.

KRED	Plate	Well	Conversion free enzyme (0.8 vol% lysate) /%	Conversion immobilized enzyme (4 vol% lysate) /%
KRED parent 2	Codexis KRED plate 1	E9	57.1	57.2
		E10	62.0	46.7
		E11	61.5	51.8
		E12	53.8	29.3
	Codexis KRED plate 2	E3	64.1	50.1
		E4	63.0	52.6
		E5	63.5	53.2
		E6	61.7	54.3
	Average		**60.9 ± 3.6**	**49.4 ± 8.7**

Most notably, we frequently opted to use Integra 8 row or 12 column reservoirs when setting up 2D screenings and more complex immobilization/reaction varying parameters. For example, when screening the immobilization conditions in step 3 of our workflow we prepared glutaraldehyde stock solutions in an 8-row reservoir and used it instead of the standard reservoir to add the reagent to the reaction plate (method A0), resulting in a glutaraldehyde gradient from top to bottom. Following this we prepared enzyme dilutions in a 12-column reservoir (method A1) which resulted in an enzyme gradient from left to right. The results of this approach can be found in main paper's figure 5.

If not otherwise mentioned in the experimental part, default values for each parameter were used. With regards to the settling time before removing the supernatant, we found that for all particles used in the screening 20 seconds of settling time is sufficient after mixing, this value might be adapted based on the resin properties. In case multiple reaction plates (up to 8) are processed in a method the plates are stacked to allow for a streamlined handling and maximize deck space utilization. Glutaraldehyde activation and Methanol quenching steps were performed on an Biomek i7 liquid handling robot under the fumehood. All other steps were performed on an integrated Biomek i7 liquid handling robot with UV/VIS spectrophotometer and sealing device.

3.2. Method A0

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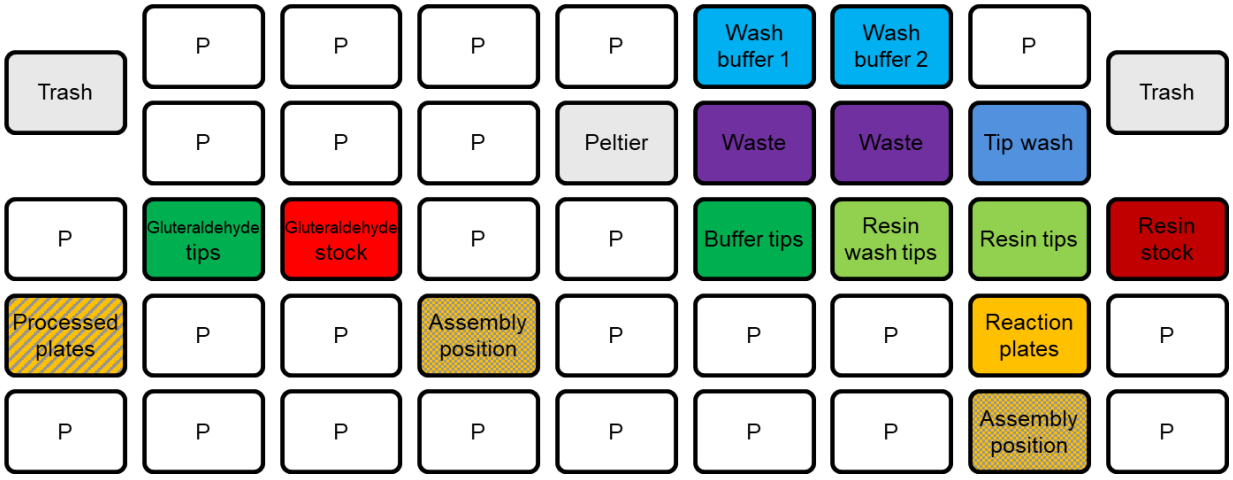

Variables

Materials		**Variables *default value (value range)***	
Reaction plates	Corning 96 well round bottom PP plates	NumberofReactionPlates	8 (1-8)
Wash buffer 1 & 2	Seahorse Reservoir	SealPlates *only on integrated Biomek i7*	yes (yes v no)
Tip wash	Seahorse Reservoir	VolumeActivationReagent	100 (20-200)
Waste	Seahorse Reservoir	VolumeResin	40 (4-60)
Glutaraldehyde stock	Seahorse Reservoir or Integra column/row reservoir	VolumeResinWash	150 (20-200)
Resin stock	Seahorse Reservoir or Integra column/row reservoir	VolumeMix	80 (20-100)
Resin tips	Beckman Coulter Biomek i-series 190 µL pipette tips, wide bore, filtered		
Resin wash tips	Beckman Coulter Biomek i-series 190 µL pipette tips, wide bore, sterile, filtered		
Buffer tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile		
Glutaraldehyde tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile		

3.3. Method A1

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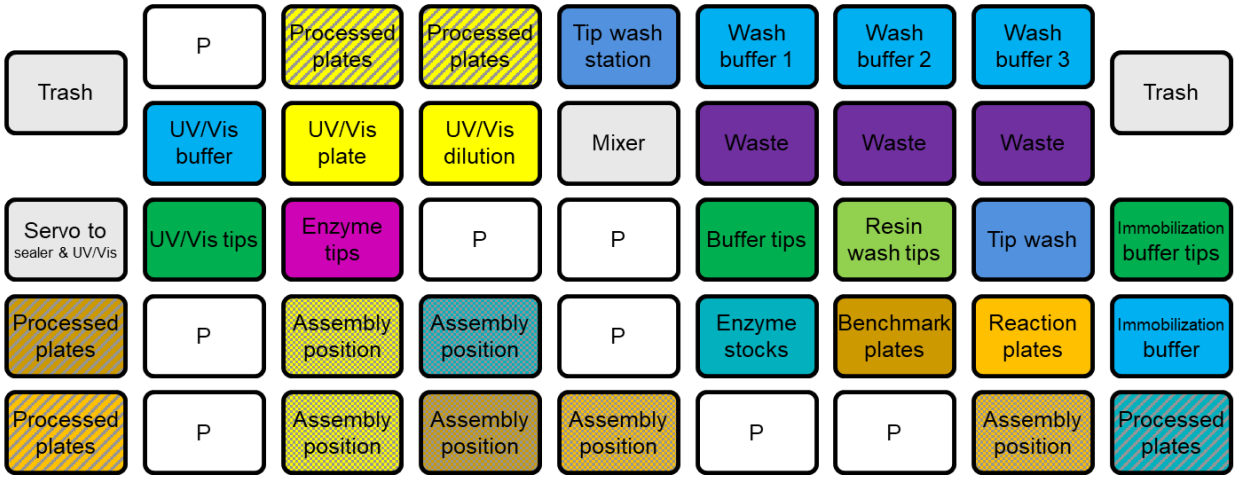

Variables

Materials		**Variables *default value (value range)***	
Reaction plates	Corning plate 96 well round bottom PP	NumberofReactionPlates	8 (1-8)
Benchmark plates	Corning plate96 well round bottom PP	SealPlates *only on integrated Biomek i7*	yes (yes v no)
Enzyme stocks	Biorad plate 96 well PCR hard-shell	DilutionFactor1	10 (5-20)
UV/Vis plates	Nunc plate 96 well flat bottom, UV transparent	DilutionFactor2	10 (2-20)
UV/Vis dilutions	Nunc plate 96 well flat bottom, non-UV transmissible	OverallVolumeDilution1	200 (100-250)
Wash buffer 1, 2 & 3	Seahorse Reservoir	OverallVolumeDilution2	200 (100-250)
Tip wash	Seahorse Reservoir	VolumeActivation	125 (45-225)
Waste	Seahorse Reservoir	VolumeResinWash	150 (20-200)
Immobilization buffer	Seahorse Reservoir or Integra column/row reservoir	VolumeMix	80 (20-100)
UV/Vis buffer	Seahorse Reservoir	VolumeImmoBuffer	50 (10-200)
Resin wash tips	Beckman Coulter Biomek i-series 190 µL pipette tips, wide bore, sterile, filtered	VolumeLysateImmo	50 (10-80)
Buffer tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile	VolumeLysateBM	10 (10-80)
Immobilization buffer tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile		
Enzyme tips	Beckman Coulter Biomek i-series 90 µL pipette tips, non-sterile		
UV/Vis tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile		

3.4. Method B1

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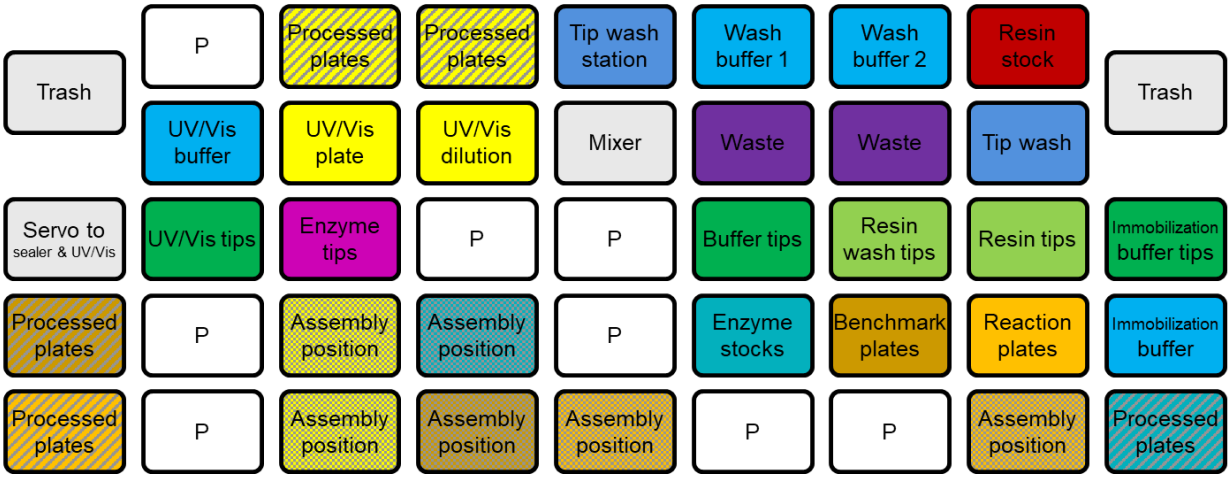

