Supplementary Information

DERA in flow: Synthesis of a statin side chain precursor in continuous flow employing deoxyribose-5-phosphate aldolase immobilized in alginate-luffa matrix

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Content

1. SDS-Page DERA

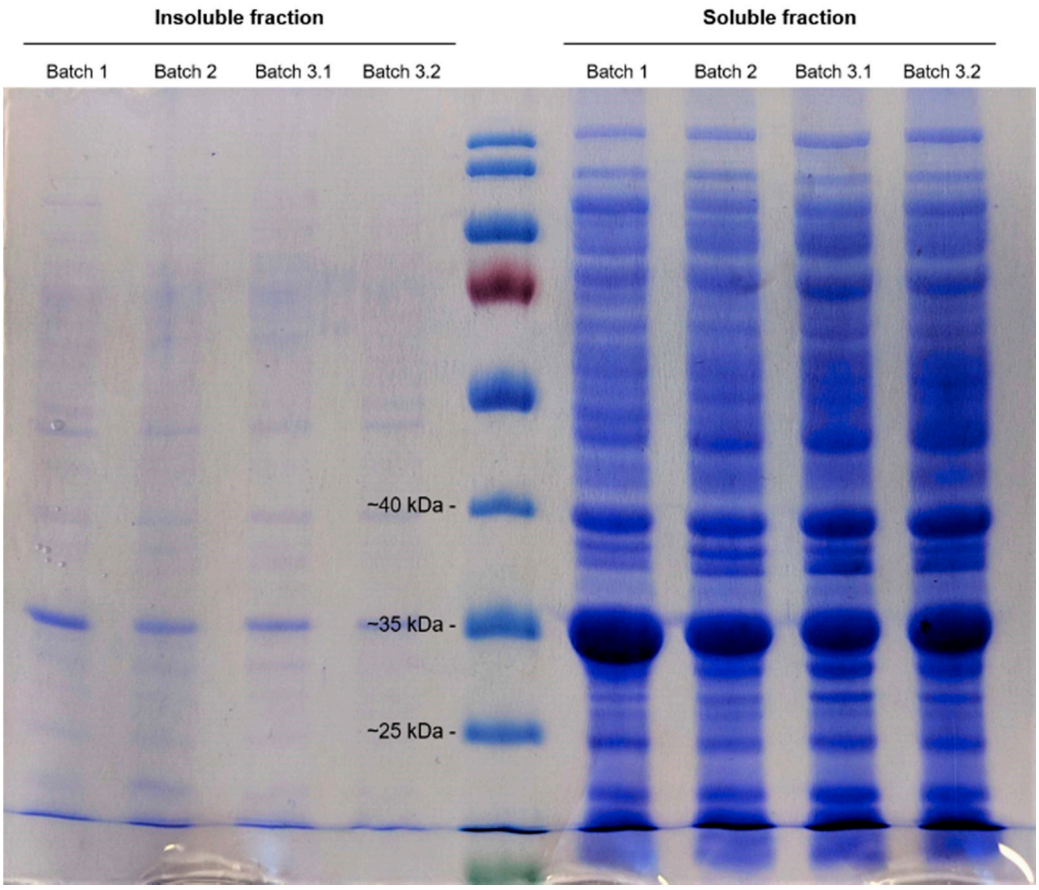

Figure S1. SDS-PAGE of freeze-dried E. coli BL21 (DE3), containing overexpressed DERA (27.7 kDa)

2. Experimental design for optimization of pH and temperature in batch

Table S1. Conducted experiments (process parameters: temperature and pH-value) for DoE to optimize the process parameters in batch

Entry	pH code	T code	pH	T / °C	r_{dimer} / μmol min^{-1}	r_{lactol} / μmol min^{-1}
				1st circuit		
4	-1	-1	6.0	28.0	1.0	0.1
5	-1	0	6.0	32.5	21.9	0.1
6	-1	1	6.0	37.0	13.3	0.1
7	-0.5	-1	6.5	28.0	18.6	0.0
8	-0.5	0	6.5	32.5	1.0	0.1
9	-0.5	1	6.5	37.0	29.8	0.1
10	0	-1	7.0	28.0	6.4	0.0
11	0	0	7.0	32.5	16.0	1.1
12	0	1	7.0	37.0	5.1	0.0
13	0.5	-1	7.5	28.0	21.8	2.7
14	0.5	0	7.5	32.5	92.4	53.0
15	0.5	1	7.5	37.0	7.8	0.1
16	1	-1	8.0	28.0	24.5	0.7
17	1	0	8.0	32.5	74.5	32.8
18	1	1	8.0	37.0	50.9	0.5
				2nd circuit		
19	0.25	0.44	7.25	30.0	150.1	9.4
20	0.25	0.89	7.25	32.0	40.4	45.3
21	0.25	1.33	7.25	34.0	88.2	59.6
22	0.5	0.44	7.50	30.0	154.6	24.8
23	0.5	0.89	7.50	32.0	88.6	46.0
24	0.5	1.33	7.50	34.0	102.4	71.5
25	0.75	0.44	7.75	30.0	15.8	0.0
26	0.75	0.89	7.75	32.0	66.0	46.1
27	0.75	1.33	7.75	34.0	75.2	66.3

Reaction conditions: 500 μL of 0.1 M TEOA buffer containing 1.5 M acetaldehyde, 7 μL DMSO, stir at 200 rpm for 1 h. Sampling: 200 μL of reaction mixture diluted with 800 μL of acetonitrile. Analysis by means of GC-FID

