Enzymatic cascade of DERA and ADH for lactone synthesis

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Table S1. List of primers

*Lb*DERA_fwd _E78K	GCTATGGCGACCGAATCT**AAA**ATCTTCGAAGCGACCACC
*Lb*DERA_rev _E78K	GGTGGTCGCTTCGAAGAT**TTT**AGATTCGGTCGCCATAGC
*Lb*DERA_fwd _C42M	GCTAAAAAATTCAACACCGCGTCTGTT**ATG**GTTAACTCTTACTGGATCCCGTTC
*Lb*DERA_rev _C42M	GAACGGGATCCAGTAAGAGTTAACCAT**AAC**AGACGCGGTGTTGAATTTTTTAGC
*Lb*DERA_fwd _C42M_2	CAACACCGCGTCTGTT**ATG**GTTAACTCTTACTGGATCC
*Lb*DERA_rev _C42M_2	GGATCCAGTAAGAGTTAAC**CAT**AACAGACGCGGTGTTG

Table S2. Nucleotide and amino acid sequence of *Lb*DERA, and *Lb*DERA variants.

Histag is underlined and mutations are highlighted in red.

> lb-dera (pET28a, NcoI/XhoI restriction sites) – 5'-5' frame 3 – C-term histag is vector encoded

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AAAGCTGGTTACCTCGAG

Translated protein sequence

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> lb-dera C42M

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Translated protein sequence

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> lb-dera E78K

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Translated protein sequence

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> lb-dera C42M E78K

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Translated protein sequence

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AGASRMGVSATVAILTGDDSHAKAGYLEHHHHHH

Table S3. Purification table *Lb*DERA C42M E78K

Sample	[protein] (mg/mL)	Activity (U/mL)	Volume (mL)	Specific Activity (U/mg)	Units Enzyme	mg Protein	Purification Factor	Recovery (%)
CFE	5.15	2.45	75.00	0.48	183.81	386.36	1	100.00
FT	2.04	1.09	70.00	0.53	76.34	143.11	1.12	41.53
8%B	5.14	3.46	2.50	0.67	8.66	12.88	1.41	4.71
20%B	7.16	4.92	2.50	0.69	12.30	17.90	1.44	6.69
100%B	7.34	5.02	2.50	0.68	12.56	18.34	1.44	6.83

Purification table of *Lb*DERA C42M E78K. Enzyme concentrations were measured with BCA assay. Activity of DERA was measured with coupled DR5P assay. CFE is cell free extract, FT is flow through fraction. Buffer A: 20 mM KPi, 100 mM NaCl, pH 7.4. Buffer B: 20 mM KPi, 500 mM imidazole, 100 mM NaCl, pH 7.4.

Table S4. Screening conversion of lactol to lactone

ADH	Yield Lactone (%) with NAD^+	Yield Lactone (%) with $NADP^+$
210	0	8
260	-	33
440	-	45
441	19	12
442	22	-
380	51	-
RAS	0	0
LB	0	0
LK	0	0

Screening of most active ADH for the conversion of lactol to lactone. 20 mM lactol, 5 mM NAD(P)+, 1 mg ADH, and 1 mg NOX were mixed in 0.2 mL 50 mM KPi pH 7.5 buffer. The reactions were incubated at 30 °C for 20 hours and analysed with GC.

Table S5 Conversion lactol to lactone ADH380, ADH440

ADH	Specific activity (U/mg)	Yield Lactone (%) with 1 unit ADH
380	204	10
440	1.2	46

Screening of most active ADH for the conversion of lactol to lactone. 20 mM lactol, 5 mM $NAD(P)^+$, 1 unit ADH, and 1 mg NOX were mixed in 0.2 mL 50 mM KPi pH 7.5 buffer. The reactions were incubated at 30 °C for 20 hours and analysed with GC.

Table S6. Cost analysis of the cofactor regeneration system

The cost for the regeneration systems within the reaction has been calculated. It is important to note that this cost reflects our expenses for the reaction and serves as an indication for the cost on laboratory scale. The calculations were based on the prices provided by the suppliers: FMN[1] and catalase[2] were purchased from Sigma Merck and NOX[3] from Prozomix.