Variables

Materials		**Variables *default value (value range)***	
Reaction plates	Corning plate 96 well round bottom PP	NumberofReactionPlates	8 (1-8)
Benchmark plates	Corning plate96 well round bottom PP	SealPlates *only on integrated Biomek i7*	yes (yes v no)
Enzyme stocks	Biorad plate 96 well PCR hard-shell	VolumeResin	40 (4-60)
UV/Vis plates	Nunc plate 96 well flat bottom, UV transparent	DilutionFactor1	10 (5-20)
UV/Vis dilutions	Nunc plate 96 well flat bottom, non-UV transmissible	DilutionFactor2	10 (2-20)
Wash buffer 1, 2 & 3	Seahorse Reservoir	OverallVolumeDilution1	200 (100-250)
Tip wash	Seahorse Reservoir	OverallVolumeDilution2	200 (100-250)
Waste	Seahorse Reservoir	VolumeActivation	125 (45-225)
Immobilization buffer	Seahorse Reservoir or Integra column/row reservoir	VolumeResinWash	150 (20-200)
UV/Vis buffer	Seahorse Reservoir	VolumeMix	80 (20-100)
Resin stock	Seahorse Reservoir or Integra column/row reservoir	VolumeImmoBuffer	50 (10-200)
Resin tips	Beckman Coulter Biomek i-series 190 µL pipette tips, wide bore, filtered	VolumeLysateImmo	50 (10-80)
Resin wash tips	Beckman Coulter Biomek i-series 190 µL pipette tips, wide bore, sterile, filtered	VolumeLysateBM	10 (10-80)
Buffer tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile		
Immobilization buffer tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile		
Enzyme tips	Beckman Coulter Biomek i-series 90 µL pipette tips, non-sterile		
UV/Vis tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile		

3.5. Method 2

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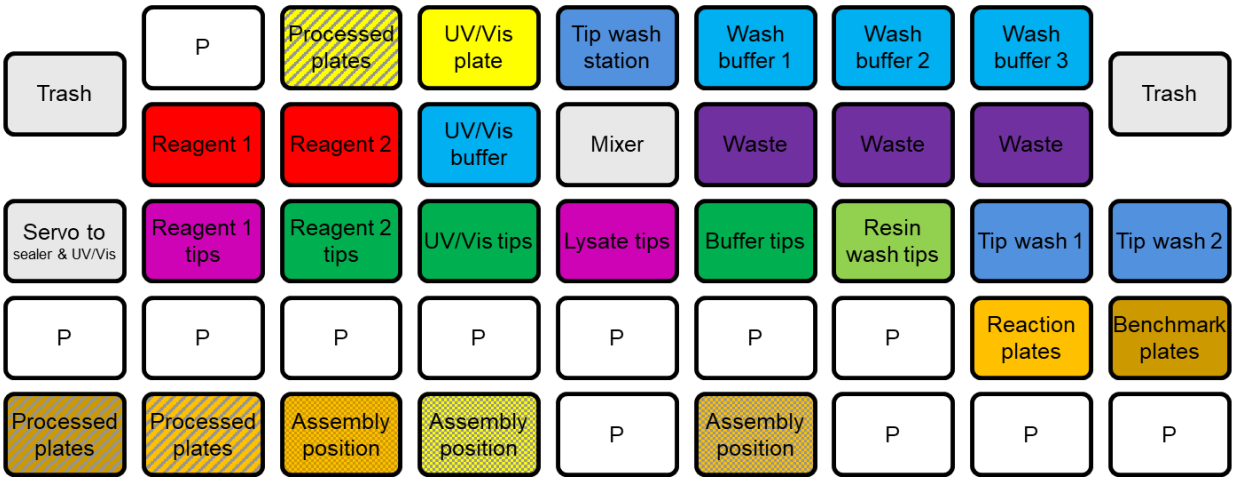

Variables

Materials		Variables *default value (value range)*	
Reaction plates	Corning plate 96 well round bottom PP	NumberofReactionPlates	8 (1-8)
Benchmark plates	Corning plate96 well round bottom PP	SealPlates *only on integrated Biomek i7*	yes (yes v no)
UV/Vis plates	Nunc plate 96 well flat bottom, UV transparent	DilutionFactor1	10 (5-20)
Reagent 1	Seahorse Reservoir or Nunc plate 96 well flat bottom, non-UV transmissible	OverallVolumeDilution1	200 (100-250)
Reagent 2	Seahorse Reservoir or Nunc plate 96 well flat bottom, non-UV transmissible	VolumeImmobilization	125 (45-225)
Wash buffer 1, 2 & 3	Seahorse Reservoir	VolumeResinWash	150 (20-200)
Tip wash	Seahorse Reservoir	VolumeMix	80 (20-100)
Waste	Seahorse Reservoir	VolumeImmoBuffer	50 (10-200)
Immobilization buffer	Seahorse Reservoir or Integra column/row reservoir	VolumeLysateImmo	50 (10-80)
UV/Vis buffer	Seahorse Reservoir	VolumeLysateBM	10 (10-80)
Reagent 1 tips	Beckman Coulter Biomek i-series 90 µL pipette tips, non-sterile	Reagent1BM	yes (yes v no)
Reagent 2 tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile	Reagent1Immo	no (yes v no)
Resin wash tips	Beckman Coulter Biomek i-series 190 µL pipette tips, wide bore, sterile, filtered	Reagent2BM	yes (yes v no)
Buffer tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile	Reagent2Immo	yes (yes v no)
Enzyme tips	Beckman Coulter Biomek i-series 90 µL pipette tips, non-sterile	VolumeReagent1Immo	0 (10-80)
UV/Vis tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile	VolumeReagent1BM	15 (10-80)
		VolumeReagent2Immo	100 (20-200)
		VolumeReagent2BM	100 (20-200)

3.6. Method 3

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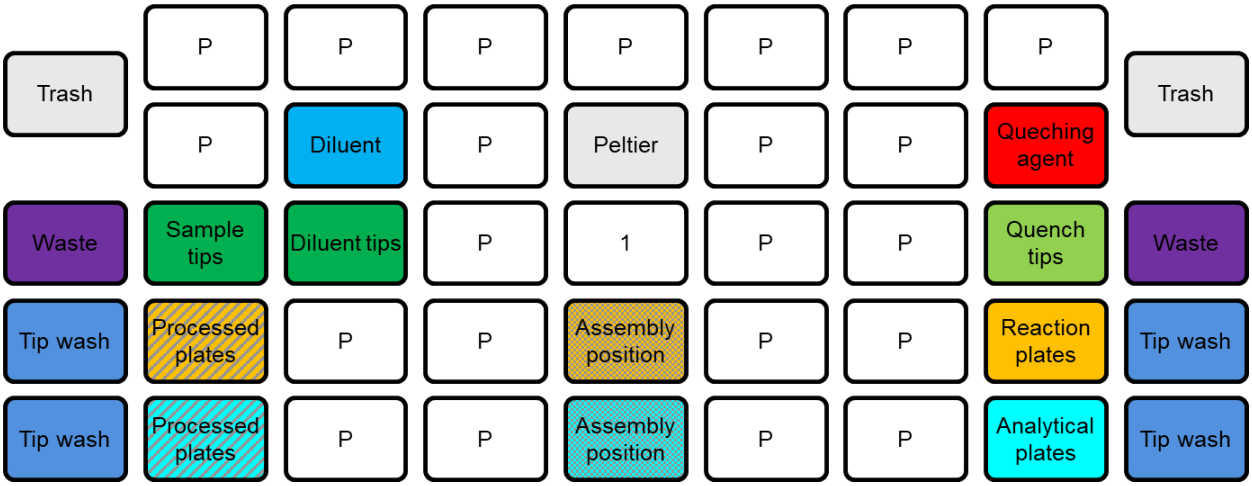

Variables

Materials		**Variables *default value (value range)***	
Reaction plates	Corning plate 96 well round bottom PP	NumberofReactionPlates	8 (1-8)
Analytical plates	Greiner plate 96 well V-bottom PP	Buffer time	0 (0-1000)
Tip wash	Seahorse Reservoir	DilutionFactor	5 (5-20)
Waste	Seahorse Reservoir	OverallVolumeDilution	200 (100-250)
Quenching agent	Seahorse Reservoir	VolumeQuench	125 (50-175)
Diluent	Seahorse Reservoir	VolumeReaction	125 (50-225)
Quench tips	Beckman Coulter Biomek i-series 190 µL pipette tips, wide bore, sterile, filtered	VolumeMix	80 (20-100)
Diluent tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile	WashSteps	3 (1-3)
Sample tips	Beckman Coulter Biomek i-series 230 µL pipette tips, non-sterile		

4. Experimental Part

4.1. Cell expression and enzyme preparation

96 well plate format

To grow the screening plates in a 96 well plate format for Stage 1 and 2 of our workflow NUNC microwell 96 flat bottom plates (Thermo scientific, 243656) were filled with 180 µL LB media (Teknova, L8000) containing 1% glucose (Teknova, G0550), 30 µg/mL Chloramphenicol and Codexis KRED Plate 1,2 and 4 was supplemented with 2 mM $MgSO_4$ (Sigma Aldrich) and Codexis KRED Plate 3 was supplemented with100 µM $ZnSO_4$.(Sigma Aldrich). Then the cells were copied from glycerol stocks of the screening plates with a disposable replica plater. Plates were sealed with breathable seal and incubated in a shaker at 200 rpm, 30 °C and 85%rH overnight.

For subculturing Costar Assay block 2 ml 96 well square V bottom (VWR, 3960) were filled with 390 µL TB media (Teknova, T7060) containing 30 µg/mL Chloramphenicol and Codexis KRED Plate 1,2 and 4 was supplemented with 2 mM $MgSO_4$ and Codexis KRED Plate 3 was supplemented with100 µM $ZnSO_4$. Then 10 µL of overnight culture was added. Plates were sealed with breathable seal (Adhesive film for culture plates, porous, VWR) and put into shaker at 250 rpm, 30 °C and 85%rH until OD 0.4-0.8 was reached. For induction 40 µL of 10 mM IPTG solution (Teknova, I3502, 1M) was added to reach a final concentration of 1 mM. Plates were sealed with breathable seal and incubated at 250 rpm, 30 °C and 85%rH for 16-20 hours.

For harvesting the plates were centrifuged for 10 min at 4000g and 4 °C. Media were removed and the plates stored at -80 °C until further use.

For cell lysis plates were thawed and 300 uL lysis buffer (20 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ or 100 µM $ZnSO_4$, 1mg/mL lysozyme (Lysozyme from chicken egg white, Sigma Aldrich, LL6876-100g), 0.5 mg/mL PMBS (Polymixin B Sulphate, Apollo Scientific, BIP0145) 0.1uL/mL DNAseI (New England Biolabs, M030L, 2000 U/mL)) was added before incubation for 2 hours. Plates are centrifuged for 30 min at 4000g and 4 °C and lysate is diluted according to the immobilization protocol.

Shake flask powder (SFP) production

To produce shake flask powder for stage 2, 3 and 4 of our workflow a 100 mL baffled shake flask filled with 25 mL LB media containing 1% glucose, 30 µg/mL Chloramphenicol and was supplemented with 2 mM $MgSO_4$ (as all of them originate from Codexis plate 1, 2 or 4). The shake flasks were incubated at 250 rpm and 30 °C overnight.