3. GC-spectra

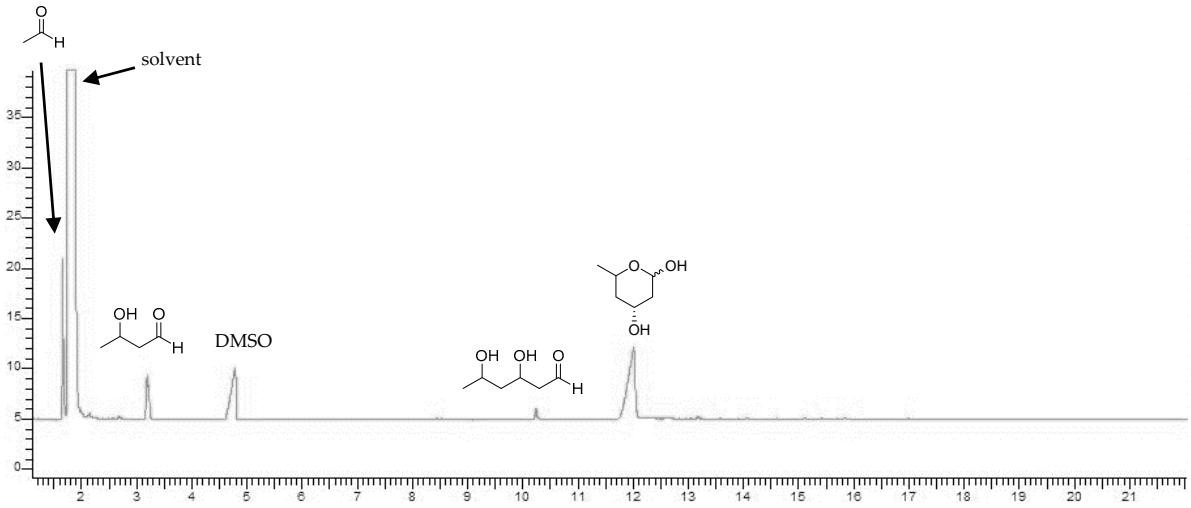

Figure S2. GC spectrum from a sample (t = 120 min) of a batch aldol addition of three molecules of acetaldehyde.

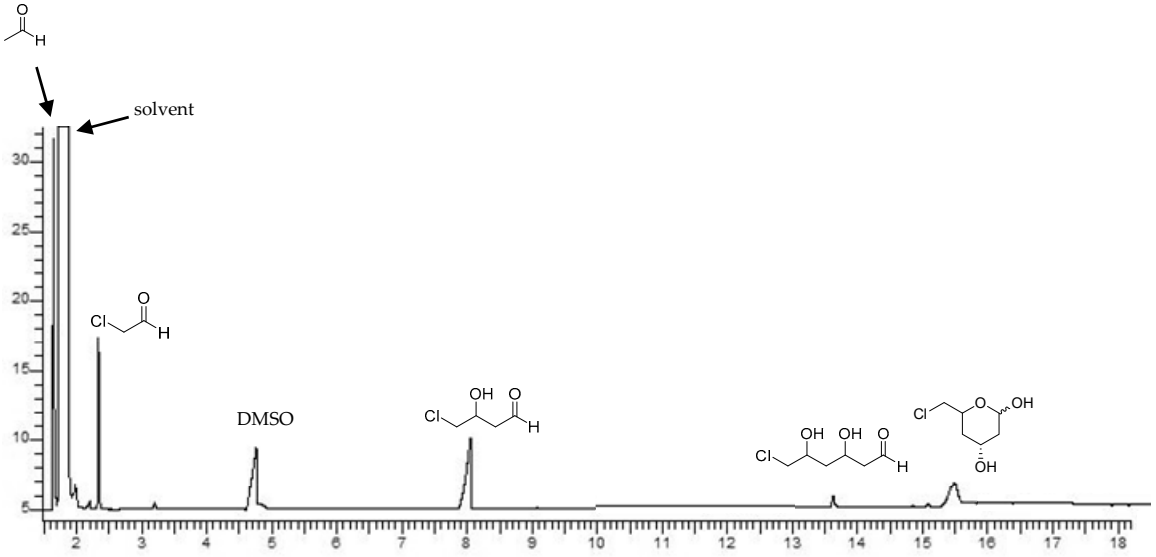

Figure S3. GC spectrum from a sample (t = 30 min) of a batch aldol addition of chloroacetaldehyde and two molecules of acetaldehyde.

