Supplementary Information

Cascade enzymatic synthesis of L-homoserine – mathematical modelling as a tool for process optimisation and design

M. Česnik,[1] M. Sudar,[1] K. Hernández,[2] ~~P. Clapés,~~[2] S. Charnock[3], Đ. Vasić-Rački,[1] P. Clapés,[2] Z. Findrik Blažević[1,*]

[1]University of Zagreb, Faculty of Chemical Engineering and Technology, Savska c. 16, HR-10000 Zagreb, Croatia

[2] Institute of Advanced Chemistry of Catalonia, Biological Chemistry Department, Biotransformation and Bioactive Molecules Group, IQAC-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

[3] Prozomix Ltd, United Kingdom

[*]corresponding author, e-mail: zfindrik@fkit.hr, tel: +385 1 4597 157, fax: +385 1 4597 133

Contents

S1. The kinetics of TA 039-catalysed reaction

TA 039 catalyses an equilibrium reaction in which L-homoserine is synthesised by transamination of L-alanine to 4-hydroxy-2-oxobutanoate. A typical Michaelis-Menten dependence was obtained for the influence of L-alanine concentration on the specific activity (Fig. S1A). Mild substrate inhibition dependence was obtained for the influence of 4-hydroxy-2-oxobutanoate concentration on the specific enzyme activity (Fig. S1B). The influence of coenzyme PLP on the specific TA 039 activity can be described by the Michaelis-Menten equation (Fig. S1C). These results reveal that PLP concentrations above ca 1 mM should be used in the reactor to obtain the maximum TA 039 activity. The reaction products, i.e. L-homoserine and pyruvate, both inhibit the forward transamination reaction (Fig. S1D and E), as does formaldehyde; the reactant for YfaU 013 in the cascade system (Fig. S1F). Commercial formaldehyde contains up to 4.8 mol% of methanol for stabilisation, which can be a non-competitive inhibitor.[1] It was found that methanol mildly inhibits TA 039 (Fig. S1G). The estimated apparent kinetic parameters in the forward reaction are presented in Table S1.

The kinetics of the reverse reaction is presented in Figs. S1H-N. The influence of L-homoserine, pyruvate and PLP on the specific enzyme activity show typical Michaelis-Menten dependencies (Figs. S1H-J). Formaldehyde, L-alanine, 4-hydroxy-2-oxobutanoate and methanol inhibit the enzyme in the reverse reaction (Figs. S1K-N).

The estimated kinetic parameters for TA 039-catalysed reaction system (Table S1) show 2.9-fold higher maximum reaction rate for the unwanted reverse reaction (V_{m2}, Table S1), than for the forward reaction (V_{m1}, Table S1). This implies a poor position of the reaction equilibrium and the need to shift it by coupling the reaction with an efficient pyruvate recycling system. The estimated apparent Michaelis constants (Table S1) show higher affinity of the enzyme towards 4-hydroxy-2-oxobutanoate ($K_{m1,\ \text{4-hydroxy-2-oxobutanoate}}$, Table S1) and pyruvate ($K_{m2,\ \text{pyruvate}}$, Table S1) than towards L-alanine ($K_{m1,\ \text{L-alanine}}$, Table S1) and L-homoserine ($K_{m2,\ \text{L-homoserine}}$, Table S1). The substrate inhibition constant for ($K_{i1,\ \text{4-hydroxy-2-oxobutanoate}}$, Table S1) implies mild inhibition. However, accumulation of 4-hydroxy-2-oxobutanoate in the reactor will slow down the synthesis of L-homoserine. Thus, besides its consumption, it is very important to adjust the rate of its synthesis by adjusting the activity of YfaU 013. Even though the transamination products, pyruvate and L-homoserine inhibit the enzyme in the forward reaction, their inhibition is relatively mild according to the parameter values ($K_{i1,\ \text{pyruvate}}$ and $K_{i1,\ \text{L-homoserine}}$, Table S1). The same can be stated for the inhibition by methanol ($K_{i1,\ \text{methanol}}$,

Table S1), whereas this is not the case for formaldehyde ($K_{i1,\ \text{formaldehyde}}$, Table S1) which severely inhibits transamination reaction. The experimental data (Fig. S1F) show that even at formaldehyde concentrations in the range of 10 mM, a significant activity drop in the range of 90% can be expected. This indicates that only very low concentrations of formaldehyde should be present in the reactor, otherwise TA 039 activity will be too low, and it will not be possible to synthesise L-homoserine. L-Alanine, 4-hydroxy-2-oxobutanoate and methanol mildly inhibit the reverse reaction ($K_{i2,\ \text{L-alanine}}$, $K_{i2,\ \text{4-hydroxy-2-oxobutanoate}}$ and $K_{i2,\ \text{methanol}}$, Table S1), whereas formaldehyde acts as a more serious inhibitor ($K_{i2,\ \text{formaldehyde}}$, Table S1).