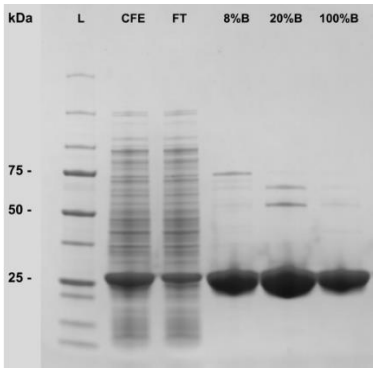
	Amount supplier (mg)	Price supplier (€)	Amount reaction (mg/mL)	Price reaction (€/mL)
NOX	1000	495.0	5	2.5
FMN	100	211	0.46	0.97
catalase	1000	54.90	0.0015	$80\ 10^{-6}$

[1] Sigma Merck, „Riboflavin 5′-monophosphate sodium salt hydrate," Sigma Aldrich Merck, 2023. [Online]. Available: https://www.sigmaaldrich.com/NL/en/product/sigma/f2253. 11 08 2023].

[2] Sigma Merck, „Catalase from bovine liber," Sigma Merck, [Online]. Available: https://www.sigmaaldrich.com/NL/en/product/sigma/c1345?gclid=CjwKCAiA1MCrBhAoEiwAC2d64RDCfajphL4AfeN1nXclU7_-AY0sY1MsE9se24nAb2bn9L5sU5Lf4BoCI5gQAvD_BwE. [06 12 2023].

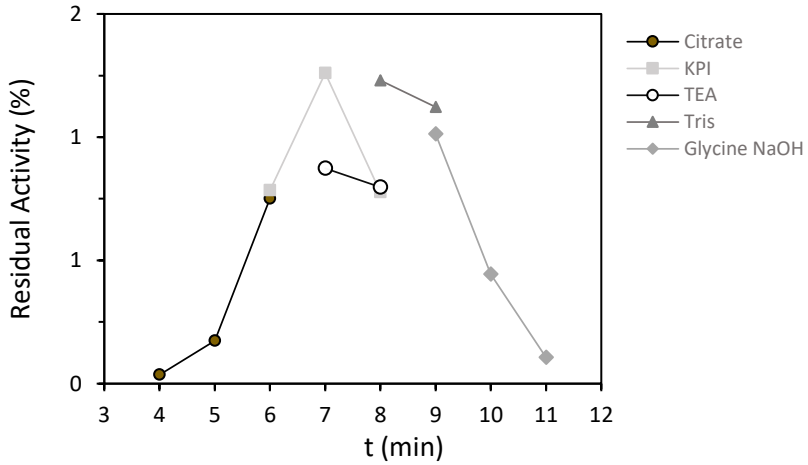
[3] Prozomix, „NAD(P)H Oxidase," Prozomix, 2023. [Online]. Available: http://www.prozomix.com/products/view?product=1892. [11 08 2023].

Figure S1. SDS PAGE *Lb*DERA C42M E78K



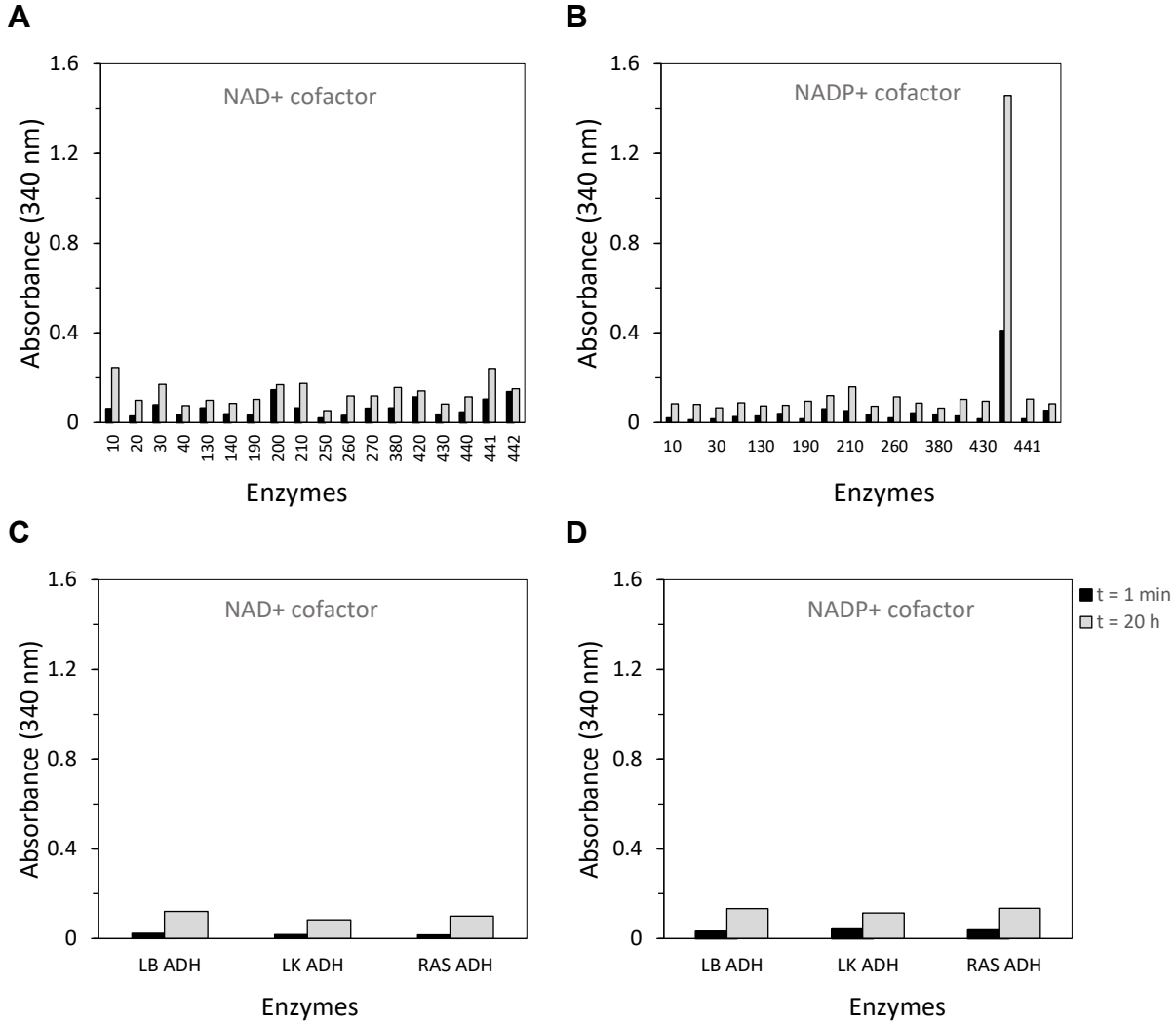
L: ladder, CFE: cell free extract, FT: flow through, 8%B: elution at 8% elution buffer, 20%B: elution at 20% elution buffer. 100%B: elution at 100% elution buffer. MW DERA: 25 kDa.

