Supporting information

Cascade synthesis of L-homoserine catalyzed by lyophilized whole cells containing transaminase and aldolase activity - the mathematical modeling approach.

Morana Česnik Katulić,[1] Martina Sudar,[1] Karel Hernández,[2] Yuyin Qi,[3] Simon J. Charnock,[3] Đurdica Vasić-Rački,[1] Pere Clapés,[2] Zvjezdana 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, Biotransformation and Bioactive Molecules Group, IQAC-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

[3] Prozomix Ltd, West End Ind. Estate, Station Court, Haltwhistle, Northumberland, NE49 9HA, United Kingdom

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

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S0. Introduction

Kinetics of whole cell biocatalyst YfaU(013)/PRO TRANS(039) was determined for each reaction step, i.e. aldol addition catalyzed by YfaU 013 and transamination catalyzed by TA 039 as well as for their corresponding reverse reactions. Based on the kinetic measurements conducted with enzymes provided as cell free extract (CFE) reported in our previous work,[1] some kinetic measurements were excluded from this work. Such was the influence of PLP concentration on the activity of TA 039 because cells naturally contain PLP, and it was not added to the cascade reaction or during determination of enzyme kinetics. It was assumed that the concentration of PLP essential for the cascade was constant over the course of reaction. Kinetic measurements for compounds that did not exhibit significant influence on enzyme activity in our previous work were also omitted. This was the case of methanol, present as formaldehyde stabilizer, as inhibitor of enzymes. Similar results were expected for both forms of biocatalyst (CFE enzymes and co-expressed enzymes within the cells) as the same enzymes were used in both cases.

S1. The kinetics of transaminase-catalyzed reaction

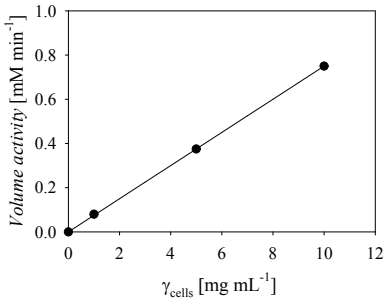

Fig S.1 The effect of cell concentration on the volume activity of TA 039 (50 mM sodium phosphate buffer pH 7.0, 25 °C, $c_{\text{L-alanine}}$ = 100 mM, $c_{\text{4-hydroxy-2-oxobutanoate}}$ = 100 mM, V_{reactor} = 1 mL).

The effect of cell concentration on volume activity of transaminase within the cells was measured (Fig S.1) to determine the optimal concentration for the kinetic measurements which was found to be 5 mg mL^{-1}. This was enough to keep the conversion below 10% during the initial reaction rate experiments. The freeze-dried cells were always freshly thawed before use.

Figure S.2 presents the results of the kinetics of the reductive transamination of 4-hydroxy-2-oxobutanoate by L-alanine catalyzed by TA 039 in lyophilized cells. The estimated apparent kinetic parameters are shown in Table S.1 and are compared to the values of kinetic constants obtained for the TA 039 CFE.[1] It can be observed that cells show higher affinity towards substrate L-alanine ($K_{m1,\ \text{L-alanine}}$) and lower towards substrate 4-hydroxy-2-oxobutanoate ($K_{m1,\ \text{4-hydroxy-2-oxobutanoate}}$) than CFE. Also, there is no substrate-inhibiting effect (with 4-hydroxy-2-oxobutanoate) within the cells observed with TA 039 CFE. A product-inhibiting effect of pyruvate ($K_{i1,\ \text{pyruvate}}$) is comparable in both cases, while inhibition with formaldehyde is higher within the cells ($K_{i1,\ \text{formaldehyde}}$).