4. GC-FID spectra and selection of results

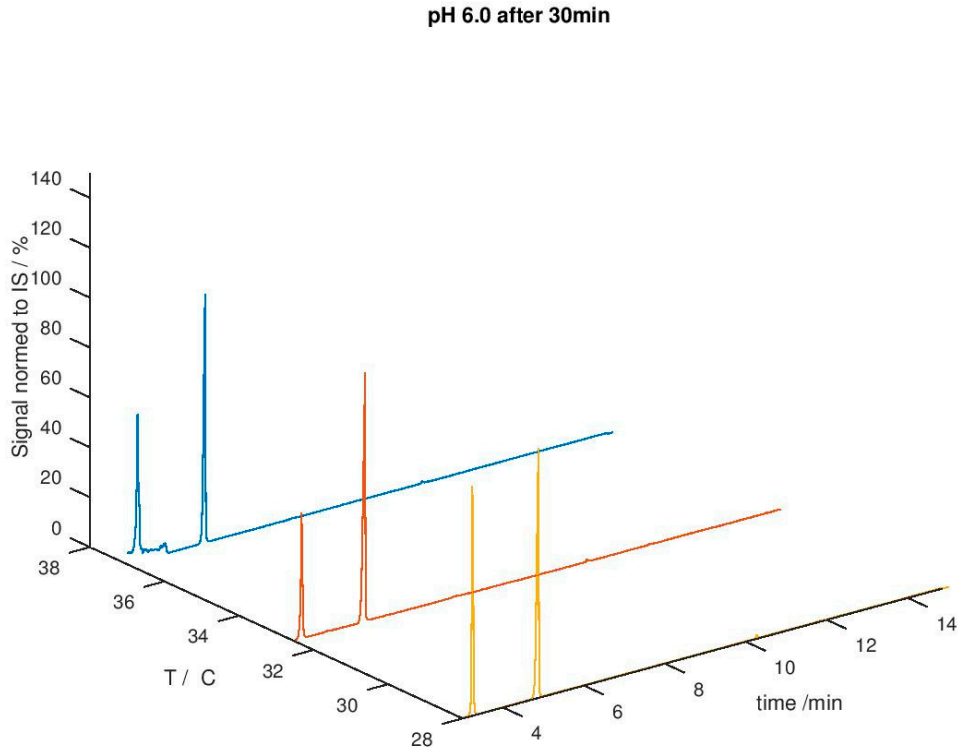

Figure S4. GC spectra for DoE runs at pH 6.0, T = 28 – 38 °C, t = 30 min

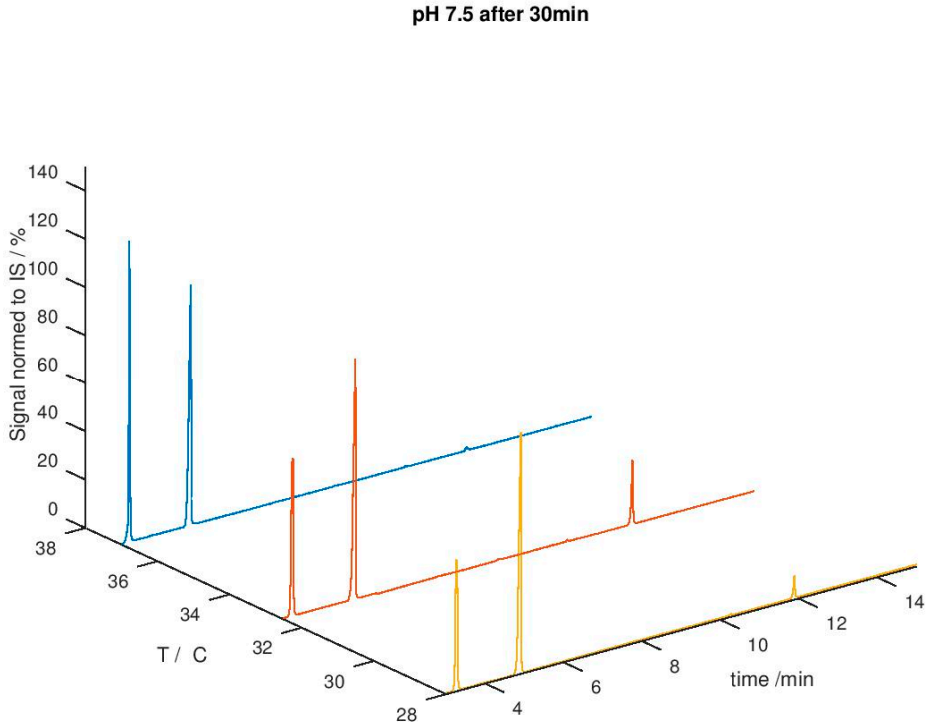

Figure S5. GC spectra for DoE runs at pH 7.5 T = 28 – 38 °C, t = 30 min

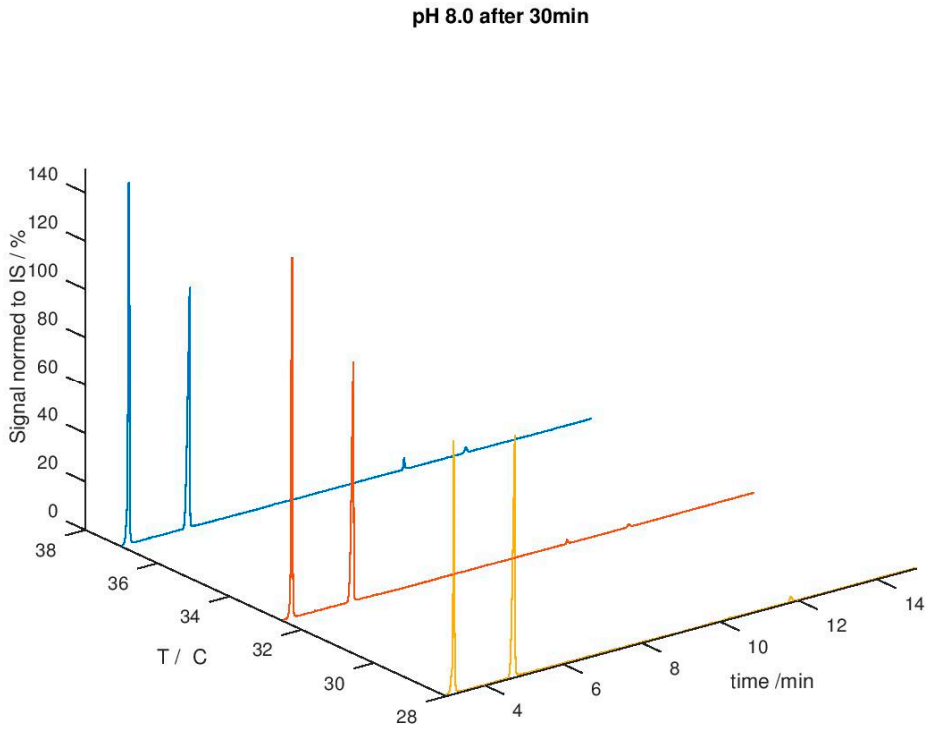

Figure S6. GC spectra for DoE runs at pH 8.0 T = 28 – 38 °C, t = 30 min

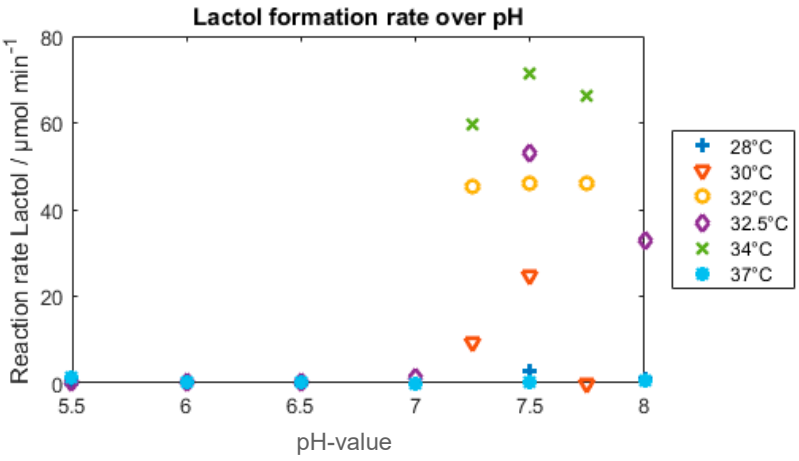

Figure S7. Result of DoE (product formation rate) for DoE over pH

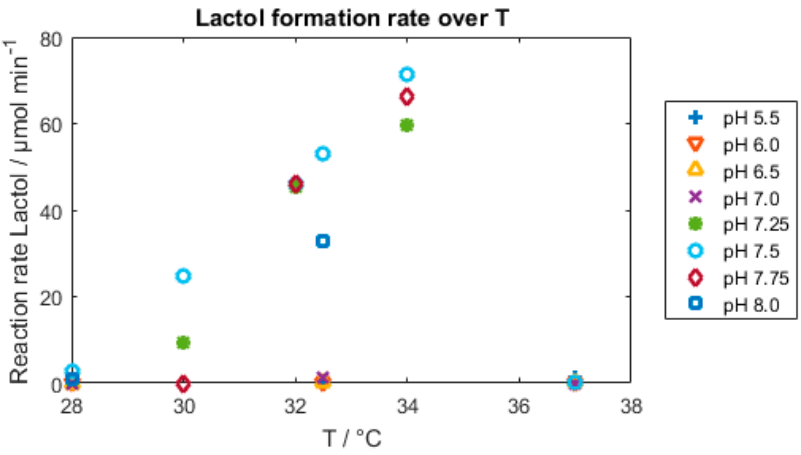

Figure S8. Result of DoE (product formation rate) for DoE over reaction temperature