Figure S2. Buffer and pH profile of *Lb*DERA.



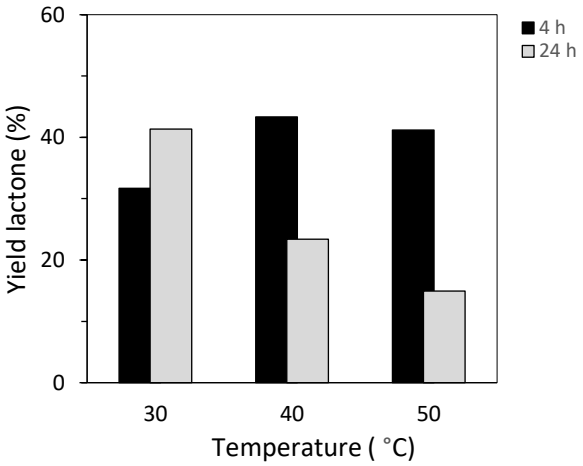
Overview of specific activity of *Lb*DERA at different pH values. The enzyme assay was performed using the standard assay procedure in the following buffers (100 mM): (1) citrate (pH 4.2, 5.0 and 6.2), (2) potassium phosphate (pH 6.2, 7.0 and 8.2), (3) Triethanolamine (pH 7.0, 8.0 and 8.3) and (4) Gly–NaOH (pH 8.6, 9.0, 10.0 and 10.6).

Figure S3. Screening ADH.