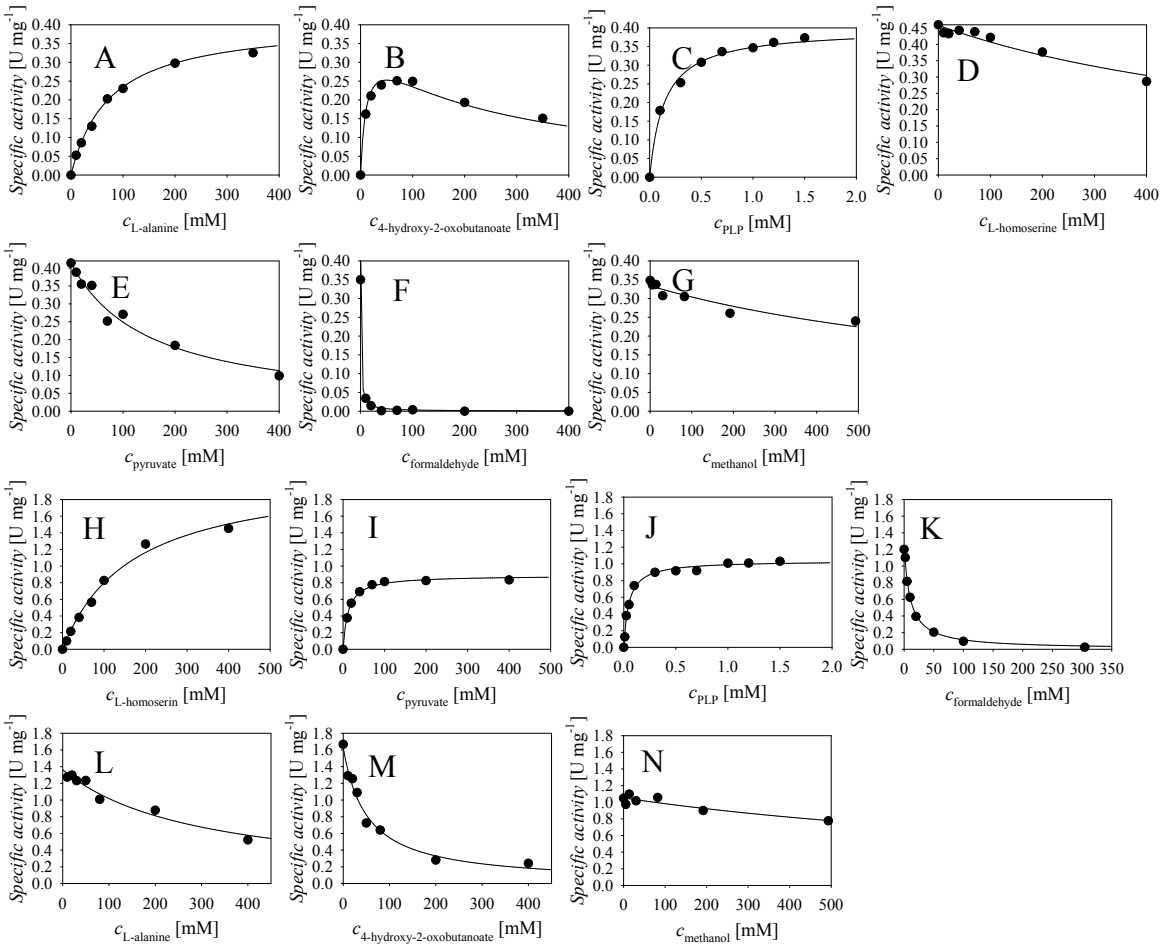

Figure S1 Dependence of TA 039 specific activity (50 mM sodium phosphate buffer pH 7.0, 25 °C, 1000 rpm, $\gamma_{\text{TA 039}}$ = 1 mg mL^{-1}, V_{reactor} = 0.5 mL) in the forward reaction on the concentration of **A**. L-alanine ($c_{\text{4-hydroxy-2-oxobutanoate}}$ = 100 mM, c_{PLP} = 1 mM), **B**. 4-hydroxy-2-oxobutanoate ($c_{\text{L-alanine}}$ = 100 mM, c_{PLP} = 1 mM), **C**. PLP ($c_{\text{L-alanine}}$ = 300 mM, $c_{\text{4-hydroxy-2-oxobutanoate}}$ = 40 mM), **D.** L-homoserine ($c_{\text{L-alanine}}$ = 300 mM, $c_{\text{4-hydroxy-2-oxobutanoate}}$ = 70 mM, c_{PLP} = 1 mM), **E.** pyruvate ($c_{\text{L-alanine}}$ = 300 mM, $c_{\text{4-hydroxy-2-oxobutanoate}}$ = 70 mM, c_{PLP} = 1 mM), **F.** formaldehyde ($c_{\text{L-alanine}}$ = 300 mM, $c_{\text{4-hydroxy-2-oxobutanoate}}$ = 70 mM, c_{PLP} = 1 mM), **G.** methanol ($c_{\text{L-alanine}}$ = 300 mM, $c_{\text{4-hydroxy-2-oxobutanoate}}$ = 70 mM, c_{PLP} = 1 mM). Dependence of TA 039 specific activity in the reverse reaction on the concentration of **H.** L-homoserine (c_{pyruvate} = 100 mM, c_{PLP} = 1 mM), **I.** pyruvate ($c_{\text{L-homoserine}}$ = 100 mM, c_{PLP} = 1 mM), **J.** PLP ($c_{\text{L-homoserine}}$ = 100 mM, c_{pyruvate} = 100 mM), **K.** formaldehyde ($c_{\text{L-homoserine}}$ = 300 mM, c_{pyruvate} = 100 mM, c_{PLP} = 1 mM), **L.** L-alanine ($c_{\text{L-homoserine}}$ = 300 mM, c_{pyruvate} = 100 mM, c_{PLP} = 1 mM), **M.** 4-hydroxy-2-oxobutanoate ($c_{\text{L-homoserine}}$ = 300 mM, c_{pyruvate} = 100 mM, c_{PLP} = 1 mM), **N.** methanol ($c_{\text{L-homoserine}}$ = 300 mM, c_{pyruvate} = 100 mM, c_{PLP} = 1 mM).