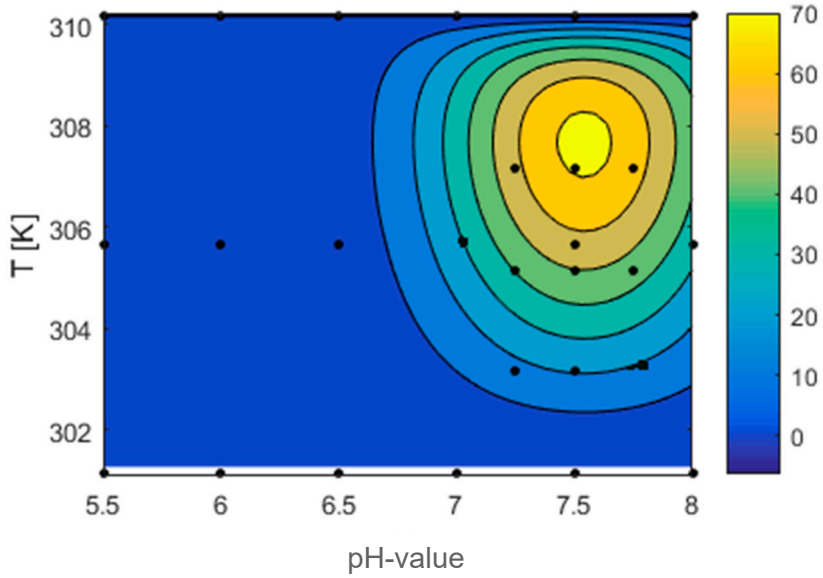

Figure S9. Response surface for the experimental design – view from top

5. Coating procedure

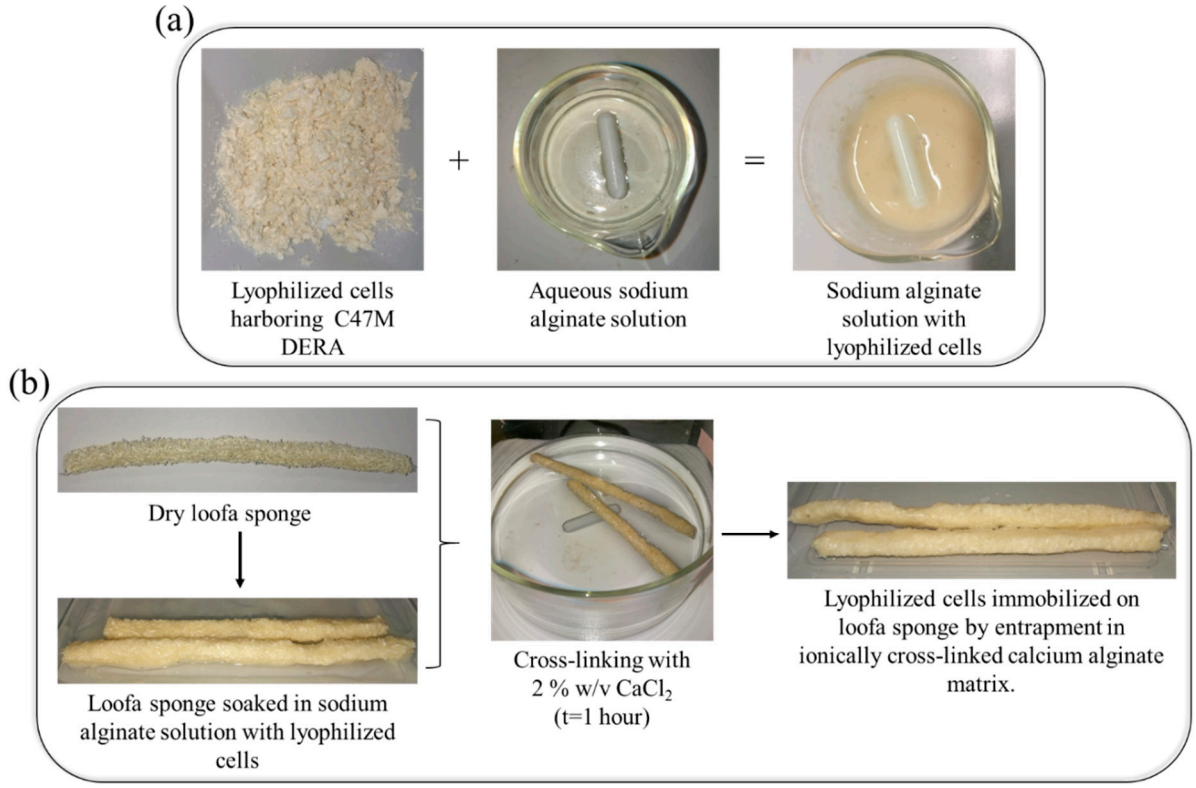

Figure S10. Immobilization of the lyophilized cells harboring C47M DERA mutant illustrating (a) preparation of the sodium alginate solution with the lyophilized cells and (b) cross-linking after soaking the sponge in the sodium alginate solution with the lyophilized cells and the final immobilized product.

6. Determination of the residence time distribution (RTD) in the flow reactor.

An HPLC (High Performance Liquid Chromatography) column (20 cm × 0.8 cm) packed with loofa sponge was connected to two HPLC pumps (Knauer, Azura P4.1 S) flushed with either solvent (ethanol:water 6:4 v/v) or tracer (0.4 % v/v anisole in ethanol:water 6:4 v/v). The outlets of the HPLC pumps and the inlet of the column were connected via a 6-port injection valve (IDEX Health & Science LLC, V-450) used for efficient solvent switching during a step input. The column was placed inside the plug & play reactor connected to a Lauda P18 Proline thermostat. The tracer signal was detected by an inline UV-vis flow cell (Avantes, Cell-Z-10) with 10 mm optical path length at the outlet of the reactor. UV-vis measurements were performed with an Avantes system equipped with a detector (Avantes, AvaSpec-ULS2048) and a deuterium lamp (Avantes, AvaLight-DS-DUV) light source. The RTD set-up is shown in Figure .