For subculturing 1 L baffled shake flasks were filled with 250 mL TB media containing 30 µg/mL Chloramphenicol and was supplemented with 2 mM $MgSO_4$ (as all of them originate from Codexis plate 1, 2 or 4). The shake flasks were inoculated with 5 mL preculture and incubated at 250 rpm until OD 0.6-0.8 is reached. For induction 250 µL of 1 M IPTG solution (final concentration 1 mM) was added and the cultures were incubated at 250 rpm, 30 °C for 16-20 hours.

For harvesting the cultures were centrifuged for 10 min at 4000g and 4 °C. Cell pellets are resuspended in 50 mM TEoA buffer pH 7 and processed in the microfluidizer (Microfluidizer LM200) for cell lysis. Cell fragments are separated from the lysate by centrifugation for 30 min at 12000g and 4 °C. The lysate is lyophilized (VirTis SP Scientific Advantage Pro with Intellitronics) overnight and stored at -20 °C until further use. We typically achieved 30 wt% protein content after lyophilization that is used to calculate the protein loading for ethe enzyme immobilization.

4.2. Workflow overview: Stage 1-4

An overview over the full workflow is given in figure S3.

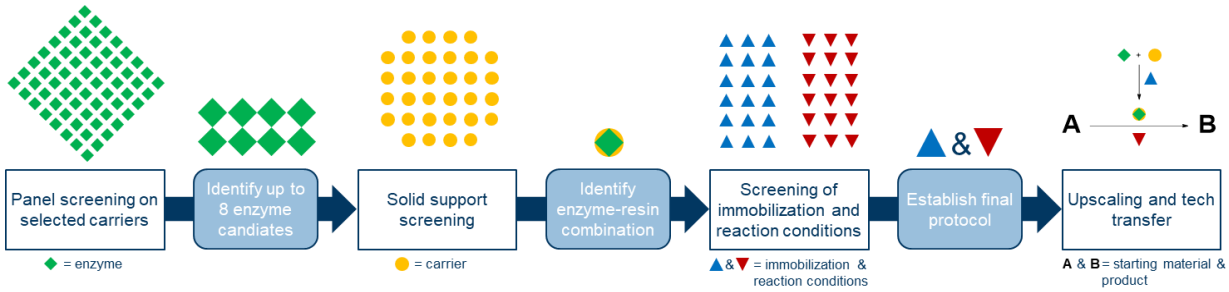

Figure S3: Individual steps of the streamlined high throughput workflow for rapid application of immobilized enzymes in pharma process development. First step is the panel screening to identify suitable enzyme candidates (green diamond) based on selected parameters, e.g., conversion, selectivity or recovered activity. Up to eight candidates are carried forward in the second stage of the workflow where differently functionalized supports (yellow circles) are investigated. Upon identification of the final enzyme-carrier combination the immobilization (blue triangle) and reaction (red triangle) parameters are optimized in the penultimate stage before a final scale-up gives verification of the parameters identified in the high-throughput screening prior to a potential tech transfer.

For the automated high throughput workflow, we obtained the conversion for the immobilized enzyme and for a corresponding free enzyme benchmark reaction. Since we use less volume of enzyme solution in the benchmark (10 µL) than in the immobilization (50 µL) this must be considered. We therefore determine the recovered activity (activity recovery) using the following formula.

$$Recovered\ activity\ [\%] = \frac{conversion_{immobilized\ enzyme}[\%] \cdot Volume_{enzyme\ solution\ for\ free\ enzyme\ reaction}}{conversion_{immobilized\ enzyme}[\%] \cdot Volume_{enzyme\ solution\ for\ immobilization}} \cdot 100$$

In addition, we can quantify the protein content of the supernatant using UV/Vis spectroscopy to estimate the immobilization yield. While this value can be of interest in some cases, we observed a color change of the supernatant when using glutaraldehyde activation which renders the results inconclusive for this study. In general, we consider the recovered activity more important for process development because it directly translate to costs while immobilization yield and efficiency is more generally informative.

$$Immobilization\ yield\ [\%] = \frac{protein\ conc._{supernatant\ after\ immobilization}\ [mg/mL] \cdot dilution\ factor}{protein\ conc._{supernatant\ before\ immobilization}\ [mg/mL] \cdot dilution\ factor} \cdot 100$$

Having determined these two values one could calculate the immobilization efficiency as the last of the three values frequently reported in enzyme immobilization studies.[1]

$$Immobilization\ efficiency\ [\%] = \frac{Immobilization\ yield\ [\%]}{Recovered\ activity\ [\%]} \cdot 100$$

4.3. Stage 1: panel screening

Table S5: Individual experimental steps carried out by the liquid handling robot for panel screening (stage 1 of the presented workflow) using method A0, A1, 2 and 3.

Step	Method	Reagents	Description
1	A0	Purolite ECR8304F Resin (suspension in destilled water)	Distributes resin (25 mg) from reservoir to reaction plate
2	A0	Destilled water	Washes resin 4 times
3	A0	25 g/L Gluteraldehyde in water	Adds 100 µL 25 g/L glutaraldehyde to reaction plate Final activation conditions: 200 mg/mL Resin 10 wt% Glutaraldehyde loading based on resin in water Final immobilization volume 125 µL
4	Incubation		Seal plate and incubate 2h at 250 rpm and 25 °C
5	A1	50 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$/100 µM $ZnSO_4$	Washes resin 4 times
6	A1	50 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$/100 µM $ZnSO_4$	Adds 50 µL Immobilization buffer
7	A1	Cell lysate (100% or 10%)	Adds 50 µL (diluted) cell lysate to reaction plates Transfer 10 uL sample to lysate benchmark plates <u>Final immobilization conditions:</u> 200 mg/mL Resin lysate concentration of 40 vol% or 4 vol% in 50 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$/100 µM $ZnSO_4$ Final immobilization volume 125 µL
8	Incubation		Seal reaction plate and incubate 18h at 250 rpm and 25 °C Seal lysate benchmark plate and store at 4°C until the reaction is setup in Step 2
9	2	*Wash buffer 1:* 500 mM sodium chloride solution *Wash buffer 2:* 20 mM 20 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$/100 µM $ZnSO_4$ *Wash buffer 3:* 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$/100 µM $ZnSO_4$	Washes resin in total 5 times, 2x wash buffer 1, 2x wash buffer 2, 1x wash buffer 3
10	2	*Reagent 1:* 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$/100 µM $ZnSO_4$ *Reagent 2:* 12.5 g/L Acetophenone 0.625 g/L $NADP^+$ disodium salt 12.5 vol% isopropanol in 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$/100 µM $ZnSO_4$	Adds 15 µL reagent to ONLY to the benchmark plates Adds 100 µL reagent to reaction AND benchmark plates Final reaction conditions: 10 g/L Acetophenone 0.5 g/L $NADP^+$ disodium salt 10 vol% isopropanol in 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$/100 µM $ZnSO_4$ Final reaction volume 125 µL
11	Incubation		Seal reaction plate and incubate 4h at 250 rpm and 25 °C
12	3	MeOH	Quenches reaction plate with 125 µL MeOH (1:1 dilution)
13	3	MeOH	Dilutes the reaction mixture in the analytical plate (1:10) for a final analytical dilution auf 1:20
14	Analytics		Run sample analysis on SFC

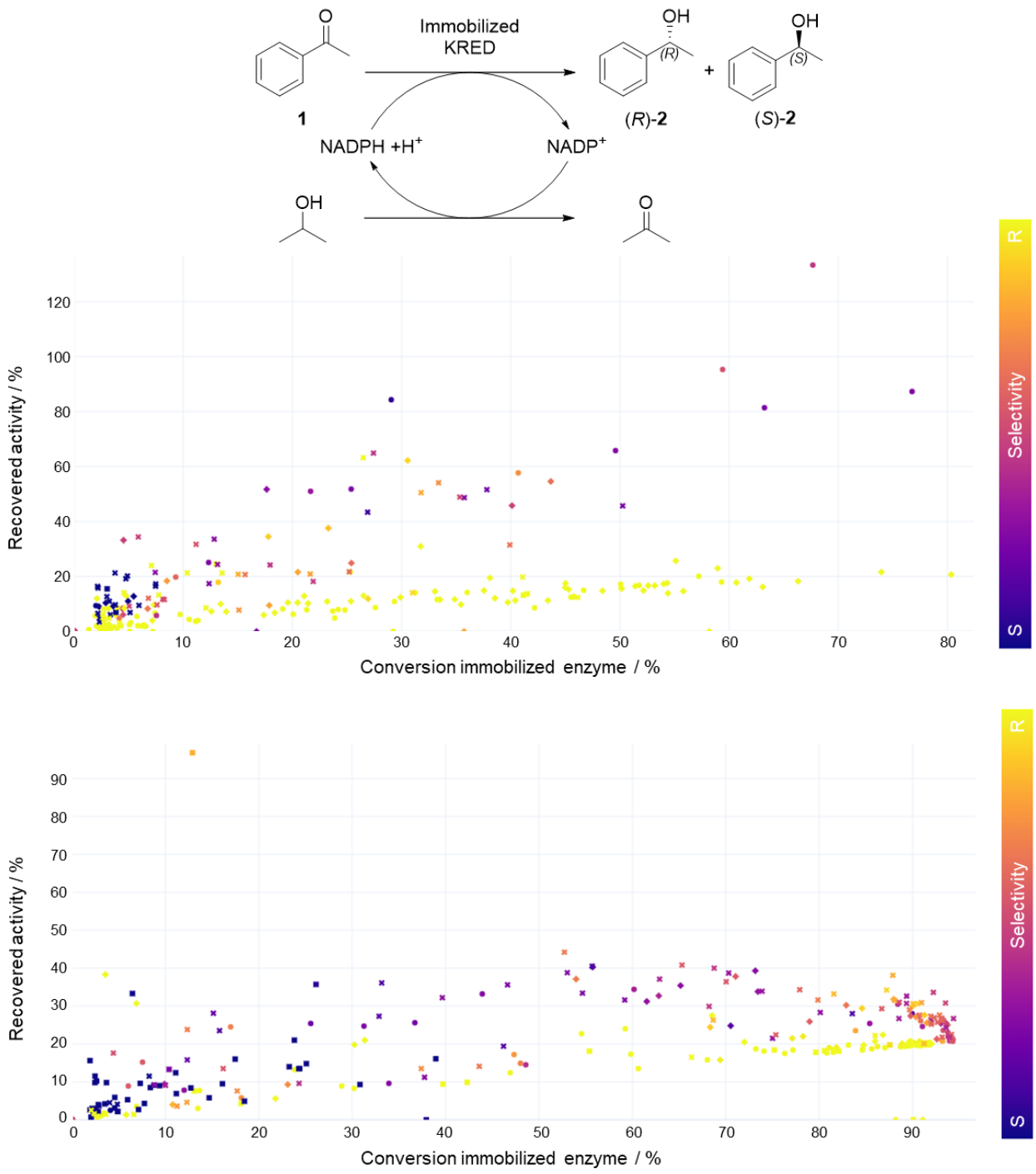

Figure S4: Selectivity, recovered activity and conversion of screened Ketoreductases for the reduction of acetophenone (1) to phenylethanol (S)-2 and (R)-2. For the cofactor regeneration of NADPH isopropanol was used as co-substrate. The screening was conducted in a 96 well plate with 4 vol% (top) and 40 vol% (bottom) lysate concentration. Recovered activity was calculated by comparison of product-related conversion of immobilized enzyme vs. free enzyme and does not take immobilization efficiency into account. The colour represents the enantioselectivity of the enzyme (R = yellow, S = blue).