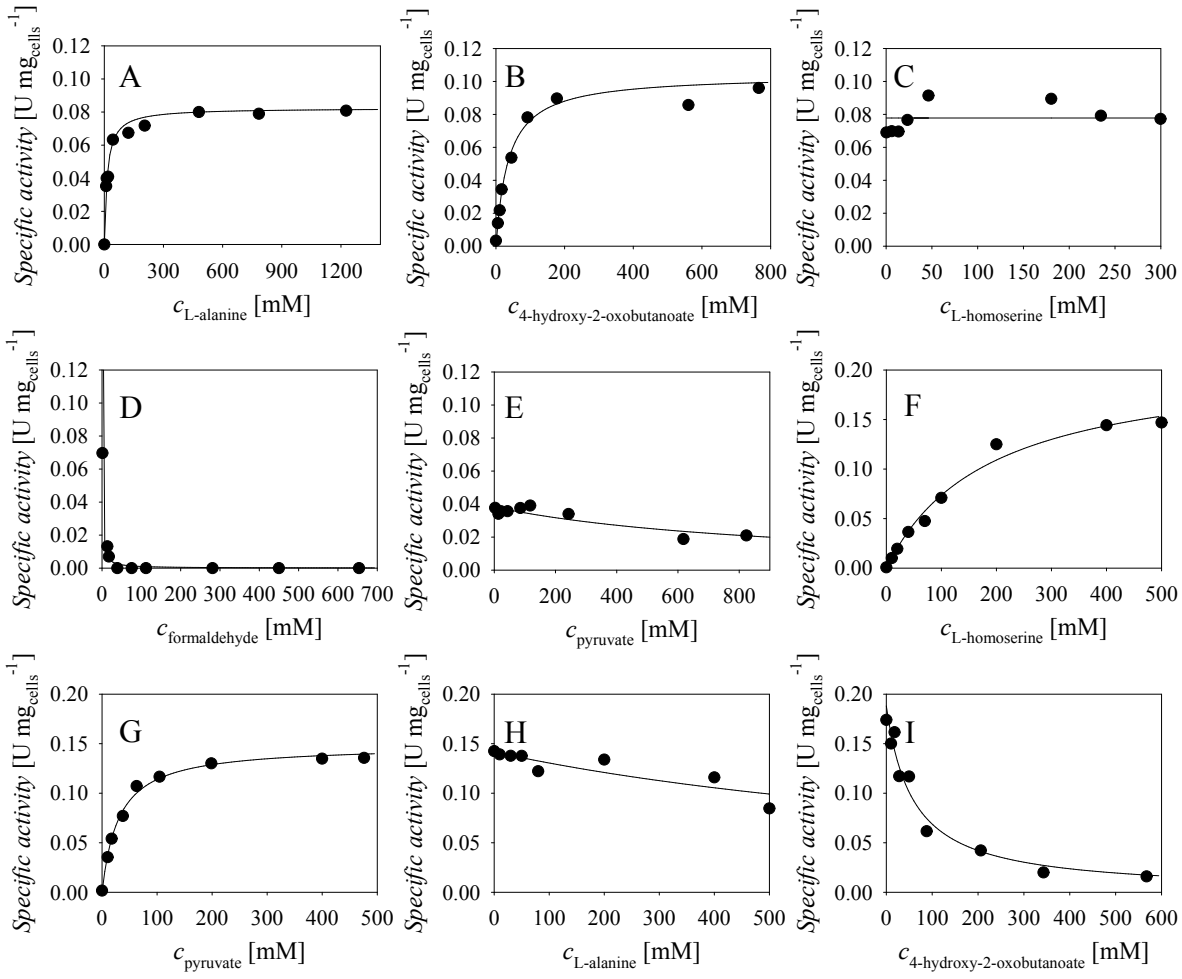

Figure S.2 A.-E. Kinetics of reductive transamination of 4-hydroxy-2-oxobutanoate by L-alanine catalyzed by TA 039 in cells (50 mM sodium phosphate buffer pH 7.0, 25 °C, γ_{cells} = 5 mg mL^{-1}, $V_{reactor}$ = 1 mL) on the concentration of **A**. L-alanine ($c_{\text{4-hydroxy-2-oxobutanoate}}$ = 100 mM), **B**. 4-hydroxy-2-oxobutanoate ($c_{\text{L-alanine}}$ = 100 mM), **C**. L-homoserine ($c_{\text{L-alanine}}$ = 300 mM, $c_{\text{4-hydroxy-2-oxobutanoate}}$ = 100 mM), **D**. formaldehyde ($c_{\text{L-alanine}}$ = 300 mM, $c_{\text{4-hydroxy-2-oxobutanoate}}$ = 100 mM), **E**. pyruvate ($c_{\text{L-alanine}}$ = 300 mM, $c_{\text{4-hydroxy-2-oxobutanoate}}$ = 100 mM). **F.-I.** Kinetics of reverse transamination catalyzed by TA 039 in cells (50 mM sodium phosphate buffer pH 7.0, 25 °C, γ_{cells} = 5 mg