Loofa sponge was soaked in 0.9 % w/v NaCl buffer for one hour. The HPLC column was packed with the soaked loofa sponge and connected to an HPLC pump flushed with 60 % v/v EtOH in H_2O (RTD solvent). The column was flushed vertically with the RTD solvent to get rid of air bubbles. The column was placed inside the plug & play reactor and connected to the UV-vis flow cell with Avantes system. Both pumps were set to identical flowrates of either 0.5 mL/min, 0.25 mL/min, or 0.1 mL/min. After equilibration to a constant signal, a baseline correction was performed by measuring the absorption at 500 nm - 506 nm wavelength. The flow to the column was switched from RTD solvent to tracer (0.4 % v/v anisole in RTD solvent) manually with the 6-port injection valve for the step input. The tracer absorption was measured at 268 nm - 274 nm wavelength. Detected signal values were saved every 500 ms.

Theoretical mean residence time, $\bar{t}_{th}$, was calculated by the following equation:

$$\bar{t}_{th} = \frac{(V_R - V_S)}{v} \qquad \text{Eq. 1}$$

where V_R is the empty reactor volume in mL, V_S is the volume of the loofa sponge soaked in RTD solvent (EtOH) in mL, and v is the flow velocity in mL/min. Volume of a loofa sponge was determined by applying the Archimedes' principle. A loofa sponge was placed in graduated cylinder filled with water and the volume of the displaced water was read from the graduated cylinder marks. Soaked loofa sponge was used to account for the sponge expansion.

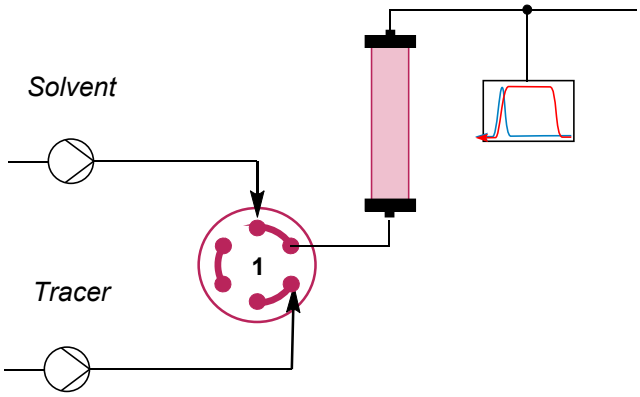

Figure S11. Experimental set-up for the RTD determination in flow. The step input is made by switching the manual 6-port injection valve from HPLC Pump 1 (Solvent) to HPLC Pump 2 (Tracer) position, allowing a constant flow of tracer through the column and subsequently the flow cell.

7. Product synthesis in semi-batch

Semi-batch reaction was performed based on the protocol described by Ručigaj A. and Krajnc M. [1] The enzyme suspension (0.1 M pH 7.5 TEOA buffer, 1.4 % v/v DMSO, and lyophilized cells) preparation and the reaction conditions were identical to the batch reaction. Chloroacetaldehyde (acceptor) and acetaldehyde (donor) were used as substrates for the semi-batch reaction. Both substrates were added to the enzyme suspension of 5 mL initial volume continuously by the polyvalent programmable syringe pumps (Lambda Laboratory Instruments, VIT-FIT) at different rate. Each pump was equipped with one 20 mL syringe. The syringes were filled with either donor or acceptor in 0.1 M pH 7.5 TEOA buffer at an initial concentration of 2.8 M and 1.8 M, respectively.

For 120 minutes of the semi-batch reaction, chloroacetaldehyde and/or acetaldehyde were added to the enzyme suspension at various rates as described in Table S2. After feeding, the reaction was left to proceed for an additional hour. The total reaction time was 3 hours and the final volume of the reaction mixture was 9 mL.

Table S2. Semi-batch reaction regime of acetaldehyde (donor) and chloroacetaldehyde (acceptor) feeding.

Feeding Time [min]	**Feeding Rate [mL/h]**		**Amount Fed [mmol]**		**Final Concentration [mol/L]**	
	Donor	Acceptor	Donor	Acceptor	Donor	Acceptor
0-30	2.6	2.9	3.7	2.5	0.50	0.33
30-60	0.9	0.0	1.3	0.0	0.64	0.32
60-120	0.8	0.0	2.0	0.0	0.83	0.29

8. Product isolation and purification

The purification procedure steps were executed according to procedure outlined by Ošlaj *et al*.[2] with slight modifications. The purification procedure was optimized and tailored to account for the differences between the reaction conditions.

Intermediate and product purified were synthesized by batch and semi-batch, respectively. After 180 minutes of semi-batch 3 vol. acetone was added to the reaction and left standing at room temperature for 30 minutes to precipitate the enzyme. The mixture was then filtered by gravity filtration using a fluted filter paper for enzyme capturing. Acetone was evaporated under reduced pressure and the enzyme-free reaction mixture was extracted 3 times with 2 vol. ethyl acetate (EtOAc). The organic EtOAc phases were combined, dried over $MgSO_4$, and filtered with fluted filter paper. After EtOAc evaporation under reduced pressure, intermediate and/or product were obtained as a light brown-yellow oil. The intermediate and product were purified by flash chromatography in silica gel (diethylether/hexane 1:1).

9. NMR

NMR-measurement was performed using a Bruker Avance III 300 MHz spectrometer (^{1}H: 300 MHz). The chemical shift (δ [ppm]) was reported relatively to the used solvent $CDCl_3$ (7.26, s).

Product (**2c**) [4]: ^{1}H NMR ($CDCl_3$): 1.50-2.05 (m, 4H); 3.50-3.60 (m, 2H); 4.10-4.15 (m, 2H); 4.31 (br, 1H); 4.58 (br, 1H); 5.18 (m, 1H);

10. Experimental design for optimization of the flow process

Table S3. Conducted experiments (process parameters: concentration of substrate **2**, flow rate and cross-linking cation for ALM) for DoE to optimize the flow process.