Table S6: Summary of the commercially available enzyme from the Codexis panels including the variant choosen for scale-up (**bold**); Recovered activity and conversion of immobilized ADH candidates for the enantioselective reduction of acetophenone (**1**). Recovered activity is calculated by comparison of product-related conversion of immobilized enzyme vs. free enzyme and does not take immobilization efficiency into account.

Plate	Well	Synonym	4 vol% Lysate			40 vol% Lysate		
			conversion / %	ee / %	Recovered activity / %	conversion / %	ee / %	Recovered activity / %
KRED plate 1	E05	Wildtype *Lactobacillus kefir* KRED	29	100	-	91	100	20
KRED plate 1	E06		36	100	14	91	100	20
KRED plate 1	E07		21	100	8	90	100	20
KRED plate 1	E08		25	100	8	90	100	20
KRED plate 3	E07		41	100	13	89	100	20
KRED plate 3	E08		41	100	14	90	100	-
KRED plate 1	E09	KRED parent 2	57	100	20	91	100	20
KRED plate 1	E10		47	100	15	90	100	20
KRED plate 1	E11		52	100	17	90	100	20
KRED plate 1	E12		29	100	11	90	100	20
KRED plate 2	E03		50	100	16	90	100	20
KRED plate 2	E04		53	100	17	92	100	20
KRED plate 2	E05		53	100	17	92	100	20
KRED plate 2	E06		54	100	18	91	100	20
KRED plate 3	E01	KRED plate 3 parent 1	0	-	-	2	-100	-
KRED plate 3	E02		0	-	-	2	-100	16
KRED plate 3	E03		0	-	-	2	-100	10
KRED plate 3	E04		0	-	-	3	-100	10
KRED plate 3	E09	KRED plate 3 parent 2	0	-	0	25	-100	15
KRED plate 3	E10		0	-	0	24	-100	14
KRED plate 3	E11		0	-	0	23	-100	14
KRED plate 3	E12		0	-	0	24	-100	14
KRED plate 1	A04	KRED-P1A04	42	100	9	91	100	20
KRED plate 1	A12	KRED-P1A12	6	100	6	83	100	20
KRED plate 1	B02	KRED-P1B02	59	9	95	93	10	24
KRED plate 4	D03		35	1	49	93	10	26
KRED plate 1	B05	KRED-P1B05	0	-	0	26	-57	25
KRED plate 4	F03		0	-	-	15	-62	28
KRED plate 1	B10	KRED-P1B10	4	33	5	94	12	21
KRED plate 4	H03		3	100	5	90	36	29
KRED plate 1	B12	KRED-P1B12	13	80	18	84	61	24
KRED plate 4	A04		10	100	21	88	63	32
KRED plate 1	C01	KRED-P1C01	0	-	0	49	-16	15
KRED plate 4	B04		0	-	0	24	-17	10
KRED plate 1	H08	KRED-P1H08	29	-87	84	90	-66	28
KRED plate 4	F07		7	-100	17	84	-73	28
KRED plate 2	A03	KRED-P2A03	4	7	6	94	21	25
KRED plate 4	E03		8	13	10	93	23	26
KRED plate 4	E04		8	9	12	92	22	25
KRED plate 4	E05		8	5	12	93	22	26
KRED plate 4	E06		3	100	4	92	21	27
KRED plate 2	B02	KRED-P2B02	5	-100	13	73	-36	34
KRED plate 4	C09		7	-100	17	74	-36	34
KRED plate 2	C01	KRED-P2C01	21	100	10	89	97	20
KRED plate 2	C02	KRED-P2C02	4	-100	9	62	-38	31
KRED plate 4	B10		5	-100	20	70	-36	39
KRED plate 2	C11	KRED-P2C11	3	100	5	83	32	30
KRED plate 4	E10		4	100	9	80	36	32
KRED plate 2	D03	KRED-P2D03	0	-	0	79	18	26
KRED plate 4	H10		3	100	5	75	17	22
KRED plate 2	D11	KRED-P2D11	5	-100	11	71	-69	25
KRED plate 4	D11		7	-43	22	46	-72	19
KRED plate 2	D12	KRED-P2D12	0	-	-	23	45	9
KRED plate 4	E11		3	100	6	18	45	8
KRED plate 2	**F01**	**KRED-P2F01 (scale-up)**	**74**	**100**	**22**	**91**	**100**	**20**

KRED plate 2	F12	KRED-P2F12	30	100	12	92	100	20
KRED plate 2	G03	KRED-P2G03	27	100	13	92	97	20
KRED plate 2	H07	KRED-P2H07	34	100	12	90	100	20
KRED plate 2	H08	KRED-P2H08	33	100	15	91	100	20
KRED plate 3	B03	KRED-P3B03	0	-	0	5	-100	2
KRED plate 3	G09	KRED-P3G09	0	-	-	0	-	0
KRED plate 3	H02	KRED-P3H02	0	-	-	3	-100	4
KRED plate 3	H12	KRED-P3H12	3	-100	16	39	-100	16

Table S7: Summary of all screened enzymes from Codexis panels; Recovered activity and conversion of immobilized ADH candidates for the enantioselective reduction of acetophenone (**1**). Recovered activity is calculated by comparison of product-related conversion of immobilized enzyme vs. free enzyme and does not take immobilization efficiency into account.

Plate	Well	Synonym	4 vol% Lysate			40 vol% Lysate		
			conversion / %	ee / %	Recovered activity / %	conversion / %	ee / %	Recovered activity / %
KRED Plate 1	A01		59	100	18	91	100	20
	A02		24	100	5	91	100	20
	A03		3	100	1	85	100	18
	A04	KRED-P1A04	42	100	9	91	100	20
	A05		50	100	17	91	100	20
	A06		33	100	12	91	100	20
	A07		2	100	1	82	100	18
	A08		11	100	4	91	100	20
	A09		2	100	2	47	100	12
	A10		0	-	0	73	100	19
	A11		1	100	1	85	100	19
	A12	KRED-P1-A12	6	100	6	83	100	20
	B01		0	-	-	0	-	0
	B02	KRED-P1B02	59	9	95	93	10	24
	B03		19	100	8	89	100	20
	B04		7	100	7	76	100	18
	B05	KRED-P1B05	0	-	0	26	-57	25
	B06		0	-	-	12	-39	8
	B07		4	100	2	91	100	20
	B08		0	-	0	77	100	18
	B09		0	-	0	24	100	13
	B10	KRED-P1B10	4	33	5	94	12	21
	B11		6	100	2	86	97	19
	B12	KRED-P1B12	13	80	18	84	61	24
	C01	KRED-P1C01	0	-	0	49	-16	15
	C02		0	-	-	0	-	0
	C03		0	-	0	14	100	8
	C04		0	-	0	47	51	17
	C05		0	-	-	0	-	0
	C06		2	-100	9	60	-27	34
	C07		2	100	6	55	100	23
	C08		0	-	0	0	-	0
	C09		77	-46	87	94	-11	21
	C10		0	-	0	61	100	14
	C11		0	-	-	3	100	1
	C12		3	100	2	86	97	19
	D01		5	100	3	89	100	19
	D02		3	100	3	68	100	16
	D03		10	100	6	81	97	19
	D04		0	-	-	31	-47	25
	D05		50	-44	66	91	-24	25
	D06		0	-	-	6	15	9
	D07		0	-	-	8	14	15
	D08		5	100	3	60	100	17
	D09		2	100	1	74	100	18
	D10		20	100	6	89	100	20
	D11		0	-	0	7	100	4
	D12		3	100	1	88	100	v
	E01	Neg Control	0	-	-	0	-	-
	E02	Neg Control	0	-	-	0	-	-
	E03	Neg Control	0	-	-	0	-	-
	E04	Neg Control	0	-	-	0	-	-
	E05	KRED parent 1	29	100	-	91	100	20
	E06	KRED parent 1	36	100	14	91	100	20
	E07	KRED parent 1	21	100	8	90	100	20

	E08	KRED parent 1	25	100	8	90	100	20
	E09	KRED parent 2	57	100	20	91	100	20
	E10	KRED parent 2	47	100	15	90	100	20
	E11	KRED parent 2	52	100	17	90	100	20
	E12	KRED parent 2	29	100	11	90	100	20
	F01		3	100	1	89	100	19
	F02		11	100	4	91	100	20
	F03		6	100	2	90	100	20
	F04		7	100	2	91	100	20
	F05		7	100	-	90	98	20
	F06		11	100	4	91	100	20
	F07		6	100	2	81	100	18
	F08		0	-	0	30	100	8
	F09		0	-	0	29	100	9
	F10		31	100	14	90	100	20
	F11		46	100	13	91	100	20
	F12		5	100	-	90	100	20
	G01		63	-50	81	94	-16	22
	G02		24	100	7	90	100	20
	G03		0	-	0	2	100	3
	G04		28	100	9	91	100	20
	G05		0	-	0	44	-47	33
	G06		4	100	1	87	100	19
	G07		0	-	-	17	39	25
	G08		68	-9	133	94	-3	23
	G09		24	100	8	90	100	20
	G10		0	-	-	3	100	1
	G11		9	22	20	48	39	15
	G12		22	-33	51	88	-20	31
	H01		0	-	0	4	-100	3
	H02		0	-	0	18	49	6
	H03		0	-	0	13	100	3
	H04		0	-	0	3	100	2
	H05		0	-	0	34	-44	10
	H06		12	-47	25	85	-34	25
	H07		25	-50	52	91	-26	27
	H08	KRED-P1H08	29	-87	84	90	-66	28
	H09		0	-	0	37	-54	26
	H10		41	47	58	91	27	26
	H11		8	-13	6	94	11	21
	H12		0	-	0	59	100	24
KRED Plate 2	A01		0	-	0	6	100	1
	A02		23	73	38	92	52	26
	A03	KRED-P2-A03	4	7	6	94	21	25
	A04		40	100	11	92	100	20
	A05		4	2	8	90	16	27
	A06		18	79	35	91	79	28
	A07		5	100	13	88	61	32
	A08		0	-	0	4	100	38
	A09		0	-	0	11	66	4
	A10		3	100	3	69	95	16
	A11		38	100	20	87	100	22
	A12		32	100	31	92	100	20
	B01		23	100	11	91	96	21
	B02	KRED-P2B02	5	-100	13	73	-36	34
	B03		27	85	12	90	88	20
	B04		3	-100	10	73	-43	39
	B05		5	-4	33	71	14	38
	B06		4	100	6	77	94	22
	B07		0	-	-	10	-32	9
	B08		25	100	11	90	100	20
	B09		20	60	22	68	77	24