Table S1 Kinetic parameters of TA 039-catalysed equilibrium reaction

Parameter	Unit	Value
Forward reaction		
V_{m1}	U mg^{-1}	0.726 ± 0.037 (2.298)
$K_{m1,\ \text{L-alanine}}$	mM	75.186 ± 4.896
$K_{m1,\ \text{4-hydroxy-2-oxobutanoate}}$	mM	11.703 ± 1.865
$K_{m1,\ \text{PLP}}$	mM	0.141 ± 0.013
$K_{i1,\ \text{4-hydroxy-2-oxobutanoate}}$	mM	237.269 ± 35.297
$K_{i1,\ \text{L-homoserine}}$	mM	90.942 ± 10.310
$K_{i1,\ \text{pyruvate}}$	mM	30.177 ± 2.934
$K_{i1,\ \text{formaldehyde}}$	mM	0.156 ± 0.015
$K_{i1,\ \text{methanol}}$	mM	1021.619 ± 162.433
Reverse reaction		
V_{m2}	U mg^{-1}	2.137 ± 0.157 (0.2052)
$K_{m2,\ \text{L-homoserine}}$	mM	167.460 ± 26.085
$K_{m2,\ \text{pyruvate}}$	mM	12.145 ± 0.982
$K_{m2,\ \text{PLP}}$	mM	0.048 ± 0.004
$K_{i2,\ \text{formaldehyde}}$	mM	1.093 ± 0.091
$K_{i2,\ \text{L-alanine}}$	mM	31.899 ± 3.152
$K_{i2,\ \text{4-hydroxy-2-oxobutanoate}}$	mM	31.843 ± 2.738
$K_{i2,\ \text{methanol}}$	mM	1416.164 ± 281.911

S2. The kinetics of YfaU 013-catalysed reaction

YfaU 013 catalyses the aldol addition reaction of pyruvate to formaldehyde yielding 4-hydroxy-2-oxobutanoate necessary for the ensuing transamination reaction. Pyruvate formed during the reductive transamination between L-alanine and 4-hydroxy-2-oxobutanoate is recycled to the aldol addition and by consuming the pyruvate formed the reaction equilibrium of the transaminase-catalysed reaction is shifted towards the synthesis of L-homoserine. In comparison to the already published work on the synthesis of L-homoserine, which used completely purified version of MBP-YfaU,[2] YfaU 013 was chosen among the eight different YfaUs (Fig. S2) (all supplied as cell free extracts (CFE) by Prozomix, Ltd.) since it preserved its activity at high concentration of formaldehyde (up to 3 M) and had the highest value of the catalytic constant (Fig. S2). The kinetic parameters of MBP-YfaU used in the previous work[2] and of YfaU 013 used in this work were compared (Table S2). It was found that each enzyme is to some extent inhibited by formaldehyde and pyruvate. Since the affinity of YfaU 013 towards formaldehyde and pyruvate is satisfactory and considering previously mentioned superiority in activity and insensitivity towards formaldehyde, YfaU 013 was chosen as the most suitable aldolase for further research in this cascade system. Its catalytic constant was estimated to be 439.71 ± 75.51 min^{-1}, taking into account its molecular weight of 31.000 g mol^{-1}, while the catalytic constant of CFE MBP-YfaU used in previous work in purified form[2]

was estimated to be 227.78 ± 42.26 min^{-1}. The substrates, pyruvate and formaldehyde, both inhibit YfaU 013 (Figs. S2A and B); however, the estimated values of the kinetic constants (Table S2) show that this substrate inhibition is mild ($K_{i3,\ formaldehyde}$, $K_{i3,\ pyruvate}$, Table S2). Similarly to the reported MBP-YfaU,[2] YfaU 013 tolerates high concentrations of formaldehyde, even though its activity decreases with an increase of formaldehyde concentration.

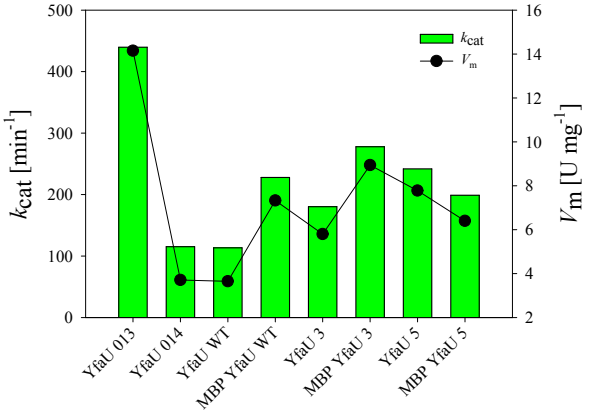

Figure S2 Comparison of different YfaU's tested for the reaction according to their k_{cat}.