mL^{-1}, $V_{reactor}$ = 1 mL) on the concentration of **F**. L-homoserine ($c_{pyruvate}$ = 200 mM), **G**. pyruvate ($c_{L\text{-homoserine}}$ = 200 mM), **H**. L-alanine ($c_{L\text{-homoserine}}$ = 200 mM, $c_{pyruvate}$= 200 mM), **I**. 4-hydroxy-2-oxobutanoate ($c_{L\text{-homoserine}}$ = 200 mM, $c_{pyruvate}$ = 200 mM). Legend: black circles – experimental data, line – model.

Table S.1 Kinetic parameters for transamination of L-alanine and pyruvate catalyzed by TA 039 in cells.

Parameter	Unit	Value cells	Value CFE[1]
Reductive transamination of 4-hydroxy-2-oxobutanoate by L-alanine			
V_{m1}	U mg_{cells}^{-1}	0.104 ± 0.003 (0.159)*	0.726 ± 0.037 (2.298)*
$K_{m1,\ 4\text{-hydroxy-2-oxobutanoate}}$	mM	36.119 ± 3.901	11.703 ± 1.865
$K_{i1,\ 4\text{-hydroxy-2-oxobutanoate}}$	mM	No inhibition	237.269 ± 35.297
$K_{m1,\ L\text{-alanine}}$	mM	14.656 ± 2.130	75.186 ± 4.896
$K_{i1,\ formaldehyde}$	mM	0.018 ± 0.001	0.156 ± 0.015
$K_{i1,\ pyruvate}$	mM	45.775 ± 9.503	30.177 ± 2.934
$K_{m1,\ PLP}$	mM	*n.a.*	0.141 ± 0.013
$K_{i1,\ L\text{-homoserine}}$	mM	No inhibition	90.942 ± 10.310
$K_{i1,\ methanol}$	mM	*n.a.*	1021.619 ± 162.433
Reverse reaction			
V_{m2}	U mg_{cells}^{-1}	0.211 ± 0.017 (0.552)*	2.137 ± 0.157 (0.2052)*
$K_{m2,\ L\text{-homoserine}}$	mM	185.968 ± 34.574	167.460 ± 26.085
$K_{m2,\ pyruvate}$	mM	30.279 ± 2.850	12.145 ± 0.982
$K_{i2,\ L\text{-alanine}}$	mM	561.628 ± 15.427	31.899 ± 3.152
$K_{i2,\ 4\text{-hydroxy-2-oxobutanoate}}$	mM	7.595 ± 0.935	31.843 ± 2.738
$K_{m2,\ PLP}$	mM	*n.a.*	0.048 ± 0.004
$K_{i2,\ methanol}$	mM	*n.a.*	1416.164 ± 281.911
$K_{i2,\ formaldehyde}$	mM	*n.a.*	1.093 ± 0.091

*The kinetic parameters in the brackets were re-estimated from the fed-batch experiments

As the reverse reaction is concerned it can be observed that both forms of biocatalyst show similar affinity towards L-homoserine ($K_{m2,\ L\text{-homoserine}}$), while the affinity for pyruvate is somewhat better for free enzyme ($K_{m2,\ pyruvate}$). Inhibition of the reverse reaction by L-alanine is very low in case of lyophilized whole cells ($K_{i2,\ L\text{-alanine}}$), which was not the case for TA 039 provided as CFE. The opposite was observed with the inhibition of the reverse reaction by 4-hydroxy-2-oxobutanoate where stronger effect was observed for the whole cells ($K_{i2,\ 4\text{-hydroxy-2-oxobutanoate}}$) than for the free enzyme. The influence of formaldehyde on the reverse reaction could not be measured because it quickly reacts with pyruvate in the YfaU 013-catalyzed reaction. As pyruvate reacts with L-homoserine in a reverse TA 039-catalyzed reaction forming 4-hydroxy-2-oxobutanoate, L-alanine is not forming as fast. Thus, the measured reaction rates were not relevant. It was assumed that similar effect of formaldehyde can be expected as with Yfau 013 CFE, and the constant was taken from the enzymatic system.