	B10		7	100	10	78	94	22
	B11		0	-	-	7	100	31
	B12		48	100	15	91	100	20
	C01	KRED-P2C01	21	100	10	89	97	20
	C02	KRED-P2C02	4	-100	9	62	-38	31
	C03		2	-100	9	65	-42	35
	C04		0	-	0	56	-72	40
	C05		17	100	6	89	100	19
	C06		43	100	11	92	100	20
	C07		66	100	18	92	100	20
	C08		35	100	10	91	100	20
	C09		46	100	13	92	100	20
	C10		54	100	14	91	100	20
	C11	KRED-P2C11	3	100	5	83	32	30
	C12		7	37	8	93	21	26
	D01		45	100	18	90	100	20
	D02		18	-43	52	63	-22	33
	D03	KRED-P2D03	0	-	0	79	18	26
	D04		8	54	18	90	54	27
	D05		0	-	0	54	37	37
	D06		0	-	0	2	100	1
	D07		21	100	13	72	100	21
	D08		20	100	11	86	100	21
	D09		52	100	15	91	100	20
	D10		55	100	26	90	100	21
	D11	KRED-P2D11	5	-100	11	71	-69	25
	D12	KRED-P2D12	0	-	-	23	45	9
	E01	Neg Control	0	-	-	0	-	-
	E02	Neg Control	0	-	-	0	-	-
	E03	KRED parent 2	50	100	16	90	100	20
	E04	KRED parent 2	53	100	17	92	100	20
	E05	KRED parent 2	53	100	17	92	100	20
	E06	KRED parent 2	54	100	18	91	100	20
	E07		2	100	12	30	100	20
	E08		3	100	8	69	100	28
	E09		0	-	0	31	100	21
	E10		17	-52	-	93	-20	25
	E11		3	100	-	0	-	0
	E12		5	100	9	90	71	30
	F01	KRED P02 F01	74	100	22	91	100	20
	F02		36	60	-	93	32	26
	F03		40	-2	46	94	0	26
	F04		20	100	11	92	100	20
	F05		13	100	10	89	96	20
	F06		45	100	16	91	100	20
	F07		56	100	15	91	100	20
	F08		80	100	21	90	100	20
	F09		40	100	11	91	100	20
	F10		45	100	15	91	100	20
	F11		25	27	25	94	20	22
	F12	KRED-P2F12	30	100	12	92	100	20
	G01		13	100	7	90	100	21
	G02		18	59	10	93	63	21
	G03	KRED-P2-G03	27	100	13	92	97	20
	G04		44	29	55	93	21	21
	G05		35	100	12	91	100	20
	G06		14	100	7	85	100	20
	G07		40	100	15	91	100	-
	G08		62	100	19	91	100	20
	G09		63	100	16	91	100	20
	G10		61	100	18	92	100	20
	G11		50	100	16	91	100	20
	G12		58	100	-	92	100	20

	H01		59	100	23	92	98	20
	H02		25	100	22	91	100	20
	H03		31	85	62	85	83	29
	H04		0	-	0	22	100	6
	H05		54	100	17	92	100	20
	H06		39	100	12	92	100	20
	H07	KRED-P2H07	34	100	12	90	100	20
	H08	KRED-P2H08	33	100	15	91	100	20
	H09		51	100	17	91	100	20
	H10		38	100	15	91	100	20
	H11		18	100	7	91	100	20
	H12		7	100	4	82	96	19
KRED Plate 3	A01		0	-	-	0	-	-
	A02		0	-	-	6	-100	33
	A03		0	-	0	5	-100	2
	A04		0	-	-	0	-	0
	A05		0	-	-	0	-	0
	A06		0	-	-	0	-	0
	A07		0	-	0	2	-100	2
	A08		0	-	0	81	100	18
	A09		0	-	0	26	-87	36
	A10		0	-	0	0	-	0
	A11		0	-	0	18	100	4
	A12		0	-	0	9	-100	9
	B01		0	-	-	7	-100	10
	B02		0	-	-	0	-	0
	B03	KRED-P3B03	0	-	0	5	-100	2
	B04		0	-	-	0	-	0
	B05		0	-	-	0	-	0
	B06		0	-	-	0	-	0
	B07		0	-	-	0	-	0
	B08		0	-	0	82	100	18
	B09		0	-	-	10	-50	13
	B10		0	-	0	80	100	18
	B11		0	-	-	0	-	0
	B12		0	-	0	13	64	97
	C01		0	-	-	11	-100	12
	C02		0	-	-	3	-100	4
	C03		0	-	0	18	-100	5
	C04		0	-	-	0	-	0
	C05		0	-	-	0	-	0
	C06		0	-	0	0	-	0
	C07		0	-	-	0	-	-
	C08		0	-	0	5	-100	3
	C09		0	-	0	16	-100	10
	C10		0	-	-	0	-	-
	C11		2	100	1	82	100	19
	C12		0	-	0	2	-100	1
	D01		0	-	-	4	-100	4
	D02		0	-	0	6	-100	5
	D03		0	-	0	5	-100	6
	D04		0	-	-	0	-	0
	D05		0	-	-	0	-	0
	D06		0	-	0	8	-100	4
	D07		0	-	0	40	100	9
	D08		0	-	-	0	-	0
	D09		0	-	-	0	-	-
	D10		0	-	0	0	-	0
	D11		0	-	-	0	-	0
	D12		0	-	-	2	-100	3
	E01	KRED plate 3 parent 1	0	-	-	2	-100	-
	E02	KRED plate 3 parent 1	0	-	-	2	-100	16

	E03	KRED plate 3 parent 1	0	-	-	2	-100	10
	E04	KRED plate 3 parent 1	0	-	-	3	-100	10
	E05	Neg Control	0	-	-	0	-	-
	E06	Neg Control	0	-	-	0	-	-
	E07	KRED parent 1	41	100	13	89	100	20
	E08	KRED parent 1	41	100	14	90	100	-
	E09	KRED plate 3 parent 2	0	-	0	25	-100	15
	E10	KRED plate 3 parent 2	0	-	0	24	-100	14
	E11	KRED plate 3 parent 2	0	-	0	23	-100	14
	E12	KRED plate 3 parent 2	0	-	0	24	-100	14
	F01		0	-	-	9	-100	9
	F02		0	-	-	2	-100	3
	F03		0	-	0	24	-100	21
	F04		0	-	0	0	-	-
	F05		0	-	-	0	-	0
	F06		0	-	-	0	-	0
	F07		3	100	2	55	100	18
	F08		0	-	0	38	-91	-
	F09		4	100	2	75	100	18
	F10		0	-	0	13	100	8
	F11		0	-	-	0	-	0
	F12		0	-	0	15	-100	6
	G01		0	-	-	2	-100	12
	G02		0	-	0	8	-100	9
	G03		0	-	0	17	-100	16
	G04		0	-	0	7	-100	3
	G05		0	-	0	3	-100	2
	G06		0	-	-	0	-	0
	G07		0	-	0	88	98	20
	G08		0	-	-	0	-	0
	G09	KRED-P3G09	0	-	-	0	-	0
	G10		0	-	-	0	-	-
	G11		0	-	0	0	-	0
	G12		4	-100	7	31	-100	9
	H01		0	-	-	13	-100	8
	H02	KRED P03 H02	0	-	-	3	-100	4
	H03		0	-	-	0	-	0
	H04		0	-	0	0	-	0
	H05		0	-	-	2	-100	3
	H06		0	-	-	0	-	0
	H07		0	-	0	3	100	1
	H08		0	-	0	11	-100	7
	H09		0	-	-	4	-100	10
	H10		0	-	0	42	100	10
	H11		0	-	-	0	-	0
	H12	KRED-P3H12	3	-100	16	39	-100	16
KRED Plate 4	A01		5	1	9	89	5	31
	A02		7	26	12	91	32	28
	A03		2	-100	16	33	-69	27
	A04	KRED-P1B12	10	100	21	88	63	32
	A05		0	-	0	4	-100	5
	A06		0	-	-	12	36	24
	A07		0	-	0	7	100	1
	A08		22	-6	18	94	10	21
	A09		4	100	3	66	100	17
	A10		0	-	-	0	-	0
	A11		15	65	21	91	55	31
	A12		32	62	51	93	35	28
	B01		3	-100	11	59	-46	32
	B02		0	-	0	80	-40	28
	B03		27	-83	43	92	-43	26
	B04	KRED-P1C01	0	-	0	24	-17	10

	B05		36	-48	49	92	-28	26
	B06		27	-14	65	92	-5	34
	B07		0	-	0	2	100	2
	B08		14	100	21	92	57	26
	B09		41	100	20	86	97	22
	B10	KRED-P2C02	5	-100	20	70	-36	39
	B11		0	-	0	53	35	44
	B12		18	-13	24	94	1	31
	C01		12	100	9	91	71	20
	C02		5	-100	7	68	-9	30
	C03		0	-	-	0	-	0
	C04		0	-	-	0	-	0
	C05		0	-	-	0	-	0
	C06		0	-	0	4	100	1
	C07		0	-	0	38	-44	11
	C08		46	100	12	91	100	20
	C09	KRED-P2B02	7	-100	17	74	-36	34
	C10		3	-100	13	55	-51	33
	C11		0	-	0	0	-	0
	C12		25	23	22	94	21	23
	D01		11	9	32	78	21	34
	D02		5	100	14	82	69	33
	D03	KRED-P1B02	35	1	49	93	10	26
	D04		0	-	0	37	47	14
	D05		0	-	-	0	-	0
	D06		0	-	0	44	32	14
	D07		2	-100	4	75	-39	22
	D08		2	-100	5	87	16	29
	D09		31	86	14	88	87	20
	D10		2	-100	16	33	-73	36
	D11	KRED-P2D11	7	-43	22	46	-72	19
	D12		15	51	8	93	61	21
	E01	Neg Control	0	-	-	0	-	-
	E02	Neg Control	0	-	-	0	-	-
	E03	KRED-P2-A03	8	13	10	93	23	26
	E04	KRED-P2-A03	8	9	12	92	22	25
	E05	KRED-P2-A03	8	5	12	93	22	26
	E06	KRED-P2-A03	3	100	4	92	21	27
	E07		6	-100	10	89	-30	27
	E08		13	100	25	91	80	31
	E09		4	-100	21	56	-74	41
	E10	KRED-P2C11	4	100	9	80	36	32
	E11	KRED-P2D12	3	100	6	18	45	8
	E12		40	29	32	93	20	22
	F01		4	-100	10	69	-10	40
	F02		5	-100	19	47	-51	36
	F03	KRED-P1B05	0	-	-	15	-62	28
	F04		0	-	0	40	-41	32
	F05		38	-55	52	94	-26	24
	F06		13	-39	24	89	-22	31
	F07	KRED-P1H08	7	-100	17	84	-73	28
	F08		7	100	24	88	63	38
	F09		6	-4	34	65	14	41
	F10		16	33	21	93	22	27
	F11		12	-54	17	94	-18	27
	F12		26	100	63	87	83	34
	G01		0	-	0	16	12	14
	G02		3	100	8	70	10	36
	G03		0	-	0	10	-26	9
	G04		0	-	0	5	-100	4
	G05		0	-	-	2	100	3
	G06		0	-	0	3	-100	3
	G07		0	-	0	12	-63	16