Entry	Conc. code	Flow code	Conc. of 2 / mM	Flow rate / mL min^{-1}	Cation	Yield / %
1	1	-1	250	0.10	Ba^{2+}	37.1
2	-1	-1	750	0.10	Ba^{2+}	57.6
3	0	-1	500	0.10	Ba^{2+}	43.3
4	1	1	250	0.10	Ba^{2+}	6.5
5	-1	1	750	0.10	Ba^{2+}	23.1
6	0	1	500	0.10	Ba^{2+}	18.6
7	1	0	250	0.25	Ba^{2+}	19.0
8	-1	0	750	0.25	Ba^{2+}	36.0
9	0	0	500	0.25	Ba^{2+}	28.2
10	1	-1	250	0.10	Ca^{2+}	35.2
11	-1	-1	750	0.10	Ca^{2+}	49.1
12	0	-1	500	0.10	Ca^{2+}	41.0
13	1	1	250	0.10	Ca^{2+}	15.1
14	-1	1	750	0.10	Ca^{2+}	20.2
15	0	1	500	0.10	Ca^{2+}	17.0
16	1	0	250	0.25	Ca^{2+}	21.5
17	-1	0	750	0.25	Ca^{2+}	33.9
18	0	0	500	0.25	Ca^{2+}	26.7
19	0	0	500	0.25	Ca^{2+}	25.9

Reaction conditions: 850 mg loofa sponge, 350 mg freeze-dried E. coli cells hosting DERA, 2 mol eq. of **1** with respect to **2**. 0.1 M TEOA buffer pH 7.5, 1.4 vol% DMSO, T = 32.5 °C. total run time per experiment 120 min. Analysis by means of GC-FID

11. Details on DoE for optimizing flow process

MODDE supplies additional diagrams and information besides the contour plot of the response surface. The diagrams are shown in Figure S12 to Figure S17 and a brief explanation is given below.

a. Replicate Plot

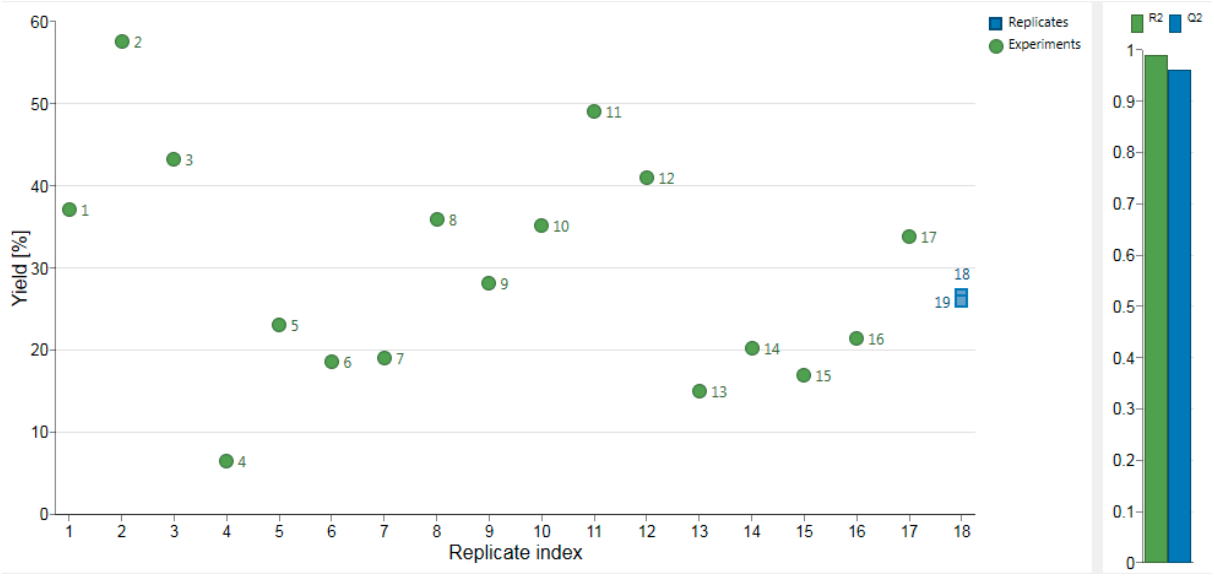

Figure S12. Replicate Plot of DoE for optimizing the flow process.

The replicate plot (Figure S12) sums up the response (yield) for all experiments conducted during DoE and the replicates. It shows that the variability of the replicates (18 and 19) is small in comparison to the variability of the other experiments. This is a hint that the resulting model will be very useful.

b. Histogram plot

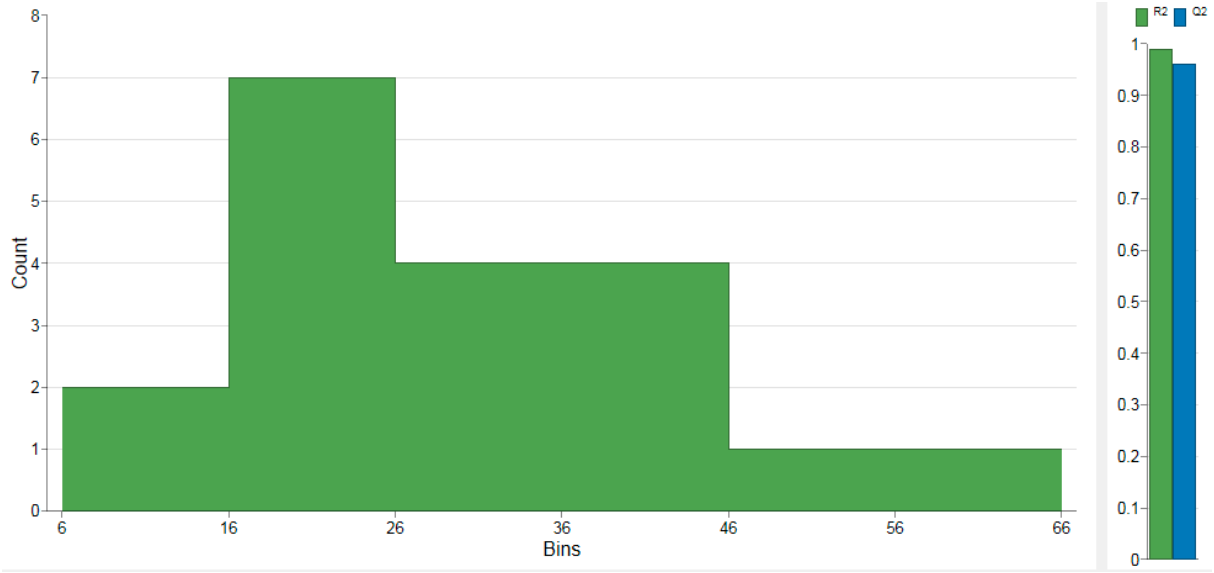

Figure S13. Histogram Plot of Doe for optimization of the flow process.