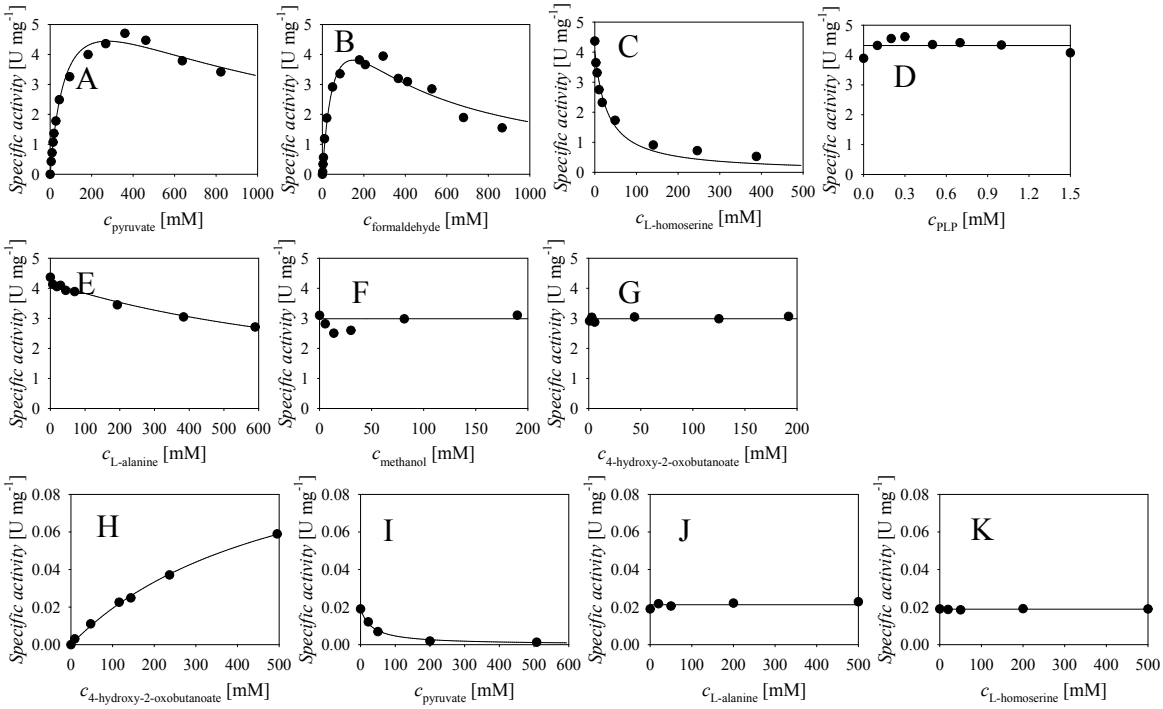

Figure S3 Dependence of YfaU 013 specific activity (50 mM sodium phosphate buffer pH 7.0, 25 °C, 1000 rpm, $\gamma_{YfaU\ 013}$ = 0.25 mg mL^{-1}, $V_{reactor}$ = 0.5 mL) on the concentration of **A.** formaldehyde ($c_{pyruvate}$ = 83.6 mM), **B.** pyruvate ($c_{formaldehyde}$ = 142.93 mM), **C.** L-homoserine ($c_{pyruvate}$ = 100 mM, $c_{formaldehyde}$ = 100 mM), **D.** PLP ($c_{pyruvate}$ = 100 mM, $c_{formaldehyde}$ = 100 mM), **E.** L-alanine ($c_{pyruvate}$ = 100 mM, $c_{formaldehyde}$ = 100 mM), **F.** methanol ($c_{pyruvate}$ = 100 mM, $c_{formaldehyde}$ = 100 mM) **G.** 4-hydroxy-2-oxobutanoate ($c_{pyruvate}$ = 100 mM, $c_{formaldehyde}$ = 100 mM) in the aldol addition, and the dependence of YfaU 013 specific activity in the retro-aldol reaction on the concentration of **H.** 4-hydroxy-2-oxobutanoate, **I.** pyruvate ($c_{4\text{-hydroxy-2-oxobutanoate}}$ = 100 mM), **J.** L-alanine ($c_{4\text{-hydroxy-2-oxobutanoate}}$ = 100 mM), **K.** L-homoserine ($c_{4\text{-hydroxy-2-oxobutanoate}}$ = 100 mM).

L-Homoserine and L-alanine present in the cascade reaction inhibit the aldol addition (Figs. S3C and E, $K_{i3,\ \text{L-homoserine}}$, $K_{i3,\ \text{L-alanine}}$, Table S2), whereas PLP, methanol and 4-hydroxy-2-oxobutanoate do not have a negative influence on the enzyme activity (Figs. S3D, F and G).

The retro-aldol reaction kinetics was also investigated. The results (Figs. S3H-K) show that the retro-aldol reaction occurs, but the estimated maximum reaction rate (V_{m4}, Table S2) is rather low in comparison to the maximum reaction rate of the aldol addition (V_{m3}, Table S2); i.e. 117-fold lower. Additionally, the apparent Michaelis constant for 4-hydroxy-2-oxobutanoate is quite high ($K_{m4,\ \text{4-hydroxy-2-oxobutanoate}}$, Table S2) and the retro-aldol reaction is inhibited by pyruvate (Fig. S3I), which means that this reaction should not be very significant.

Table S2 Kinetic parameters of YfaU 013-catalysed aldol addition of formaldehyde to pyruvate – 1st addition

Parameter	Unit	YfaU 013 - Value	MBP-YfaU - Value
Aldol addition			
V_{m3}	U mg^{-1}	14.158 ± 2.435 (38.264)	7.33 ± 1.36
$K_{m3,\ \text{formaldehyde}}$	mM	68.599 ± 17.956	18.99 ± 2.51
$K_{m3,\ \text{pyruvate}}$	mM	82.035 ± 16.444	209.18 ± 82.68
$K_{i3,\ \text{formaldehyde}}$	mM	318.278 ± 76.662	90.51 ± 11.64
$K_{i3,\ \text{pyruvate}}$	mM	933.246 ± 264.612	75.50 ± 16.63
$K_{i3,\ \text{L-alanine}}$	mM	426.055 ± 32.269	*n.a.*
$K_{i3,\ \text{L-homoserine}}$	mM	12.720 ± 2.430	*n.a.*
Retro-aldol reaction			
V_{m4}	U mg^{-1}	0.121 ± 0.008	*n.a.*
$K_{m4,\ \text{4-hydroxy-2-oxobutanoate}}$	mM	528.191 ± 56.354	*n.a.*
$K_{i4,\ \text{pyruvate}}$	mM	26.783 ± 3.346	*n.a.*

It is known that YfaU 013 can also catalyse the aldol addition of 4-hydroxy-2-oxobutanoate to a second equivalent of formaldehyde. Thus, this reaction was investigated in detail. Formaldehyde (Fig. S4A) inhibits the enzyme and the activity of YfaU 013 shows a linear dependence on the concentration of 4-hydroxy-2-oxobutanoate (Fig. S4B). This implies that this reaction can be controlled by the appropriate rate of 4-hydroxy-2-oxobutanoate consumption in the TA 039-catalysed reaction and its synthesis in the first aldol addition catalysed by YfaU 013. A mild inhibition by L-homoserine, L-alanine and pyruvate (Figs. S4C-E) was detected. All the estimated kinetic constants for the second aldol addition are presented in Table S3.