	G08		0	-	-	0	-	0
	G09		0	-	-	9	-42	9
	G10		13	-44	34	63	-22	37
	G11		0	-	-	4	7	18
	G12		0	-	-	0	-	0
	H01		3	-100	6	89	-32	33
	H02		2	-100	7	53	-49	39
	H03	KRED-P1B10	3	100	5	90	36	29
	H04		50	-52	46	94	-15	22
	H05		0	-	-	16	-73	24
	H06		0	-	0	11	53	4
	H07		33	47	54	92	29	27
	H08		0	-	-	12	58	5
	H09		22	58	21	69	68	26
	H10	KRED-P2D03	3	100	5	75	17	22
	H11		8	100	13	90	73	31
	H12		0	-	0	8	-100	12

4.4. Stage 2: carrier screening

Table S8: Individual experimental steps carried out by the liquid handling robot for carrier screening (stage 2 of the presented workflow) using method A0, A1, 2 and 3 for amino-functionalized resins.

Step	Method	Reagents	Description
1	A0	Amino-functionalized resin in Integra Column reservoir (see table S1)	Distributes resin (25 mg) from reservoir to reaction plate
2	A0	Destilled water	Washes resin 4 times
3	A0	25 g/L Gluteraldehyde in water	Adds 100 µL 25 g/L glutaraldehyde to reaction plate Final activation conditions: 200 mg/mL Resin 10 wt% Glutaraldehyde loading based on resin in water Final immobilization volume 125 µL
4	Incubation		Seal plate and incubate 2h at 250 rpm and 25 °C
5	A1	50 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$	Washes resin 4 times
6	A1	50 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$	Adds 50 µL Immobilization buffer
7	A1	2.5 g/l SFP solution of 8 selected candidates (1 candidate per row)	Adds 50 µL SFP dilution to reaction plates Transfer 10 uL sample to lysate benchmark plates <u>Final immobilization conditions:</u> 200 mg/mL Resin SFP concentration of 10 g/L (5 wt% loading based on resin) in 50 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ Final immobilization volume 125 µL
8	Incubation		Seal reaction plate and incubate 18h at 250 rpm and 25 °C Seal lysate benchmark plate and store at 4°C until the reaction is setup in Step 2
9	2	*Wash buffer 1:* 500 mM sodium chloride solution *Wash buffer 2:* 20 mM 20 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Wash buffer 3:* 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$	Washes resin in total 5 times, 2x wash buffer 1, 2x wash buffer 2, 1x wash buffer 3
10	2	*Reagent 1:* 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Reagent 2:* 12.5 g/L Acetophenone 0.625 g/L $NADP^+$ disodium salt 12.5 vol% isopropanol in 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$	Adds 15 µL reagent 1 to ONLY to the benchmark plates Adds 100 µL reagent 2 to reaction AND benchmark plates Final reaction conditions: 10 g/L Acetophenone 0.5 g/L $NADP^+$ disodium salt 10 vol% isopropanol in 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$/100 µM $ZnSO_4$ Final reaction volume 125 µL
11	Incubation		Seal reaction plate and incubate 4h at 250 rpm and 25 °C
12	3	MeOH	Quenches reaction plate with 125 µL MeOH (1:1 dilution)
13	3	MeOH	Dilutes the reaction mixture in the analytical plate (1:10) for a final analytical dilution auf 1:20
14	Analytics		Run sample analysis on SFC

Table S9: Individual experimental steps carried out by the liquid handling robot for carrier screening (stage 2 of the presented workflow) using method B1, 2 and 3 for amino-functionalized resins.

Step	Method	Reagents	Description
1	B1	Epoxy-functionalized resin in Integra Column reservoir (see table S1)	Distributes resin (25 mg) from reservoir to reaction plate
2	B1	1.6 M mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$	Washes resin 4 times
3	B1	1.6 M mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$	Adds 50 µL Immobilization buffer
4	B1	2.5 g/l SFP solution of 8 selected candidates (1 candidate per row)	Adds 50 µL SFP dilution to reaction plates Transfer 10 uL sample to lysate benchmark plates <u>Final immobilization conditions:</u> 200 mg/mL Resin SFP concentration of 10 g/L (5 wt% loading based on resin) in 50 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ Final immobilization volume 125 µL
5	Incubation		Seal reaction plate and incubate 18h at 250 rpm and 25 °C Seal lysate benchmark plate and store at 4°C until the reaction is setup in Step 2
6	2	*Wash buffer 1:* 500 mM sodium chloride solution *Wash buffer 2:* 20 mM 20 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Wash buffer 3:* 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$	Washes resin in total 5 times, 2x wash buffer 1, 2x wash buffer 2, 1x wash buffer 3
7	2	*Reagent 1:* 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Reagent 2:* 12.5 g/L Acetophenone 0.625 g/L $NADP^+$ disodium salt 12.5 vol% isopropanol in 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$	Adds 15 µL reagent 1 to ONLY to the benchmark plates Adds 100 µL reagent 2 to reaction AND benchmark plates Final reaction conditions: 10 g/L Acetophenone 0.5 g/L $NADP^+$ disodium salt 10 vol% isopropanol in 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ Final reaction volume 125 µL
8	Incubation		Seal reaction plate and incubate 4h at 250 rpm and 25 °C
9	3	MeOH	Quenches reaction plate with 125 µL MeOH (1:1 dilution)
10	3	MeOH	Dilutes the reaction mixture in the analytical plate (1:10) for a final analytical dilution auf 1:20
11	Analytics		Run sample analysis on SFC

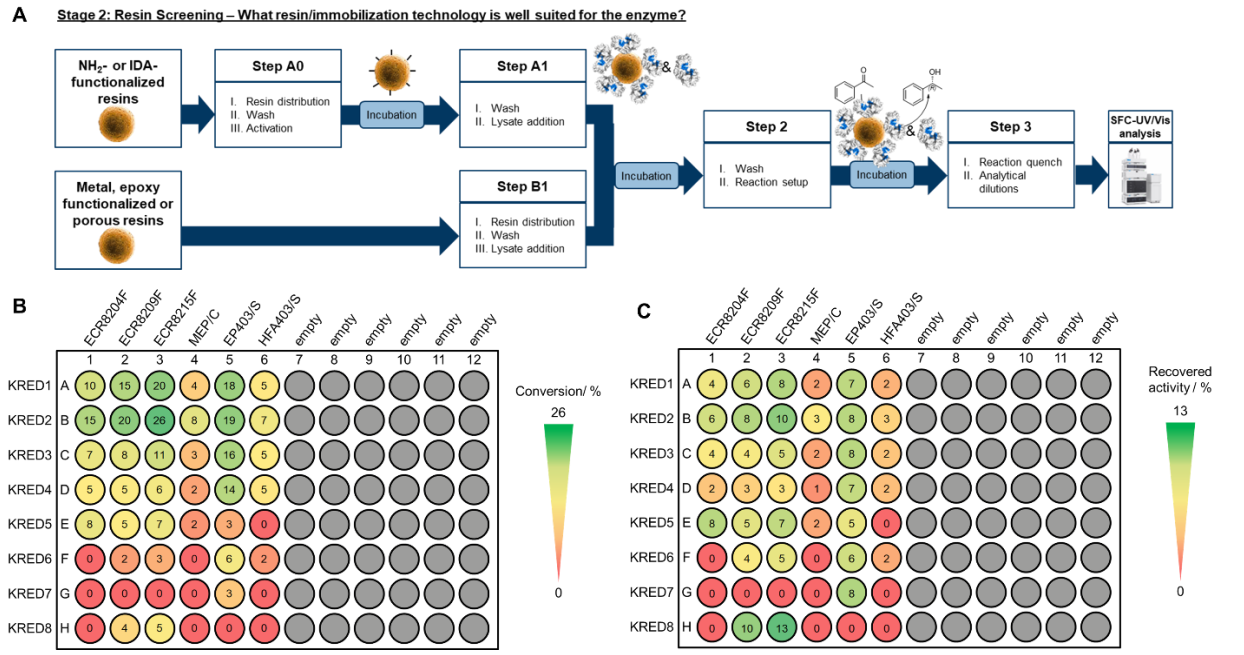

Figure S5: **Stage 2 Resin Screening – What resin and which corresponding immobilization approach is well suited for the KREDs showing the highest reduction of acetophenone (1)**. (**A**) Schematic flow chart of the different automated steps conducted. (**B**) Conversion and (**C**) recovered activity of immobilized KRED candidates 1-8 on eopxy-functionalized carriers for the enantioselective reduction. Recovered activity is calculated by comparison of product-related conversion of immobilized enzyme vs. free enzyme and does not take immobilization efficiency into account. KRED 1-5 are highly R-selective, while KRED 6-8 are non-selective (See also Figure 3)

4.5. Stage 3: immobilization and reaction condition screening

Table S10: Individual steps for experiment 1 (enzyme concentration and glutaraldehyde loading), experiment 2 (buffer type & pH and enzyme concentration) and experiment 3 (refined enzyme concentration and glutaraldehyde loading) carried out by the liquid handling robot for immobilization condition screening (stage 3 of the presented workflow) using method A0, A1, 2 and 3 for the enzyme candidate 2 on ECR8304F resin.