S2. The kinetics of YfaU 013-catalyzed reaction

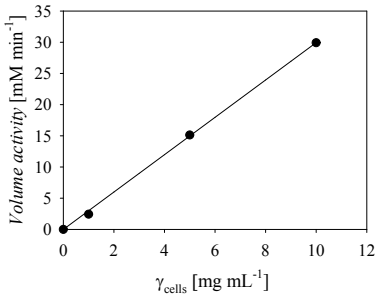

Fig S.3 The effect of cell concentration on the volume activity of YfaU 013 (50 mM sodium phosphate buffer pH 7.0, 25 °C, $c_{formaldehyde}$ = 100 mM, $c_{pyruvate}$ = 100 mM, $V_{reactor}$ = 1 mL).

The effect of cell concentration on volume activity was measured (Fig S.3) to determine the optimal concentration for the kinetic measurements. It was found that the cells contain high YfaU activity, and that the concentration of 0.2 mg mL^{-1} was enough to keep the conversion below 10% during the fast initial reaction rate experiments. The freeze-dried cells were always freshly thawed before use.

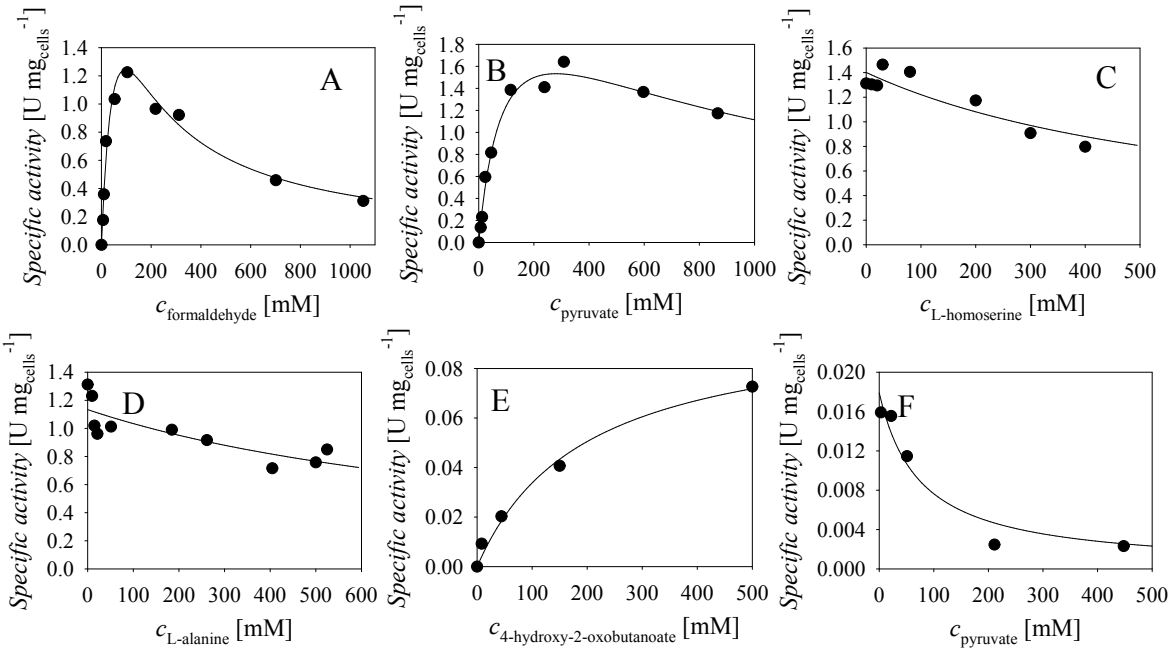

Figure S.4 A.-D. Kinetics of the aldol addition catalyzed by YfaU 013 within lyophilized whole cells (50 mM sodium phosphate buffer pH 7.0, 25 °C, γ_{cells} = 0.2 mg mL^{-1}, $V_{reactor}$ = 1 mL) on the concentration of **A**. formaldehyde ($c_{pyruvate}$ = 100 mM), **B**. pyruvate ($c_{formaldehyde}$ = 100 mM), **C**. L-homoserine ($c_{pyruvate}$ = 200 mM, $c_{formaldehyde}$ = 100 mM), **D**. L-alanine ($c_{pyruvate}$ = 200 mM, $c_{formaldehyde}$ = 100 mM). **E.-F.** Kinetics of the reverse aldol reaction catalyzed by YfaU 013 in whole cells (50 mM sodium phosphate buffer pH 7.0, 25 °C, γ_{cells} = 10 mg mL^{-1}, $V_{reactor}$ = 1 mL) on the concentration of **E**. 4-hydroxy-2-oxobutanoate, **F**. pyruvate ($c_{4\text{-hydroxy-2-oxobutanoate}}$ = 100 mM). Legend: black circles – experimental data, line – model.

Kinetics of the aldol addition of formaldehyde and pyruvate catalyzed by YfaU 013 in lyophilized cells is presented in Fig. S.4A-D and the apparent estimated kinetic parameters are shown in Table S.2. They are compared with the values obtained for YfaU 013 as CFE. The apparent affinity of the enzyme towards formaldehyde and pyruvate in both cases is the same, which can be seen from the similar K_m values for formaldehyde and pyruvate. Both substrates inhibit the enzyme within the cells as well as in the case of CFE. Inhibition is very similar for both biocatalysts in case of pyruvate ($K_{i3,\ \text{pyruvate}}$), while inhibition with