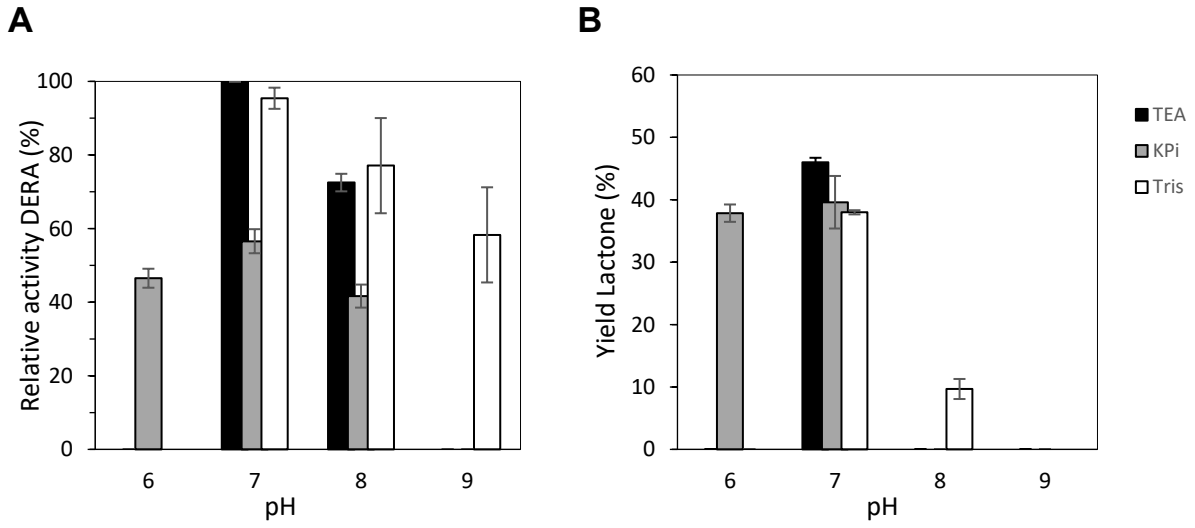
Activity assay for 18 different commercial ADH with NAD^+ (**A**) and $NADP^+$ (**B**) as cofactor. The reactions were performed in a UV 96-well microplate in 50 mM KPi pH 7 buffer with a total reaction volume of 200 µL. The reaction mixture contains 0.5 mg enzyme, 2 mM lactol, 1 mM $NAD(P)^+$. The blank measurement was done without enzyme. The absorbance value of the blank was subtracted from the other measurements. The samples were incubated at 30 C for one minute (left) and 20 hours (right) before measuring absorbance at 340 nm. And additional activity assay was performed for three ADH isolated and purified at our lab from the species *Ralstonia* (RAS), *Lactobacillus brevis* (LB), and *Lactobacillus kefir* (LK), again with NAD^+ (**C**) and $NADP^+$ (**D**) as cofactor.

Figure S4 Temperature profile ADH440.



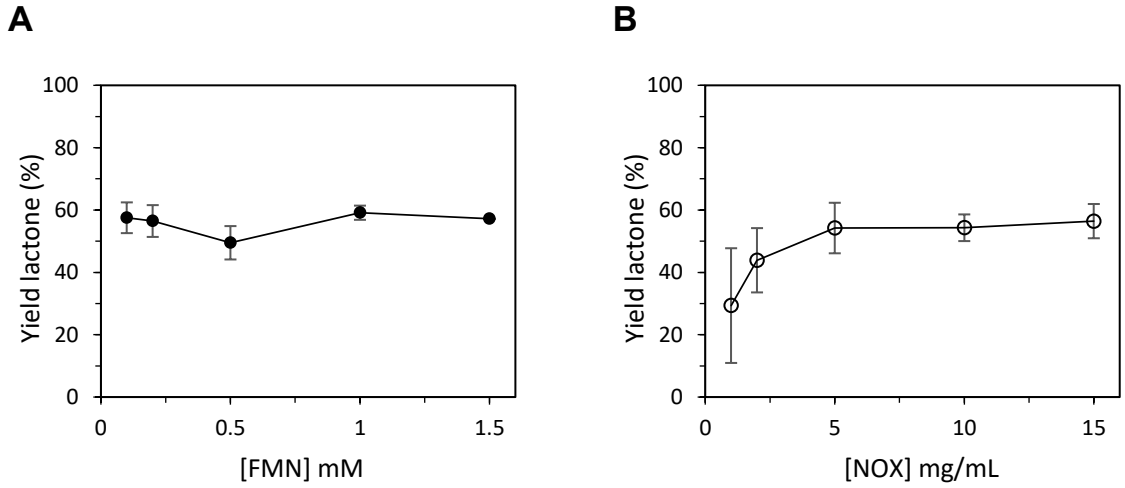
Temperature profile for the conversion of lactol to lactone by ADH440. Experimental conditions: 1 mL final volume in Eppendorf tubes, 800 rpm, 24hr on thermo shaker, substrate 20 mM, $NADP^+$ 5 mM, 440 ADH 1 mg, NOX 1 mg. Incubated at 30 °C, 40 °C and 50 °C Extraction with 2* 500 µL EtOAc. Conversions were measured by GC-FID.

Figure S5. Buffer and pH profile *Lb*DERA C42M E78K and ADH440.