The histogram (Figure S13) shows the shape of the response distribution and is used to determine whether a transformation is needed. Since the distribution already meets the requirement of being "bell shaped" no transformation is needed.

c. Coefficient plot

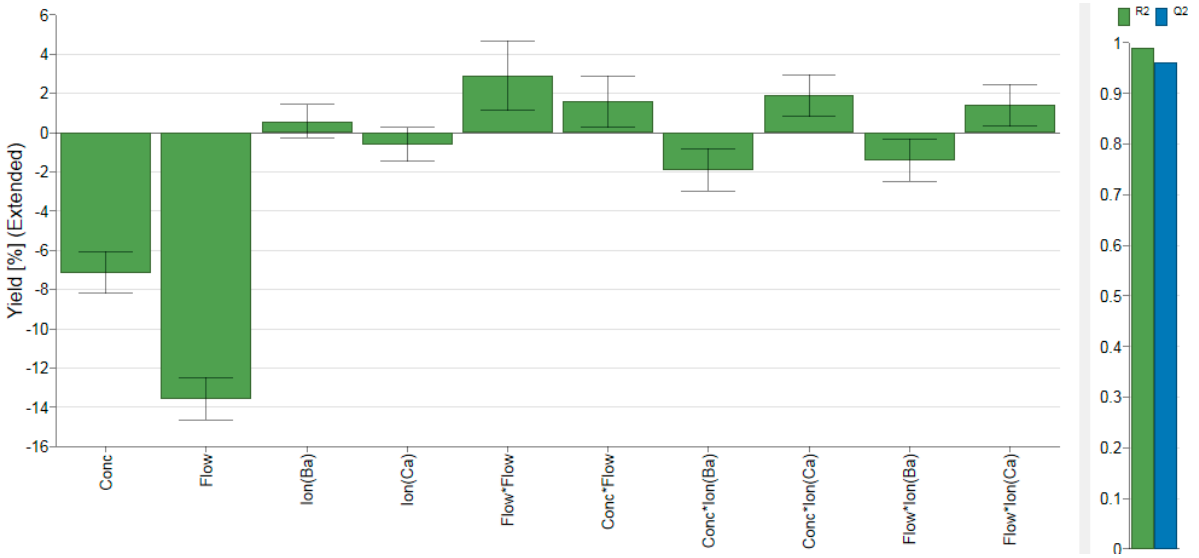

Figure S14. Coefficient plot of DoE for optimization of the flow process.

The diagram (Figure S14) shows the significance of the terms in the model. The concentration and flow rate have the biggest negative effect. With the value of these two parameters lead to a decrease in yield. The influence of the ion used for cross-linking so smaller than the variation of the experiments and thus does not need to be taken into account, when only looking at the performance of the flow set-up. There are slight interaction effects of the input parameter, but none of the them has a significant effect on the performance of the process as the variance of the experiments is almost as high as detected effect.

d. Summary plot

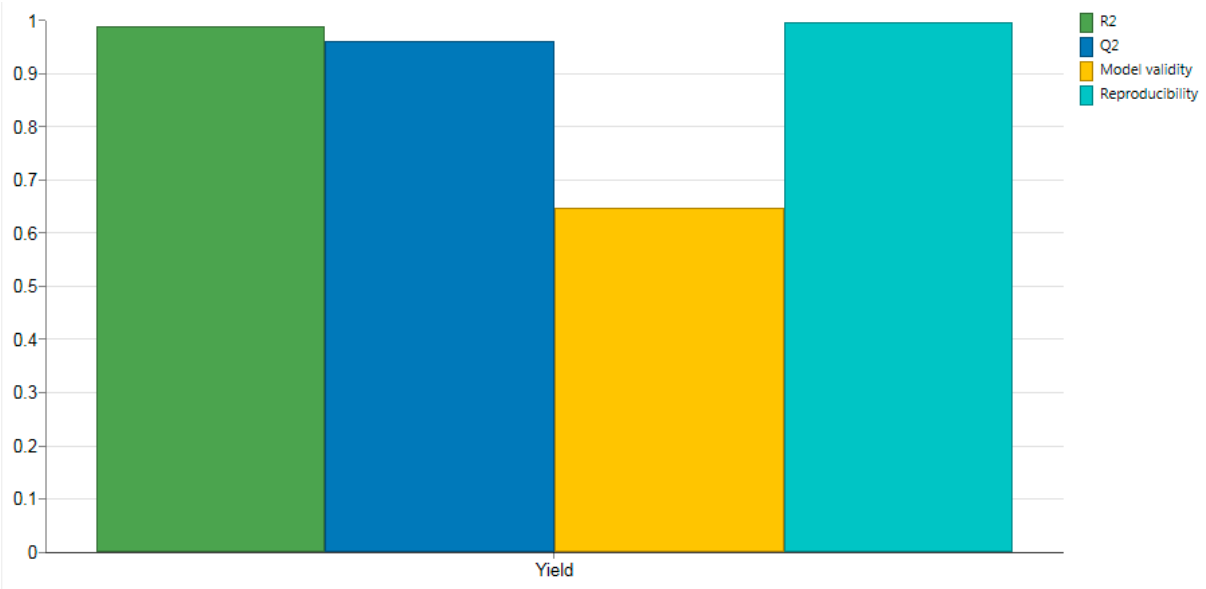

Figure S15. Summary plot of DoE for optimization of the flow process.