formaldehyde is stronger for lyophilized whole cells ($K_{i3,\ \text{formaldehyde}}$). Comparison of $K_{i3,\ \text{L-homoserine}}$ shows that there is a stronger inhibiting effect of L-homoserine on YfaU 013 in the reaction catalyzed by CFE enzyme. The inhibition by 4-hydroxy-2-oxobutanoate, methanol and PLP was not evaluated while it was expected that the result would be the same as with CFE of YfaU 013, i.e., no inhibition. Kinetics of the retro-aldol reaction catalyzed by YfaU 013 within the cells is presented in Fig. S.4E and F and estimated apparent kinetic parameters are shown in Table S.2. The apparent affinity of YfaU 013 within the cells towards 4-hydroxy-2-oxobutanoate is better than for CFE of YfaU 013 ($K_{m4,\ \text{4-hydroxy-2-oxobutanoate}}$), and in both cases the enzyme is inhibited by pyruvate ($K_{i4,\ \text{pyruvate}}$). The effect of L-alanine and L-homoserine on the reaction rate of reverse reaction was not evaluated while no inhibition was found for CFE Yfau 013.

Table S.2 Kinetic parameters for the aldol addition of formaldehyde and pyruvate catalyzed by YfaU 013 cells.

Parameter	**Unit**	**Value cells**	**Value CFE [1]**
Aldol addition			
V_{m3}	U mg_{cells}^{-1}	6.379 ± 1.514 (99.744)*	14.158 ± 2.435 (38.264)*
$K_{m3,\ \text{formaldehyde}}$	mM	70.963 ± 23.183	68.599 ± 17.956
$K_{m3,\ \text{pyruvate}}$	mM	95.751 ± 22.823	82.035 ± 16.444
$K_{i3,\ \text{formaldehyde}}$	mM	131.186 ± 41.999	318.278 ± 76.662
$K_{i3,\ \text{pyruvate}}$	mM	818.800 ± 251.327	933.246 ± 264.612
$K_{i3,\ \text{L-homoserine}}$	mM	83.444 ± 3.825	12.720 ± 2.430
$K_{i3,\ \text{L-alanine}}$	mM	289.440 ± 77.841	426.055 ± 32.269
Retro-aldol reaction			
V_{m4}	U mg_{cells}^{-1}	0.100 ± 0.010 (0.057)*	0.121 ± 0.008
$K_{m4,\ \text{4-hydroxy-2-oxobutanoate}}$	mM	196.062 ± 50.654	528.191 ± 56.354
$K_{i4,\ \text{pyruvate}}$	mM	49.386 ± 19.532	26.783 ± 3.346
2nd aldol addition			

k_5	U mg^{-1} mM^{-1}	*n.a.*	0.000515 ± 0.000133
$K_{m5,\ formaldehyde}$	mM	*n.a.*	16.116 ± 4.003
$K_{i5,\ formaldehyde}$	mM	*n.a.*	46.666 ± 11.111
$K_{i5,\ L\text{-}homoserine}$	mM	*n.a.*	1363.229 ± 117.026
$K_{i5,\ L\text{-}alanine}$	mM	*n.a.*	2751.870 ± 380.778
$K_{i5,\ pyruvate}$	mM	*n.a.*	411.716 ± 130.031