Step	Method	Reagents	Description
1	A0	Purolite ECR8304F Resin (suspension in destilled water)	Distributes resin (25 mg) from reservoir to reaction plate
2	A0	Destilled water	Washes resin 4 times
3	A0	*Experiment 1&3:* Serial dilution of 1.6-200 g/L Gluteraldehyde in water *Experiment 2:* 25 g/L Gluteraldehyde in water	Adds 100 µL glutaraldehyde solution to reaction plate Final activation conditions: 200 mg/mL Resin *Experiment 1&3:* 0.6-80 wt% Glutaraldehyde loading based on resin *Experiment 2:* 10 wt% Glutaraldehyde loading based on resin in water Final immobilization volume 125 µL
4	Incubation		Seal plate and incubate 2h at 250 rpm and 25 °C
5	A1	*Experiment 1&3:* 50 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Experiment 2:* Integra Column Revervoirs filled with different buffers 50 mM TEoA buffer pH 7/7.5/8 or 50 mM MOPS buffer pH 7/7.5 or 50 mM Tris buffer pH 7.5/8/8.5/9 or 50 mM KP_i buffer pH 7/7.5/8 + 2 mM $MgSO_4$	Washes resin 4 times
6	A1	*Experiment 1&3:* 50 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Experiment 2:* Different buffers 50 mM TEoA buffer pH 7/7.5/8 or 50 mM MOPS buffer pH 7/7.5 or 50 mM Tris buffer pH 7.5/8/8.5/9 or 50 mM KP_i buffer pH 7/7.5/8 + 2 mM $MgSO_4$	Adds 50 µL Immobilization buffer
7	A1	*Experiment 1:* Serial dilution of 0-200 g/L SFP (KRED 2) in immobilization buffer *Experiment 2:* Serial dilution of 0-100 g/L SFP (KRED 2) in immobilization buffer *Experiment 3:* Adjusted serial dilution of 0-200 g/L SFP (KRED 2) in immobilization buffer	Adds 50 µL SFP dilution to reaction plates Transfer 10 uL sample to lysate benchmark plates Final immobilization conditions: 200 mg/mL Resin *Experiment 1:* SFP concentration of 0-80 g/L (0-40 wt% SFP loading based on resin) in 50 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Experiment 2:* SFP concentration of 0-40 g/L (0-20 wt% SFP loading based on resin) in

			different buffers + 2 mM $MgSO_4$ *Experiment 3:* SFP concentration of 0-80 g/L (0-40 wt% SFP loading based on resin) in 50 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ Final immobilization volume 125 µL
8	Incubation		Seal reaction plate and incubate 18h at 250 rpm and 25 °C
9	2	*Wash buffer 1:* 500 mM sodium chloride solution *Wash buffer 2:* 20 mM 20 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Wash buffer 3:* 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$	Washes resin in total 5 times, 2x wash buffer 1, 2x wash buffer 2, 1x wash buffer 3
10	2	*Reagent 1:* 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Reagent 2:* 25 g/L Acetophenone 0.625 g/L $NADP^+$ disodium salt 25 vol% isopropanol in 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$	Adds 15 µL reagent 1 to ONLY to the benchmark plates Adds 100 µL reagent 2 to reaction AND benchmark plates Final reaction conditions: 20 g/L Acetophenone 0.5 g/L $NADP^+$ disodium salt 20 vol% isopropanol in 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ Final reaction volume 125 µL
11	Incubation		*Experiment 1&2:* Seal reaction plate and incubate 4h at 250 rpm and 25 °C *Experiment 1&2:* Seal reaction plate and incubate 20 min at 250 rpm and 25 °C
12	3	MeOH	Quenches reaction plate with 125 µL MeOH (1:1 dilution)
13	3	MeOH	Dilutes the reaction mixture in the analytical plate (1:20) for a final analytical dilution auf 1:40
14	Analytics		Run sample analysis on SFC

Table S11: Individual steps for experiment 4 (isopropanol concentration and acetophenone concentration), experiment 5 (buffer type & pH and NADP concentration) and experiment 6 (refined buffer type & pH and NADP concentration) carried out by the liquid handling robot for reaction condition screening (stage 3 of the presented workflow) using method A0, A1, 2 and 3 for the enzyme candidate 2 on ECR8304F resin.

Step	Method	Reagents	Description
1	A0	Purolite ECR8304F Resin (suspension in destilled water)	Distributes resin (25 mg) from reservoir to reaction plate
2	A0	Destilled water	Washes resin 4 times
3	A0	3.1 g/L Gluteraldehyde in water	Adds 100 µL glutaraldehyde solution to reaction plate Final activation conditions: 200 mg/mL Resin 1.3 wt% Glutaraldehyde loading based on resin in water Final immobilization volume 125 µL
4	Incubation		Seal plate and incubate 2h at 250 rpm and 25 °C
5	A1	50 mM Tris HCl buffer pH 7.5 + 2 mM $MgSO_4$	Washes resin 4 times
6	A1	50 mM Tris HCl buffer pH 7.5 + 2 mM $MgSO_4$	Adds 50 µL Immobilization buffer
7	A1	25 g/L SFP (KRED 2) in immobilization buffer	Adds 50 µL SFP dilution to reaction plates Transfer 10 uL sample to lysate benchmark plates Final immobilization conditions: 200 mg/mL Resin SFP concentration of 10 g/L (5 wt% SFP loading based on resin) in 50 mM Tris HCl buffer pH 7.5 + 2 mM $MgSO_4$ Final immobilization volume 125 µL
8	Incubation		Seal reaction plate and incubate 18h at 250 rpm and 25 °C
9	2	*Experiment 1:* *Wash buffer 1:* 500 mM sodium chloride solution *Wash buffer 2:* 20 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Wash buffer 3:* 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Experiment 1:* *Wash buffer 1:* 500 mM sodium chloride solution *Wash buffer 2:* 20 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Wash buffer 3:* destilled water	Washes resin in total 5 times, 2x wash buffer 1, 2x wash buffer 2, 1x wash buffer 3
10	2	*Experiment 1:* *Reagent 1:* Serial dilution of 50-375 g/L acetophenone in isopropanol *Reagent 2:* Serial dilution of 0-100 vol% isopropanol in 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Experiment 2:* *Reagent 1:* 50 g/L acetophenone in	*Note: Add 15 µL to benchmark plates prior to processing* Adds 50 µL reagent 1 to reaction AND benchmark plates Adds 50 µL reagent 2 to reaction AND benchmark plates Final reaction conditions: *Experiment 1:* 20 -150 g/L Acetophenone 0.5 g/L $NADP^+$ disodium salt 25-78 vol% isopropanol in 100 mM TEoA buffer pH 7.5 + 2 mM $MgSO_4$ *Experiment 2:* 20 g/L acetophenone 0-1 g/L $NADP^+$ disodium salt

		75 vol& isopronaol in water + 2.5 mM $MgSO_4$ *Reagent 2:* 0-2.5 g/L $NADP^+$ disodium salt in different buffers 125 mM TEoA buffer pH 7/7.5/8 or 125 mM MOPS buffer pH 7/7.5 or 125 mM Tris buffer pH 7.5/8/8.5/9 or 125 mM KP_i buffer pH 7/7.5/8 *Experiment 3:* *Reagent 1:* 50 g/L acetophenone in 75 vol& isopropanol in water + 2.5 mM $MgSO_4$ *Reagent 2:* 0-10 g/L $NADP^+$ disodium salt in different buffers 125 mM TEoA buffer pH 7/7.5/8 or 125 mM MOPS buffer pH 7/7.5 or 125 mM Tris buffer pH 7.5/8/8.5/9 or 125 mM KP_i buffer pH 7/7.5/8	30 vol% isopropanol in different buffers *Experiment 3:* 20 g/L acetophenone 0-4 g/L $NADP^+$ disodium salt 30 vol% isopropanol in different buffers (final concentration 50 mM) Final reaction volume 125 µL
11	Incubation		*Experiment 1:* Seal reaction plate and incubate 4h at 250 rpm and 25 °C *Experiment 2&3:* Seal reaction plate and incubate 20 min at 250 rpm and 25 °C
12	3	MeOH	Quenches reaction plate with 125 µL MeOH (1:1 dilution)
13	3	MeOH	Dilutes the reaction mixture in the analytical plate (1:20) for a final analytical dilution auf 1:40
14	Analytics		Run sample analysis on SFC

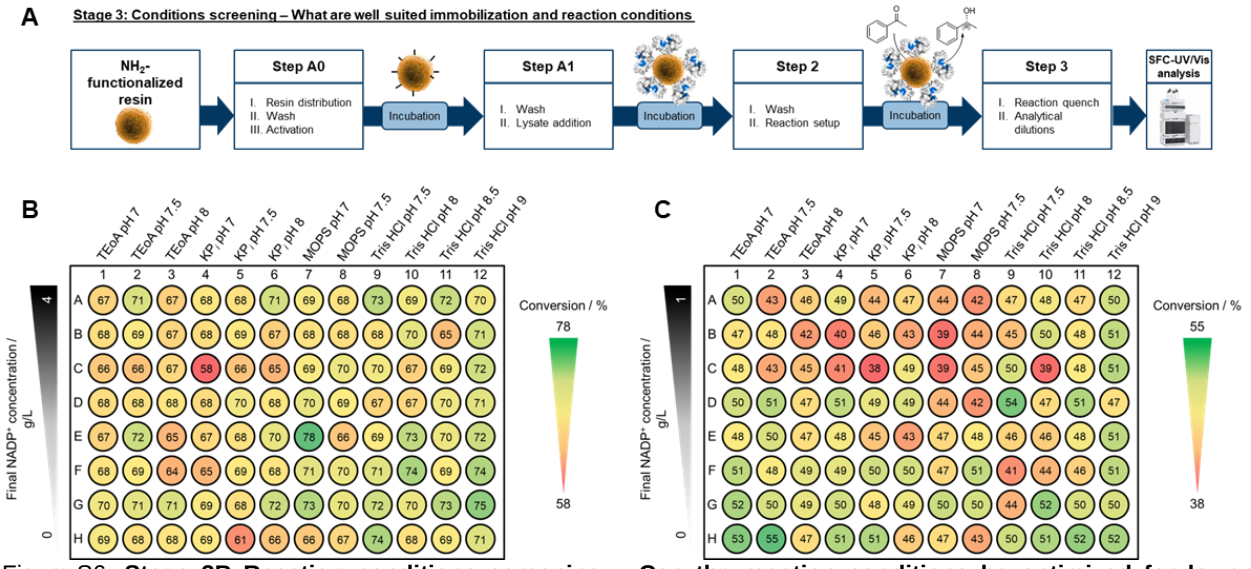

Figure S6: **Stage 3B Reaction conditions screening – Can the reaction conditions be optimized for lower cofactor concentrations and thus lower costs and carbon footprint.** 2D heat maps of different key reaction parameters after 2h incubation of immobilized KRED on ECR8304F resin with varying buffer type and pH and $NADP^+$ concentration. (**A**) Schematic flow chart of the different automated steps conducted. (**B,C**) 2D heat maps of the conversion of 20 g/L acetophenone with varying $NADP^+$ concentration and 30 vol% isopropanol with immobilized KRED 2 on ECR8304F resin at 25°C. Immobilization was conducted under optimized conditions identified in Stage 3A (0.0125 g/g_{resin} glutaraldehyde loading and 0.053 g/g_{resin} enzyme loading in 75 mM Tris HCl pH 7.5 buffer: (**B**) Conversion of acetophenone (**1**) towards the desired (*R*)-**2** product with varying $NADP^+$ concentrations from row A to H and varying buffer types and pH in column 1-12. (**C**) reproduction of previous with higher NADP+ concentration - Conversion of acetophenone (**1**) towards the desired (*R*)-**2** product with varying $NADP^+$ concentrations from row A to H and varying buffer types and pH in column 1-12.