The kinetic data (Figs. S1, S3 and S4 and Tables S1-S3) clearly illustrate the complexity of this cascade system, in which a fine tuning of substrates/products concentration is necessary for a successful optimisation outcome. That is why a kinetic model is of crucial importance to minimize the lab effort necessary for achieving the optimal synthetic process.

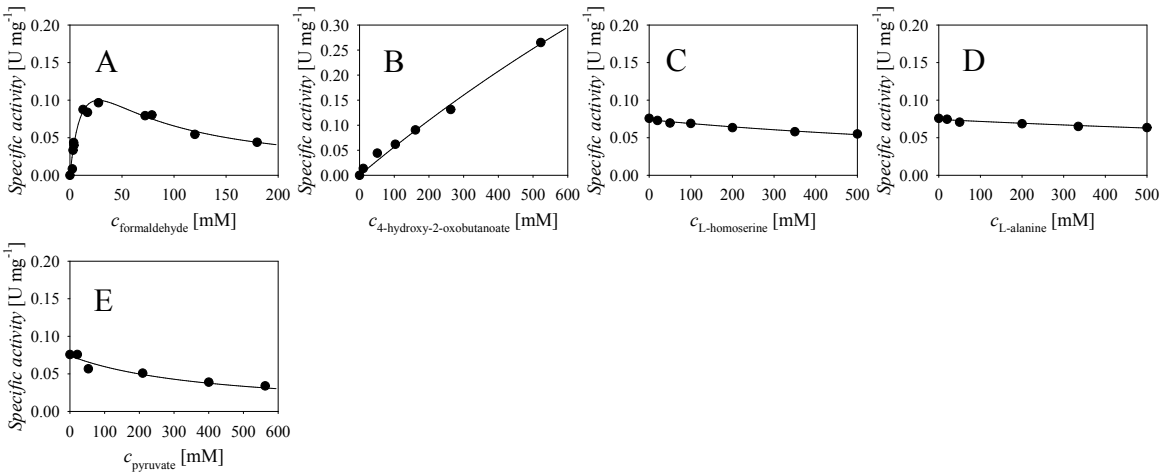

Figure S4 Kinetics of the 2nd aldol addition of formaldehyde to 4-hydroxy-2-oxobutanoate (50 mM sodium phosphate buffer pH 7.0, 25 °C, 1000 rpm, $\gamma_{\text{YfaU 013}}$ = 10 mg mL^{-1}, V_{reactor} = 0.3 mL). Dependence of YfaU 013 specific activity in the aldol addition on the concentration of **A**. formaldehyde ($c_{\text{4-hydroxy-2-oxobutanoate}}$ = 100 mM), **B.** 4-hydroxy-2-oxobutanoate ($c_{\text{formaldehyde}}$ = 100 mM), **C.** L-homoserine ($c_{\text{4-hydroxy-2-oxobutanoate}}$ = 100 mM, $c_{\text{formaldehyde}}$ = 50 mM), **D.** L-alanine ($c_{\text{4-hydroxy-2-oxobutanoate}}$ = 100 mM, $c_{\text{formaldehyde}}$ = 50 mM), **E.** pyruvate ($c_{\text{4-hydroxy-2-oxobutanoate}}$ = 100 mM, $c_{\text{formaldehyde}}$ = 50 mM).

Table S3 Kinetic parameters of YfaU 013-catalysed aldol addition of formaldehyde to 4-hydroxy-2-oxobutanoate – 2nd addition

Parameter	Unit	Value
Aldol addition		
k_5	U mg^{-1} mM^{-1}	0.000515 ± 0.000133
$K_{m5,\text{ formaldehyde}}$	mM	16.116 ± 4.003
$K_{i5,\text{ formaldehyde}}$	mM	46.666 ± 11.111
$K_{i5,\text{ L-homoserine}}$	mM	1363.229 ± 117.026
$K_{i5,\text{ L-alanine}}$	mM	2751.870 ± 380.778
$K_{i5,\text{ pyruvate}}$	mM	411.716 ± 130.031

S3. Instability of reaction compounds; i.e. 4-hydroxy-2-oxobutanoate and pyruvate

The stability of all compounds in the presence and the absence of enzymes was evaluated. It was found that 4-hydroxy-2-oxobutanoate slowly degrades (Fig. S5A). The reaction rate of this unspecific transformation (r_6, Eq. 7, Table 1a, main document) was described by the kinetics of the first order and was included in the mathematical model. The kinetic constant of the first order (k_6) was estimated to be $9.43 \cdot 10^{-5} \pm 1.15 \cdot 10^{-5}$ min^{-1}.

It was also found that pyruvate undergoes an unspecific transformation in the presence of YfaU 013 (Fig. S5B). As all enzymes were used as CFE, it is possible that other enzymes present in the mixture catalysed this transformation. The outcome of this transformation remains unknown. The reaction was described by the first order kinetics (r_7, Eq. 8, Table 1a, main document) multiplied by the concentration of YfaU 013. The kinetic constant k_7 was estimated to be $6.98 \cdot 10^{-5} \pm 6.50 \cdot 10^{-6}$ mL mg^{-1} min^{-1}. This unspecific transformation of

pyruvate is one of the reasons why the pyruvate recycling cannot be completely efficient which is discussed in the paper.