*The kinetic parameters in the brackets were re-estimated from the fed-batch experiments

The second aldol addition catalyzed by Yfau 013 within cells was not investigated, while it was expected that this reaction will not have a significant influence on the cascade reaction outcome since this was the case with CFE enzymes. However, the reaction was not neglected in the model, and the value of the constants were adopted to the mathematical model of the whole cell system.

S.3 Stability of reaction components during incubation without and with cells

The stability of 4-hydroxy-2-oxobutanoate was evaluated in the previous work [1] and it was estimated that its unspecific chemical transformation can be described by the kinetics of the first order and a kinetic constant $9.43 \cdot 10^{-5} \pm 1.15 \cdot 10^{-5}$ min^{-1}. It was expected that certain side-reactions in this system would occur, considering that the substrate such as pyruvate is an important metabolite in the cells. That is why we evaluated the activity of cells during incubation with different compounds; L-homoserine, L-alanine, pyruvate. In the absence of cells all these compounds were found stable. Competent cells YfaU(013)/PRO TRANS(039) (50 mg mL^{-1}) were incubated for three days in the presence of different concentrations of L-alanine, L-homoserine and pyruvate separately. The results have shown that the cells consume L-homoserine at a rate, which depends on its concentration. That is why further analysis was done investigating the initial reaction rates, i.e., specific activities. Figure S.5A shows that the dependence of the specific enzyme activity on the L-homoserine concentration can be simulated by the Michaelis-Menten kinetics. For L-alanine it was found that the specific activity of the cells vs L-alanine shows typical substrate inhibition kinetics (Fig. S.5B). The influence of

pyruvate concentration on the specific activity can be described by the Michaelis-Menten kinetics. The apparent values of the estimated kinetic parameters are presented in Table S.3.