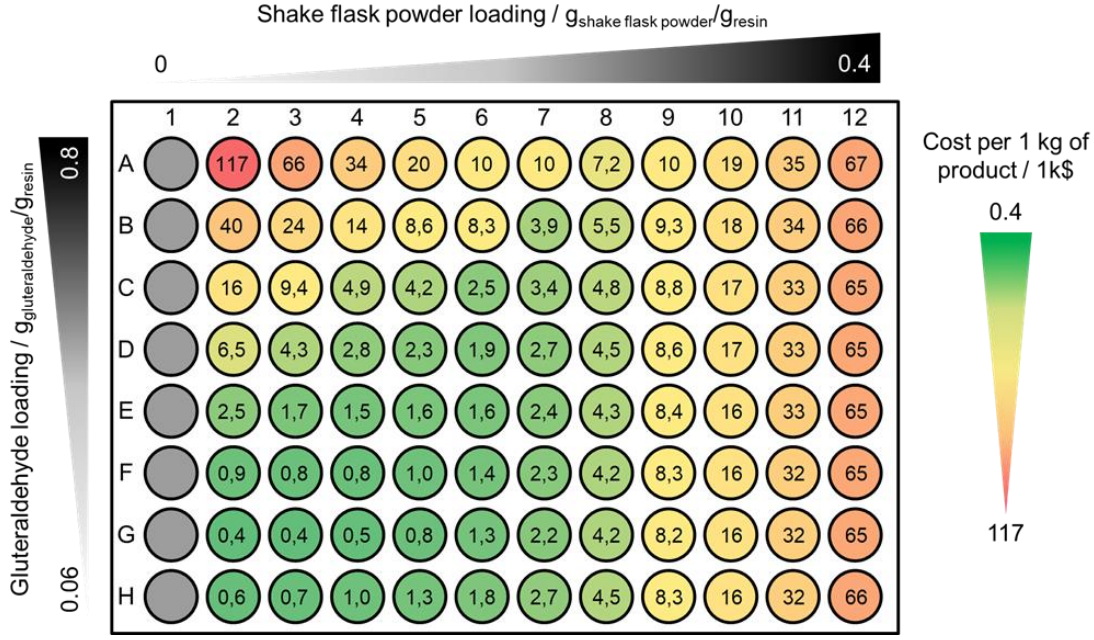

Figure S7: 2D heat maps of the cost in 1000$ per kg of product with varying glutaraldehyde and enzyme concentration analogues to the conditions described in figure 5B. Calculation is based on the cost of glutaraldehyde and enzyme in each well according to table S13.

4.6. Stage 4: Scale up of immobilization and biocatalytic reaction

4.6.1 Immobilization procedure upscaled in SPE tubes

Immobilization conditions based on literature

- 2 g wet resin (resin:buffer ratio 1:5)
- 16 g/L glutaraldehyde solution (0.08 g_{GA}/g_{resin})
- 31.2 g/L SFP (0.156 g_{SFP}/g_{resin}) of KRED 2
- 10 mL 75 mM TEoA buffer pH 7

Transfer 2 g of wet Resin to 20 mL SPE tube and wash 3 times with 10 mL of 50 mM Tris HCl pH 7.5. Prepare 10 mL 16 g/L glutaraldehyde solution by diluting 320 uL of 50 wt% glutaraldehyde stock solution with 9.68 mL water. Add glutaraldehyde solution to SPE tube and incubate vertically in Eppendorf shaker for 2 hours at 25°C. Wash 4 times with water and once with 50 mM TEoA pH 7.5. Prepare 10 mL 10.6 g/L KRED shake flask powder (KRED 2) stock solution by dissolving 312 mg of KRED SFP in 10 mL of 100 mM TEoA pH 7.5. Add 10 mL of enzyme stock solution and incubate vertically in Eppendorf shaker for 18 hours at 25°C. Remove supernatant and wash 2 times with 0.5 M NaCl solution and 3 times with 100 mM Tris HCl pH 7.5.

Immobilization conditions with optimized conditions

- 2 g wet resin (resin:buffer ratio 1:5)
- 2.5 g/L glutaraldehyde solution (0.0125 g_{GA}/g_{resin})
- 10.6 g/L SFP (0.053 g_{SFP}/g_{resin}) of KRED 2
- 10 mL 75 mM Tris HCl buffer pH 7.5

Transfer 2 g of wet Resin to 20 mL SPE tube and wash 3 times with 10 mL of 50 mM Tris HCl pH 7.5. Prepare 10 mL 2.5 g/L glutaraldehyde solution by diluting 50 uL of 50 wt% glutaraldehyde stock solution with 9.95 mL water. Add glutaraldehyde solution to SPE tube and incubate vertically in Eppendorf shaker for 2 hours at 25°C. Wash 4 times with water and once with 50 mM Tris HCl pH 7.5. Prepare 10 mL 10.6 g/L KRED shake flask powder (KRED 2) stock solution by dissolving 106 mg of KRED SFP in 10 mL of 100 mM Tris HCl pH 7.5. Add 10 mL of enzyme stock solution and incubate vertically in Eppendorf shaker for 18 hours at 25°C. Remove supernatant and wash 2 times with 0.5 M NaCl solution and 3 times with 100 mM Tris HCl pH 7.5.

4.6.2 Scale-Up of biocatalytic reaction with immobilized enzymes

Reaction conditions based on literature

- 0.1 M acetophenone
- 0.5 g/L $NADP^+$
- 800 mg enzyme-loaded resin
- 20 vol% IPA
- 100 mM TEoA pH 7.5
- Final reaction volume 4 mL
- 25 °C, 24 h
- Samples taken after 1,2 4, 8 and 24 hours

Weigh 800 mg of each wet resin into 20 mL SPE tube. Prepare 12 mL reaction solution by mixing 0.144 g acetophenone, 6 mg $NADP^+$, 2.4 mL isopropanol, 7.2 mL 125 mM TEoA pH 7.5, 24 µL 1 M $MgSO_4$ solution and 2.232 mL water in a 15 mL tube. Add 4 mL substrate stock solution to each resin and shake vertically at 25 °C and 900 rpm for 24 hours. Take 15 µL aliquots after 1, 2, 4, 8 and 24 hours and dilute in 1.5 mL MeOH (1 g/L final conc.) before analyzing via SFC.

Reaction conditions with optimized conditions

- 1.25 M acetophenone
- 800 mg enzyme-loaded resin
- 55 vol% IPA
- 100 mM Tris HCl pH 7.5
- Final reaction volume 4 mL
- 25 °C, 24 h
- Samples taken after 1,2 4, 8 and 24 hours

Weigh 800 mg of each wet resin into 20 mL SPE tube. Prepare 12 mL reaction solution by mixing 1.8 g acetophenone, 6.6 mL isopropanol, 1.8 mL 500 mM Tris HCl pH 7.5, 24 µL 1 M $MgSO_4$ solution and 1.776 mL water in a 15 mL tube. Add 4 mL substrate stock solution to each resin and shake vertically at 25 °C and 900 rpm for 24 hours. Take 15 µL aliquots after 1, 2, 4, 8 and 24 hours and dilute in 1.5 mL MeOH (1 g/L final conc.) before analyzing via SFC.

To prove that our screening resulted in a better process compared to what one could identify from existing procedures we conducted four upscaling experiments: Two immobilizations, one with our optimized immobilization conditions and another one with conditions that could be considered “standard” based on reported work. We tested both formulations in our optimized reaction conditions as well as in conditions that could be considered “standard” when starting a biocatalytic process development monitoring the conversion for 24 hours. The reaction setup following the literature conditions were completed within the first hour and observed conversion was >92 % for every sample taken with >99% ee to the desired *R*-enantiomer. The reaction setup following the optimized conditions showed a reaction kinetic over the full 24 hours independent from the

immobilization procedure. Conversions of the two reactions are given in table S12 and enantioselectivity was measured with >99% ee to the desired *R*-enantiomer in all samples.

Table S12: Time-dependent conversion of the test reaction using 1.25 M acetophenone and 20 wt% resin loading 55 vol% isopropanol in 100 mM Tris HCl buffer pH 7.5.The resin was prepared either under standard literature conditions or with optimized conditions.

Time / h	Immobilisation based on literature conditions	Immobilisation based on optimized conditions
1	40%	40%
2	52%	53%
4	62%	64%
8	69%	69%
24	73%	73%

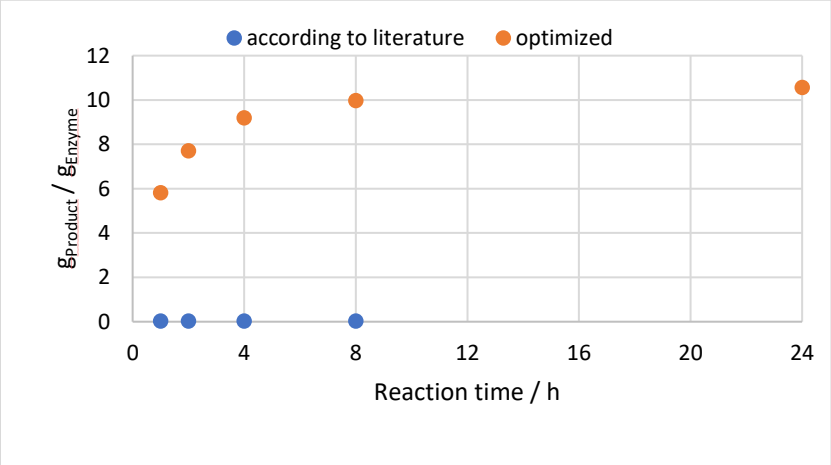

Figure S8: Time-dependent catalyst productivity plot comparing the literature conditions and the optimized conditions following our workflow. The biocatalytic reduction of acetophenone (**1**, 12/150 g/L) in 20/55 vol% isopropanol towards (*R*)-2 using immobilized KRED 2 over 24 hours (see table 1 and ESI chapter 4.6.).

Table S13: Cost contribution for reagents used for the synthesis of 1 kg of product (*R*) **2** using optimized conditions presented in Table 1. Prices are from October 2022 and estimated based on listing from chemical vendors but heavily depend on market situation and scale in all cases.

Cost related to ... for 1 kg product	**Typical conditions taken from literature**	**Optimized process using our workflow**
Gluteraldehyde (250 $/kg)	340 $	5 $
Enzyme (15000 $/kg)	38000 $	1400 $
NADP (100000 $/kg)	4300 $	0 $
Resin (900 $/kg)	15000 $	1600 $
Total	**57640 $**	**3000 $**

Literature

1 R. A. Sheldon and S. van Pelt, *Chem. Soc. Rev.*, 2013, **42**, 6223