Figure S15, the summary plot, gives the model statistics in four parameters. The higher the value for the parameters the better (1 = 100 %). R2 shows the model fit. Q2 shows an estimate of the future prediction precision. A value greater than 0.5 indicates a good model. Model validity is a test of diverse model problems. If this value was lower than 0.25 would mean that statistically significant model problems, such as the presence of outliers, an incorrect model, or a transformation problem is present. Reproducibility is a variation of the replicates compared to the overall variability.

e. Residual Normal Probability plot

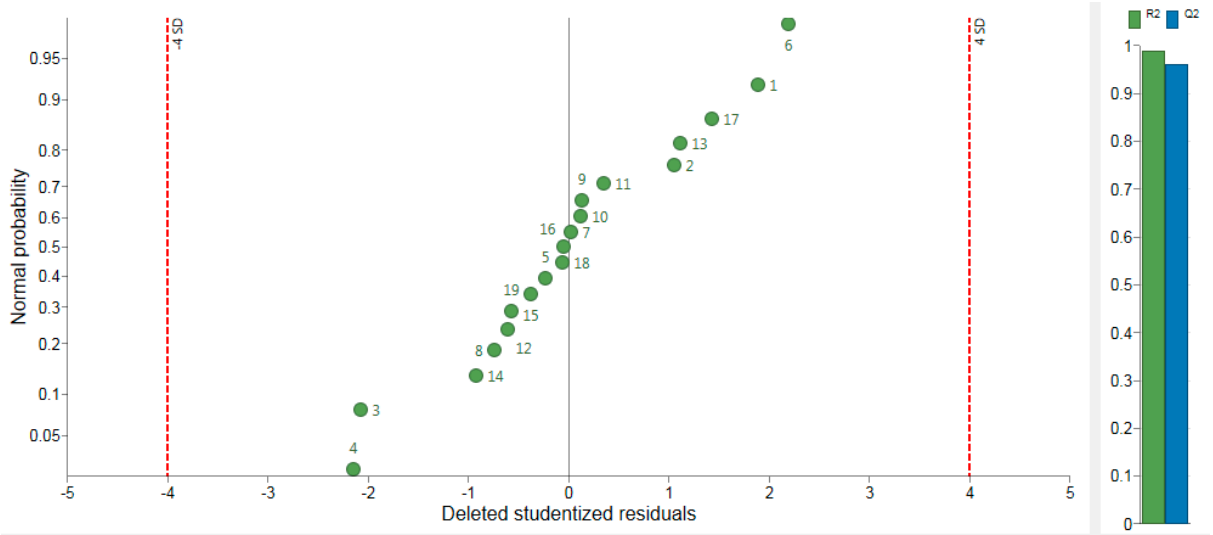

Figure S16. Residual normal probability plot of DoE for optimization of the flow process.

This plot (Figure S16) shows the residuals of a response vs. the normal probability of the distribution. Most points are on a straight line on the diagonal, indicating that the residuals are normally distrusted noise. There are no points outside the red line, which would indicate outliers.

f. Observed vs. Predicted

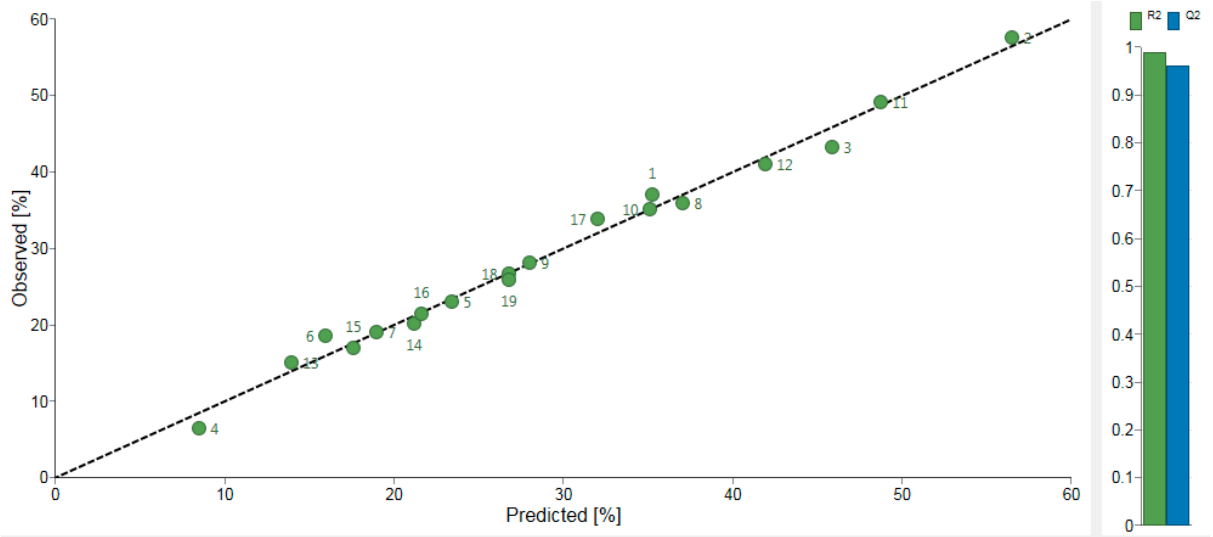

Figure S17. Observed vs. predicted plot of DoE for optimization of the flow process.

This plot (Figure S17) displays observed response vs. predicted values. For a good model, the points should be close to a straight line, as it is in this diagram.

12. References

1. Ručigaj, A.; Krajnc, M. Optimization of a Crude Deoxyribose-5-phosphate Aldolase Lyzate-Catalyzed Process in Synthesis of Statin Intermediates. *Org. Process Res. Dev.* **2013**, *17*, 854–862, doi:10.1021/op400040b.
2. Ošlaj, M.; Cluzeau, J.; Orkić, D.; Kopitar, G.; Mrak, P.; Casar, Z. A highly productive, whole-cell DERA chemoenzymatic process for production of key lactonized side-chain intermediates in statin synthesis. *PLoS ONE* **2013**, *8*, e62250, doi:10.1371/journal.pone.0062250.

3. Ručigaj, A.; Krajnc, M. Kinetic modeling of a crude DERA lysate-catalyzed process in synthesis of statin intermediates. *Chemical Engineering Journal* **2015**, *259*, 11–24, doi:10.1016/j.cej.2014.07.124.
4. Gijsen, H.J.M.; Wong, C.-H. Unprecedented Asymmetric Aldol Reactions with Three Aldehyde Substrates Catalyzed by 2-Deoxyribose-5-phosphate Aldolase. *J. Am. Chem. Soc.* **1994**, *116*, 8422–8423, doi:10.1021/ja00097a082.