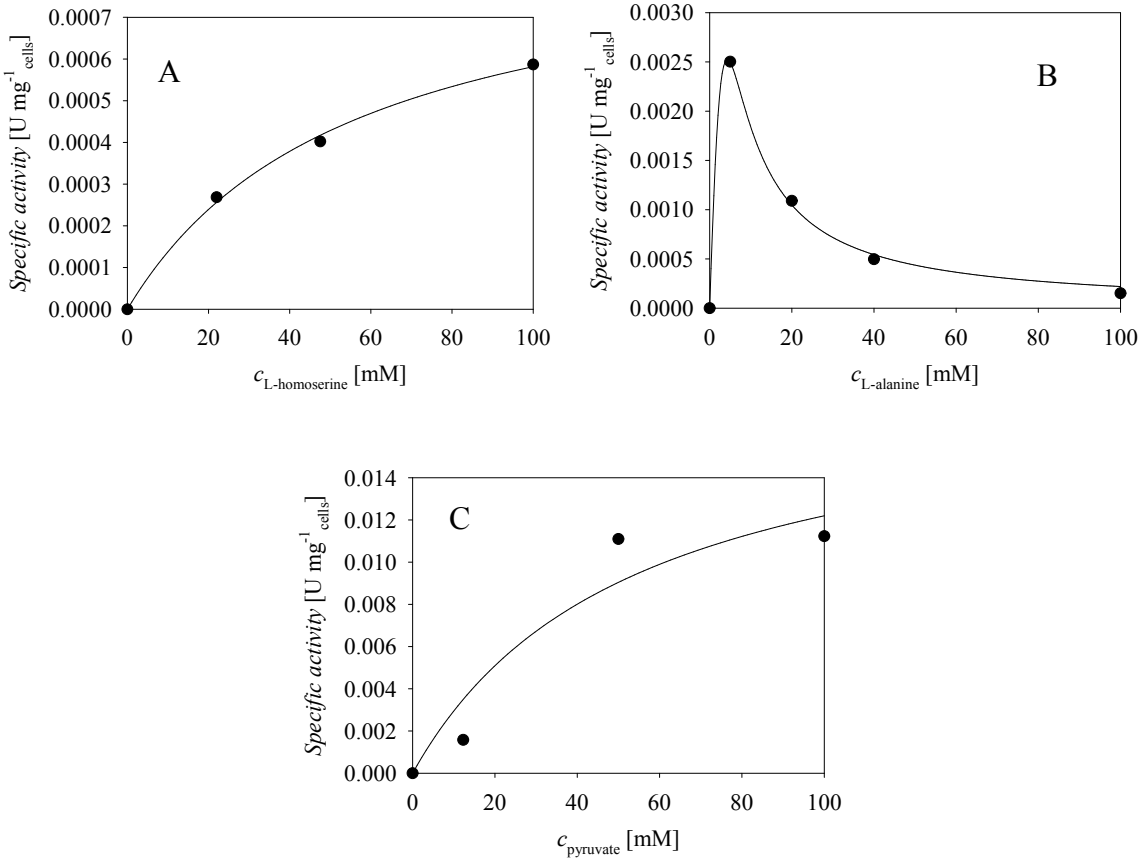

Figure S.5 The influence of **A.** L-homoserine, **B**. L-alanine and **C**. pyruvate concentration on the specific activity of cells (V = 1 mL, 1000 rpm, 25 °C, sodium phosphate buffer, 50 mg mL^{-1} of cells).

It was not the purpose of this work to describe this kinetics in detail, and that is why only several experimental points (initial reaction rates) were measured. However, even from these results it can be seen that the rates of the side-reactions are significantly lower than is the case of the studied reaction, i.e., V_{m1} = 0.726 U mg^{-1}$_{cells}$, and V_{m3} = 14.158 U mg^{-1}$_{cells}$. It could appear that the transformation of L-alanine would likely take place but considering severe substrate inhibition and the concentrations of L-alanine used in the L-homoserine cascade synthesis, this reaction should be minimized. Considering the activity of cells on pyruvate alone, it could be expected that some of the pyruvate will be lost in this side-reaction, as it was quickly consumed at all concentrations.

Table S.3 Kinetic parameters for the side-reactions catalyzed by the cells with L-homoserine, L-alanine and pyruvate.

Parameter	Unit	Value cells
Pyruvate		
V_{m7}	U mg_{cells}^{-1}	0.019 (0.024)*
$K_{m7,\ pyruvate}$	mM	53.527 (881.958)*
L-alanine		
V_{m8}	U mg_{cells}^{-1}	0.177
$K_{m8,\ L\text{-}alanine}$	mM	148.843
$K_{i8,\ L\text{-}alanine}$	mM	0.125
L-homoserine		
V_{m9}	U mg_{cells}^{-1}	0.00091
$K_{m9,\ L\text{-}homoserine}$	mM	55.710

*The kinetic parameters in the brackets were re-estimated from the fed-batch experiments

Further incubation experiments revealed that pyruvate transforms to L-alanine which means that lyophilized cells have an active alanine metabolism[2] and the presence of an enzyme able to transform pyruvate to L-alanine. Experiments were carried out with pyruvate without L-alanine, and pyruvate with L-alanine (Fig. S.6). The results show that pyruvate concentration decreases, while L-alanine concentration increases. The experiment presented in Fig. S.6A shows that L-alanine is formed from pyruvate, as there was no L-alanine in the beginning of this experiment. Figure S.6B also shows an increase of L-alanine concentration and consumption of pyruvate.