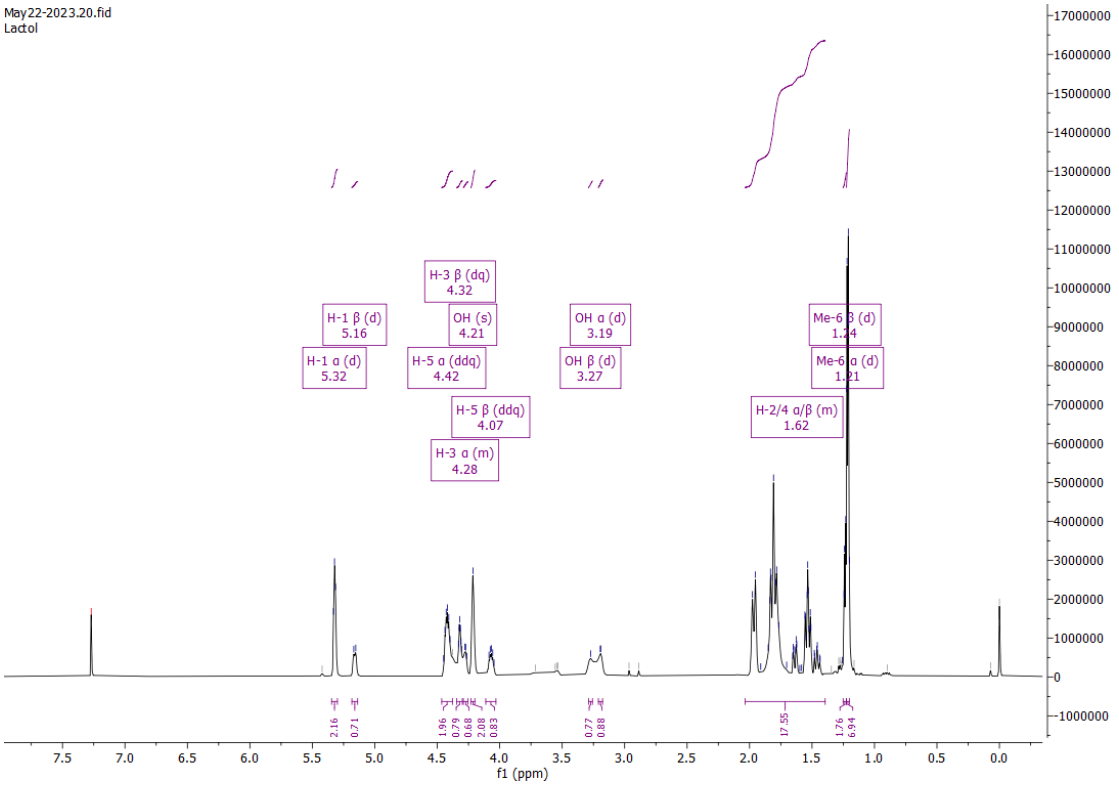
Buffer Assay *Lb*DERA C42M E78K and ADH440. 100 mM TEA pH 7/8, 100 mM Tris pH 7/8/9, and 100 mM KPi pH 6/7/8. **A.** Relative activity of DERA in different buffers. 0.4 mM DR5P, 0.2 mM NADH, 4U GDH/TPI, 10 µL DERA (3.8 U/mL) in different buffers. Absorbance at 340 nm detected. Activity measurements were done in duplicates. **B.** Conversion of lactol to lactone by ADH440. 10 mM lactol, 5 mM NADP+, 1 mg NOX, 1.2 U ADH440, 0.2 mL 30 °C, 800 rpm, 15h. Reactions were done in duplicates.