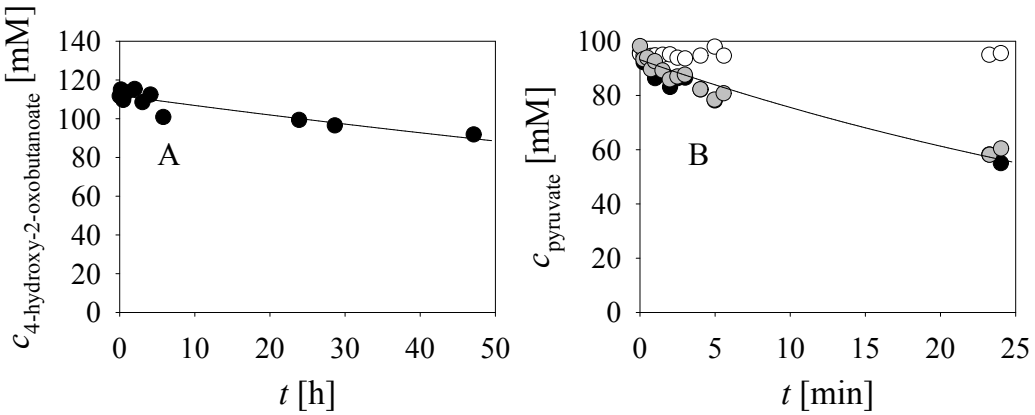

Figure S5 Stability (50 mM sodium phosphate buffer pH 7.0, 25 °C, 1000 rpm) of **A**. 4-hydroxy-2-oxobutanoate without the presence of enzymes, **B**. pyruvate; grey circles – in the presence of enzymes ($\gamma_{YfaU\ 013}$ = 5 mg mL^{-1}, $\gamma_{TA\ 039}$ = 5 mg mL^{-1}), white circles – in the presence of L-alanine without enzymes, black circles – in the presence of L-alanine without enzymes ($\gamma_{YfaU\ 013}$ = 5 mg mL^{-1}, $\gamma_{TA\ 039}$ = 5 mg mL^{-1}).

S4. The influence of formaldehyde on enzyme activity – enzyme stability during incubation with formaldehyde

TA 039 activity was measured in the reverse reaction. The reaction mixture of 325 μL volume containing 300 mM of L-homoserine, 300 mM of pyruvate, and 1 mM of coenzyme PLP was prepared for that purpose. The reaction was started by adding the sample from the incubation solution which contained specified concentration of formaldehyde and 25 mg mL^{-1} of enzyme. Sample (20 μL) withdrawn from the incubation solution to test the enzyme activity was prepared as follows: the 20 μL of solution was filtered through Amicon® Ultra filter unit to remove formaldehyde (0.5 mL vial, ultrafiltration unit with cut off 10 000 Da). The enzyme was resuspended in 175 μL of buffer and this solution was used to start the reaction (total reaction volume 500 μL, which gives an enzyme concentration in the assay of 1 mg mL^{-1}). The reaction was monitored by taking samples for 15 minutes. The initial reaction rate was calculated from the linear increase of 4-hydroxy-2-oxobutanoate concentration and used to calculate the specific enzyme activity (Eq. 1, main document).

To evaluate the effect of formaldehyde on YfaU 013, 15 mg mL^{-1} of YfaU 013 was incubated with 0, 11.4, 63.2 and 116.7 mM of formaldehyde (incubation solution). Samples (10 μL) were taken from the incubation solution to determine the enzyme activity. The enzyme activity was monitored during 24 hours of incubation by using the following assay: the reaction mixture (300 μL) contained 50 mM of formaldehyde and 50 mM of pyruvate. The reaction was started by adding the sample from the incubation solution which was prepared as follows: 10 μL of the incubation solution was filtered through Amicon® Ultra filter unit to

remove formaldehyde (0.5 mL vial, ultrafiltration unit with cut off 10 000 Da). The enzyme was resuspended in 200 μL of buffer and this solution was added to start the reaction (total reaction volume 500 μL). Thus, the enzyme concentration in the assay was 0.3 mg mL^{-1}. The reaction was monitored by taking samples for 15 minutes. The initial reaction rate was calculated from the linear increase of 4-hydroxy-2-oxobutanoate concentration and used to calculate the specific enzyme activity (Eq. 1, main document).

The effect of formaldehyde on this cascade system was observed by Hernandez et al.[2] where they assumed inhibitory or inactivation effect. In this work, both effects are experimentally confirmed. Fig. S6 presents the results of TA 039 (Fig. S6A) and YfaU 013 (Fig. S6B) specific activity drop during incubation at different concentration of formaldehyde. Even after a very short contact with formaldehyde both enzymes lose their initial activity, and as the concentration of formaldehyde increases, the activity drop is faster. The same can be expected in the reactor. Thus, besides being an inhibiting substrate for both enzymes, formaldehyde also inactivates the enzyme. When formaldehyde is absent from the solution, TA 039 shows better stability than YfaU 013 (Fig. S6). While TA 039 loses only 3-5% of its initial activity in the absence of formaldehyde after 24 hours, YfaU 013 loses nearly 25% of its initial activity. At formaldehyde concentration of ca 100 mM, both enzymes lose 79% of their initial activity after 24 hours. The similarity in enzyme activity loss depending on the concentration of formaldehyde can be observed at other concentrations as well. Hence, as expected the concentration of formaldehyde has to be minimized and well-adjusted during the cascade reaction.