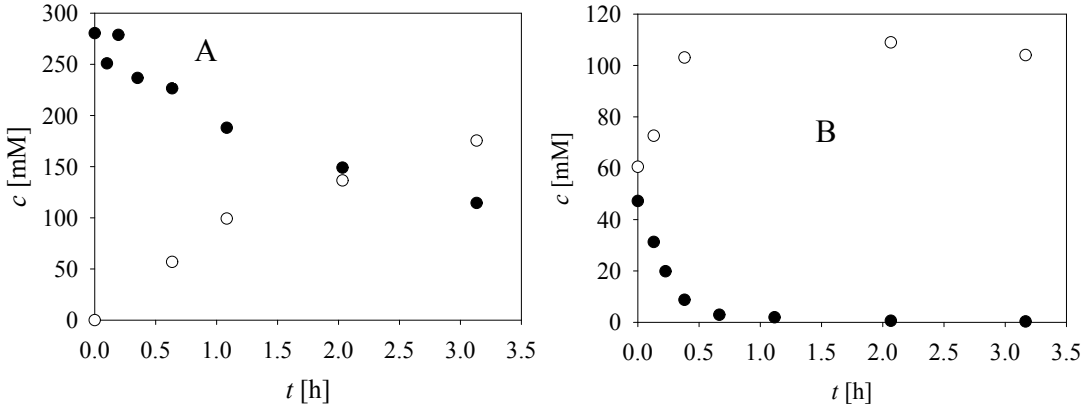

Figure S.6 Incubation of pyruvate **A**. in the absence and **B**. together with L-alanine (V = 1 mL, 1000 rpm, 25 °C, buffer, 50 mg mL^{-1} of cells). Legend: white circles – L-alanine, black circles – pyruvate.

S4. The statistical output for the simulated experiments

Table S.4 presents the statistical output of the model simulations presented in Fig. 5.

Table S.4 The statistical output of the SCIENTIST software for the goodness-of-fit of the model to the experimental data.

Figure	R^2	Coefficient of determination	Correlation	Model selection criterion
5A	0.9868	0.9761	0.9895	3.4398
5B	0.9821	0.9833	0.9832	3.3825
5B (part 2)	0.9632	0.8958	0.9469	2.1722
5C	0.9460	0.9132	0.9636	2.4436
5C (part 2)	0.9954	0.8721	0.89430	2.1150

S5. The apparatus for the fed-batch reactor experiments

S6. The influence of the enzyme activity ratio on the final concentration of L-homoserine

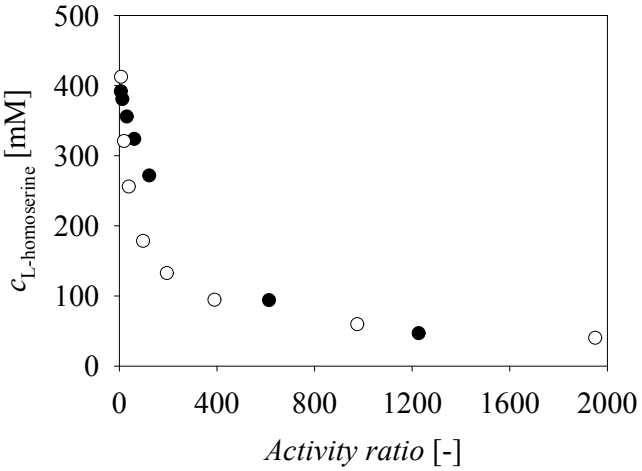

Figure S.7 Simulations of the cascade reactions in the fed-batch reactor by using CFE (white circles) and LWCB (black circles) (V_0 = 7.8 mL, $c_{\text{formaldehyde, feed}}$ = 3.1 M, $q_{\text{feed, formaldehyde}}$ = 3.0 µL min^{-1}, $c_{\text{formaldehyde,0}}$ = 0 mM, 12 hours of formaldehyde feed). **A**. $c_{\text{L-alanine}}$ = 600 mM, c_{pyruvate} = 250 mM, t = 24 h.

Figure S.7 presents the influence of aldolase/transaminase activity ratio on the final concentration of L-homoserine in the fed-batch reactor. Simulations were done for the CFE, as well as LWCB system based on the mathematical model. High level of similarity can be observed between the simulations indicating that the data and model obtained for CFE can be applied to prepare the cells with the required level of enzyme activities.

References:

(1) Česnik, M.; Sudar, M.; Hernández, K.; Charnock, S.; Vasić-Rački, Đ.; Clapés, P.; Findrik Blažević, Z., Cascade enzymatic synthesis of L-homoserine – mathematical modelling as a tool for process optimisation and design. *React. Chem. Eng.* **2020,** *5*, 747-759.

(2) https://www.genome.jp/kegg-bin/show_pathway?org_name=ecj&mapno=00250&show_description=show (accessed 2021-06-01)