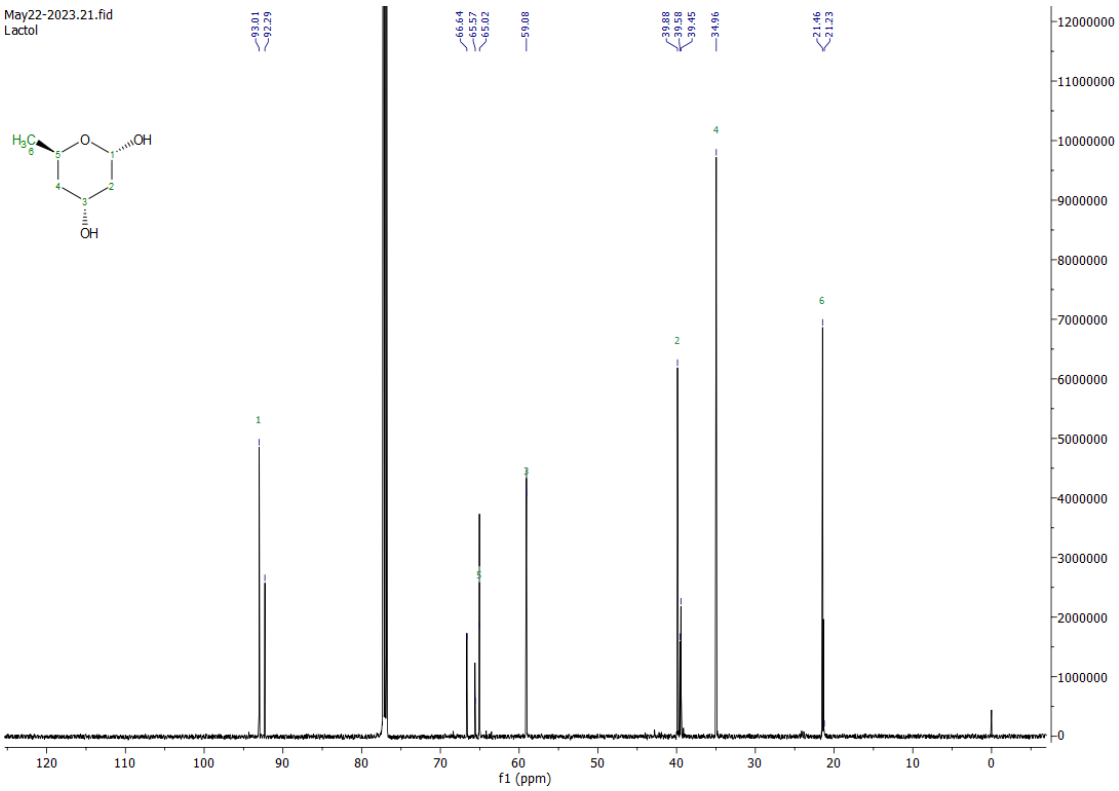
Figure S6. FMN and NOX concentration



Oxidation of lactol to lactone with ADH440 and regeneration system FMN and NOX for different concentrations of FMN and NOX. **A**. FMN. 27 mM crude lactol, 5 mM $NADP^+$, 0.1, 0.2, 0.5, 1, 1.5 mM FMN, 2.5 U ADH440 in 100 mM TEA pH 7.0, 800 rpm, 30 °C. Volume: 0.2 mL in 1,5 mL glass vials. **B**. NOX. 22 mM crude lactol, 5 mM $NADP^+$, 1, 2, 5, 10, 15 mg/mL NOX, 2.5 U ADH440 in 100 mM TEA pH 7.0, 800 rpm, 30 °C. Volume: 0.2 mL in 2 mL Eppendorf tubes. Reactions were done in duplicates.