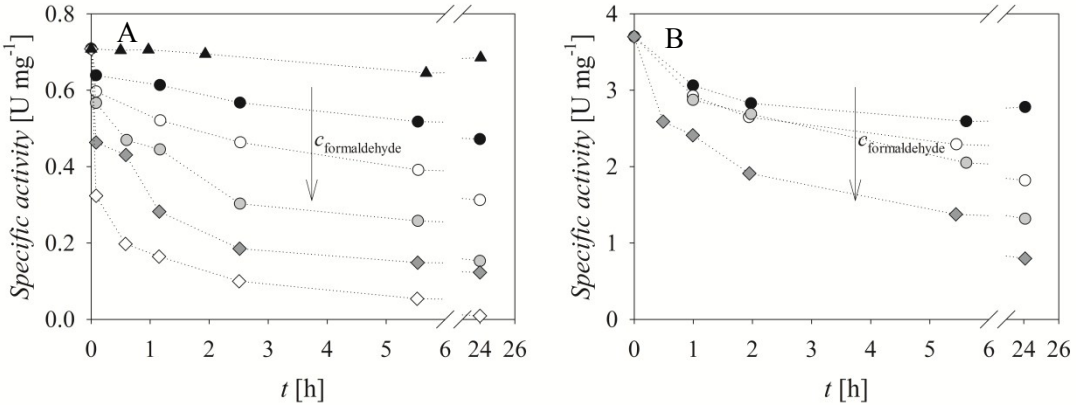

Figure S6 The influence of concentration of formaldehyde on the specific activity of **A**. TA 039 ($\gamma_{TA\ 039}$ = 25 mg mL^{-1}) and **B**. YfaU 013 ($\gamma_{YfaU\ 013}$ = 15 mg mL^{-1}) during incubation (50 mM sodium phosphate buffer pH 7.0, 25 °C, 1000 rpm). Legend: **A**. black triangles – $c_{formaldehyde}$ = 0 mM; black circles – $c_{formaldehyde}$ = 26.2 mM; white circles – $c_{formaldehyde}$ = 47.7 mM; grey circles – $c_{formaldehyde}$ = 94.7 mM; grey diamonds – $c_{formaldehyde}$ = 189.9 mM; white diamonds – $c_{formaldehyde}$ = 458.8 mM. **B**. black circles – $c_{formaldehyde}$ = 0 mM; white circles – $c_{formaldehyde}$ = 11.3 mM; grey circles – $c_{formaldehyde}$ = 63.2 mM; grey diamonds – $c_{formaldehyde}$ = 113.6 mM.

S5. Equilibrium constant of the transaminase-catalysed reaction

The simulated data at different initial reaction conditions (Fig. S7, Table S4) were used for the estimation of the equilibrium constant. Fig. S7B presents the calculation of the concentration quotients at the beginning and after 24 hours of simulation. Hyperbolic equation was used to describe this dependency and the obtained equation was used to calculate the value of K_{eq} at the point where Q_{24h}/Q_{0h} equals 1. K_{eq} was estimated to be 0.67 and it can be seen in Fig S7B that experimental, as well as simulated data, converge to this value. This only confirms the arguments of the position of the equilibrium for TA 039-catalysed reaction discussed earlier from the kinetic point of view (Chapter S1). Therefore, the recycling of pyruvate in the aldol addition reaction catalysed by YfaU 013 is needed to shift the equilibrium towards L-homoserine. Similar value of K_{eq} was experimentally determined by Hernandez et al.[2] for the transamination reaction between L-alanine and 4-hydroxy-2-oxobutanoate catalysed by transaminase TA 051, also from Prozomix library.

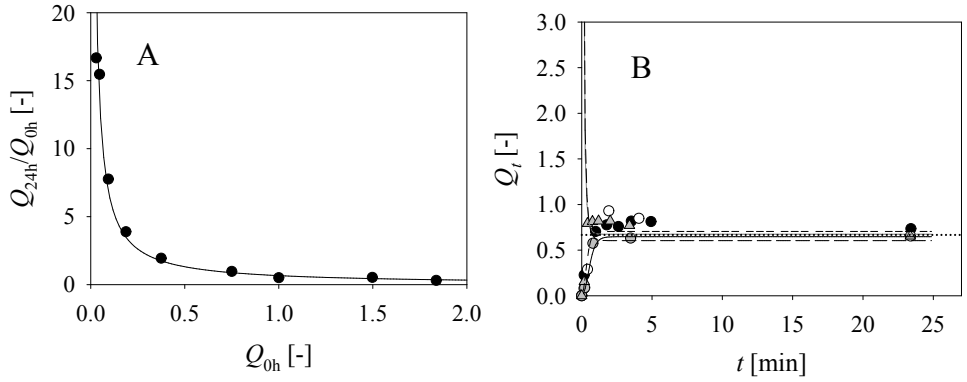

Figure S7 A. Model-based determination of K_{eq} for transamination of 4-hydroxy-2-oxobutanoate with L-alanine to yield L-homoserine. The simulations were done at different initial concentrations of reactants and products and set for 24 hours. Quotients Q_{24h}/Q_{0h} were plotted against Q_{0h}. Data were fitted to a hyperbolic equation $Q_{24h}/Q_{0h} = 0.6619 \cdot Q_{0h} - 0.992$, $R^2 = 0.9858$. **B**. Data that show convergence of the experimental and the model-based data towards the estimated $K_{eq} = 0.67$ (Legend: black circles – $c_{\text{L-alanine}} = 100$ mM, $c_{\text{4-hydroxy-2-oxobutanoate}} = 68$ mM, $c_{PLP} = 2$ mM, $\gamma_{\text{TA 039}} = 10$ mg mL^{-1}, short dash line – model simulation; white circles – Fig. 1B, black line - model simulation, grey circles - $c_{\text{L-alanine}} = 50.6$ mM, $c_{\text{4-hydroxy-2-oxobutanoate}} = 34.8$ mM, $c_{PLP} = 2$ mM, $\gamma_{\text{TA 039}} = 10$ mg mL^{-1}, grey triangles - $c_{\text{L-alanine}} = 282.0$ mM, $c_{\text{4-hydroxy-2-oxobutanoate}} = 100.0$ mM, $c_{PLP} = 2$ mM, $\gamma_{\text{TA 039}} = 10$ mg mL^{-1}, dark grey line – simulation Fig. 1C; long dash line – simulation Fig. 1D).