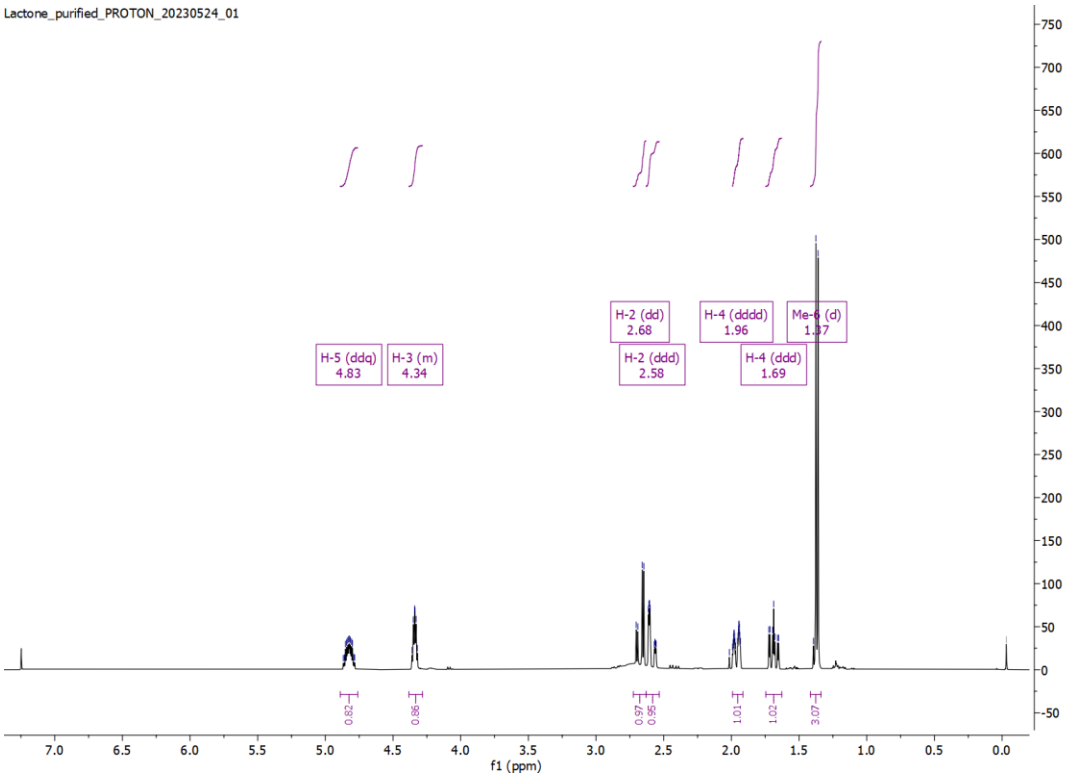
Figure S7. NMR lactol

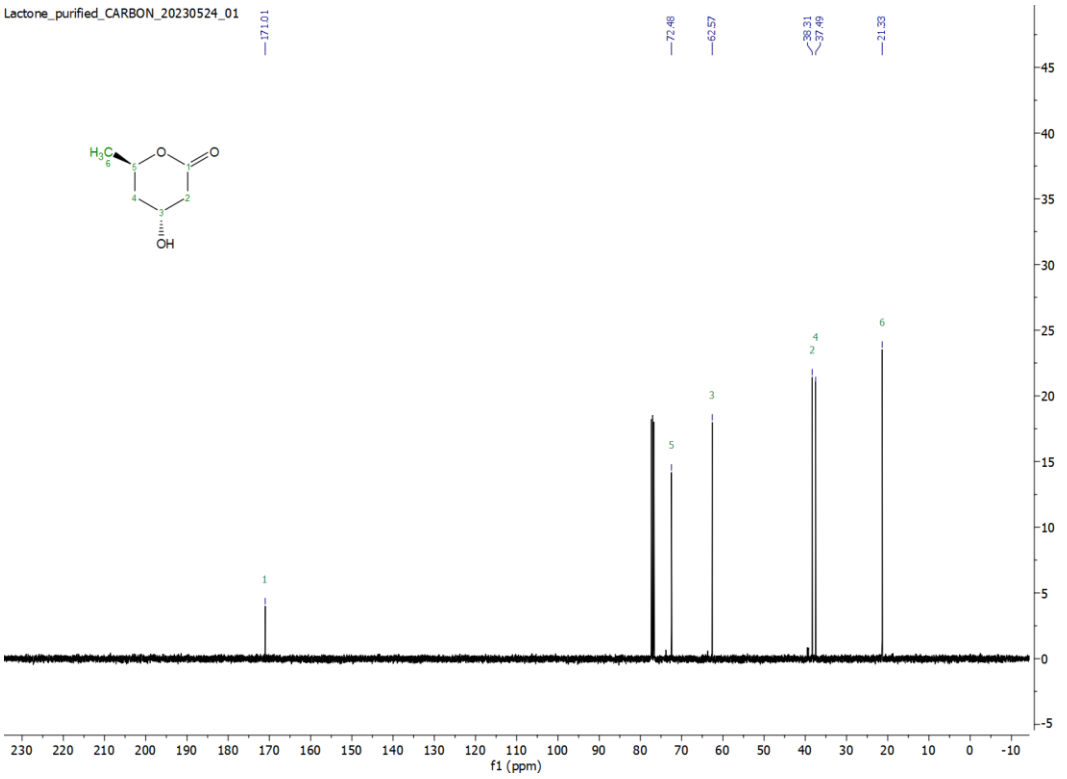




NMR spectra are in accordance with prior study: Gijsen, Harrie JM; Wong, Chi-Huey, "Unprecedented asymmetric aldol reactions with three aldehyde substrates catalyzed by 2-deoxyribose-5-phosphate aldolase," *Journal of the American Chemical Society,* vol. 116, no. 18, pp. 8422-8423, 1994.

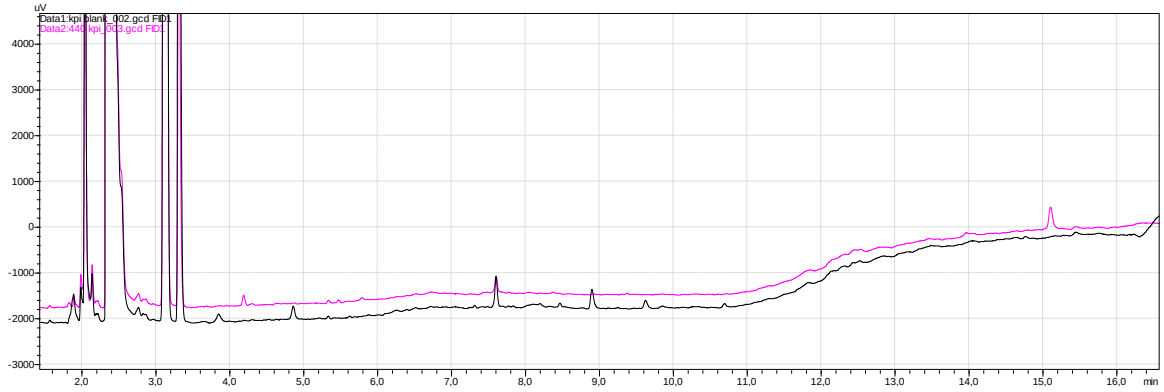
Figure S8. NMR lactone





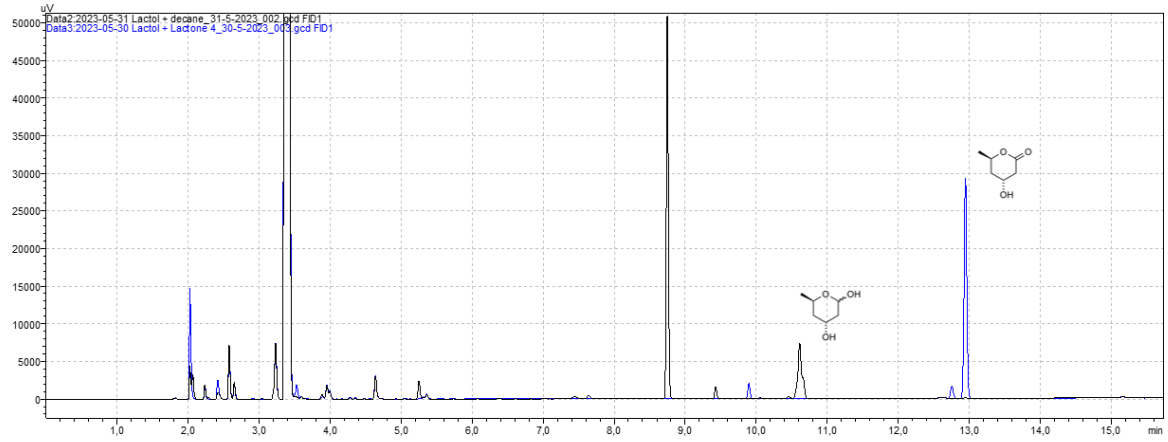
NMR spectra are in accordance with prior study: Gijsen, Harrie JM; Wong, Chi-Huey, "Unprecedented asymmetric aldol reactions with three aldehyde substrates catalyzed by 2-deoxyribose-5-phosphate aldolase," *Journal of the American Chemical Society,* vol. 116, no. 18, pp. 8422-8423, 1994.

Figure S9. GC analysis lactone screening



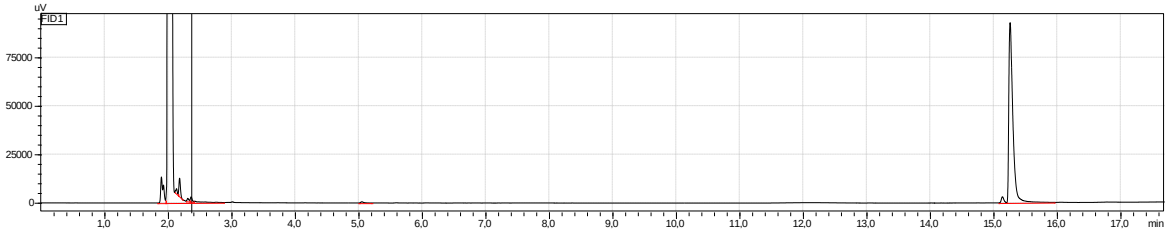
GC analysis for screening ADH. Lactol rt = 8.9 minutes (pink). Lactone rt = 15.1 minutes (black). 5 mM of decane was used as internal standard. 200 µL samples were extracted 3x with 1:1 ethyl acetate with 5 mM decane. Extraction was dried over $MgSO_4$ and subjected to GC.

Figure S10. GC analysis lactol and lactone



GC analysis of lactol rt = 10.6 minutes (black) and lactone rt = 12.9 minutes (blue). 5 mM of decane is used as internal standard (rt = 8.7 minutes). 200 µL samples were extracted 3x with 1:1 ethyl acetate with 5 mM decane. Extraction was dried over $MgSO_4$ and subjected to GC.

Figure S11. Chiral GC analysis lactone



Chiral GC analysis of lactone. 1 mg of pure lactone was dissolved in 1 mL ethyl acetate and subjected to GC.