Table S4 The simulation data used to determine the value of the equilibrium constant (Fig. S7A).

	Initial conditions of the simulations					Conditions of the simulations after 24 h					
Sim No	$c_{\text{4-hydroxy-2-oxobutanoate}}$ [mM]	$c_{\text{L-alanine}}$ [mM]	$c_{\text{L-homoserine}}$ [mM]	c_{pyruvate} [mM]	Q_{0h}	$c_{\text{4-hydroxy-2-oxobutanoate}}$ [mM]	$c_{\text{L-alanine}}$ [mM]	$c_{\text{L-homoserine}}$ [mM]	c_{pyruvate} [mM]	Q_{24h}	Q_{24h}/Q_{0h}
1	250	600	220	20	0.029	152.28	502.28	317.72	117.72	0.489	16.671
2	70	300	492.2	2	0.047	51.45	281.45	510.75	20.55	0.725	15.466
3	60	250	351.6	4	0.094	43.76	233.76	367.84	20.24	0.728	7.761
4	50	200	234.4	8	0.188	37.35	187.35	247.05	20.65	0.729	3.887
5	40	150	140.6	16	0.375	32.88	142.88	147.72	23.12	0.727	1.939
6	30	100	70.3	32	0.750	30.35	100.35	69.95	31.65	0.727	0.969
7	100	600	100	600	1.000	128.67	628.67	71.33	571.33	0.504	0.504
8	20	50	23.4	64	1.498	25.01	55.01	18.39	58.99	0.789	0.527
9	35	27	56	31	1.837	45.22	37.22	45.78	20.78	0.565	0.308

S6. Explanation of the meaning of the statistical output presented in Table 2 [3]

R-squared is defined by the formula:

$$R^2 = \frac{\left| \sum_{i=1}^{n} w_i \cdot {Y_{obs_i}}^2 - \sum_{i=1}^{n} w_i \left(Y_{obs_i} - Y_{cal_i} \right)^2 \right|}{\sum_{i=1}^{n} w_i \cdot {Y_{obs_i}}^2}$$

where n is the number of points and w_i are the weights applied to each point. The expression is similar to that of the coefficient of determination, with the sum of the squares of the observed values occupying a similar position to that of the variance in the coefficient of determination formula.

The **coefficient of determination** is defined by the formula:

$$\text{Coefficient of determination} = \frac{\sum_{i=1}^{n} w_i \left(Y_{obs_i} - \bar{Y}_{obs} \right)^2 - \sum_{i=1}^{n} w_i \left(Y_{obs_i} - Y_{cal_i} \right)^2}{\sum_{i=1}^{n} w_i \left(Y_{obs_i} - \bar{Y}_{obs} \right)^2}$$

where n is the number of points, w_i are the weights applied to each point and $\bar{Y}_{obs}$ is the weighted mean of the observed data. The coefficient of determination is a measure of the fraction of the total variance accounted for by the model and is an appropriate measure of the goodness-of-fit.

The **correlation** between two variables X and Y is defined by the expression:

$$\text{Correlation} = \frac{\sum_{i=1}^{n} w_i \left(x_i - \bar{x} \right)\left(y_i - \bar{y} \right)}{\sqrt{\sum_{i=1}^{n} w_i \left(x_i - \bar{x} \right)^2} \sqrt{\sum_{i=1}^{n} w_i \left(y_i - \bar{y} \right)^2}}$$

where $\bar{x}$ and $\bar{y}$ are the weighted means of X and Y, n is the number of points, and wi are the weights applied to the points. This may be interpreted as an indication on how the changes in one variable are correlated with changes in the other. The value reported by SCIENTIST is the correlation between observed and calculated values of the dependent variables.

Model Selection Criterion (MSC) is defined by the following expression:

$$MSC = \ln\left(\frac{\sum_{i=1}^{n} w_i \left(Y_{obs_i} - \overline{Y}_{obs}\right)^2}{\sum_{i=1}^{n} w_i \left(Y_{obs_i} - \overline{Y}_{cal_i}\right)^2}\right) - \frac{2p}{n}$$

It is based on the modified Akaike Information Criterion (AIC). The original AIC is dependent on the magnitude of the data points, as well as the number of observations. The most appropriate model according to AIC is the one with the smallest value of AIC. SCIENTIST uses a modified AIC called MSC. The most appropriate model will be the one with the largest MSC.

References

1. M. Sudar, Đ. Vasić-Rački, M. Müller, A. Walter and Z. Findrik Blažević, *J. Biotechnol.*, 2018, **268**, 71-80.
2. K. Hernández, J. Bujons, J. Joglar, S. J. Charnock, P. Domínguez de María, W. D. Fessner and P. Clapés, *ACS Catal.*, 2017, **7**, 1707-1711.
3. *SCIENTIST handbook*, Micromath, Salt Lake City, 1986-1995.