Supplemental Information for "Metabolic division of labor in microbial systems"

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Table S1. Dimensionless variable relationships and interpretations (related to Figure 1). ND signifies non-dimensionalized (dimensionless), or without units

Variable	Description	Biological value	ND variable (dimensionless)	ND Value (unitless)	ND Value Range (unitless)
K_M	Michaelis-Menten constant for intermediate synthesis	1×10^{-4} M [1]			
K_P	Michaelis-Menten constant for product synthesis	1×10^{-4} M [1]			
M	Concentration of intracellular intermediate in the SC population.		$m = \frac{M}{K_M}$		
M_1	Concentration of intracellular intermediate in the first DOL population		$m_1 = \frac{M_1}{K_M}$		
M_e	Concentration of extracellular intermediate		$m_e = \frac{M_e}{K_M}$		
M_2	Concentration of intracellular intermediate in the second DOL population		$m_2 = \frac{M_2}{K_M}$		
P	Concentration of the final product		$p = \frac{P}{K_M}$		
	Time		$\tau = td_P$		
E_1	Concentration of the first enzyme	2 μM [2, 3]	$e_1 = \frac{E_1}{K_M}$	0.02	$0 - 0.16$
E_2	Concentration of the second enzyme	2 μM [2, 3]	$e_2 = \frac{E_2}{K_P}$	0.02	$0 - 0.16$
k_{diff}	Diffusivity of the intermediate	0-2.41 s^{-1}	$\eta = \frac{k_{diff}}{d_P}$		$0 - 2.5 \times 10^5$
V	Total volume of the SC population		$v = \frac{V}{V_e}$		
V_1	Total volume of the first DOL population		$v_1 = \frac{V_1}{V_e}$		
V_2	Total volume of the second DOL population		$v_2 = \frac{V_2}{V_e}$		

k_1	Synthesis rate of the intermediate	10 s^{-1} [1]	$\alpha_1 = \frac{k_1}{d_P}$	1.04×10^6	$10^4 - 10^8$
k_2	Synthesis rate of the intermediate	10 s^{-1} [1]	$\alpha_2 = \frac{k_2}{d_P}$	1.04×10^6	$10^4 - 10^8$
d_{M_e}	Turnover rate of the intermediate outside the cell	0.1 hr^{-1}	$\delta_{m_e} = \frac{d_{M_e}}{d_P}$	2.88	$0 - 30$
d_P	Turnover rate of the final product	0.0347 hr^{-1} [4-6][2]			
d_N	Turnover rate of the SC population	0.1 hr^{-1}	$\delta_v = \frac{d_N}{d_P}$	2.88	$0 - 10$
d_{N_1}	Turnover rate of the first DOL population	0.1 hr^{-1}	$\delta_{v_1} = \frac{d_{N_1}}{d_P}$	2.88	$0 - 10$
d_{N_2}	Turnover rate of the second DOL population	0.1 hr^{-1}	$\delta_{v_2} = \frac{d_{N_2}}{d_P}$	2.88	$0 - 10$
k_{grow}	Growth rate of the SC population	0.5 hr^{-1}	$\mu_{max} = \frac{k_{grow}}{d_P}$	14.4	$3.5 - 20$
$k_{grow,1}$	Growth rate of the first DOL population	0.5 hr^{-1}	$\mu_{1,max} = \frac{k_{grow,1}}{d_P}$	14.4	$3.5 - 20$
$k_{grow,2}$	Growth rate of the second DOL population	0.5 hr^{-1}	$\mu_{2,max} = \frac{k_{grow,2}}{d_P}$	14.4	$3.5 - 20$
V_e	Volume of the extracellular space	1 mL			
V_{max}	Carrying capacity of the SC population	1 μL	$\rho = \frac{V_{max}}{V_e}$	10^{-3}	$10^{-5} - 10^{-2}$
$V_{max,1}$	Carrying capacity of the first DOL population	0.5 μL	$\rho_1 = \frac{V_{max,1}}{V_e}$	5×10^{-4}	$10^{-5} - 10^{-2}$
$V_{max,2}$	Carrying capacity of the second DOL population	0.5 μL	$\rho_2 = \frac{V_{max,2}}{V_e}$	5×10^{-4}	$10^{-5} - 10^{-2}$
n	Hill coefficient of burden	1		1	$1 - 10$

[1]Estimated from yeast, comparable to bacteria
[2]Average half life of proteins

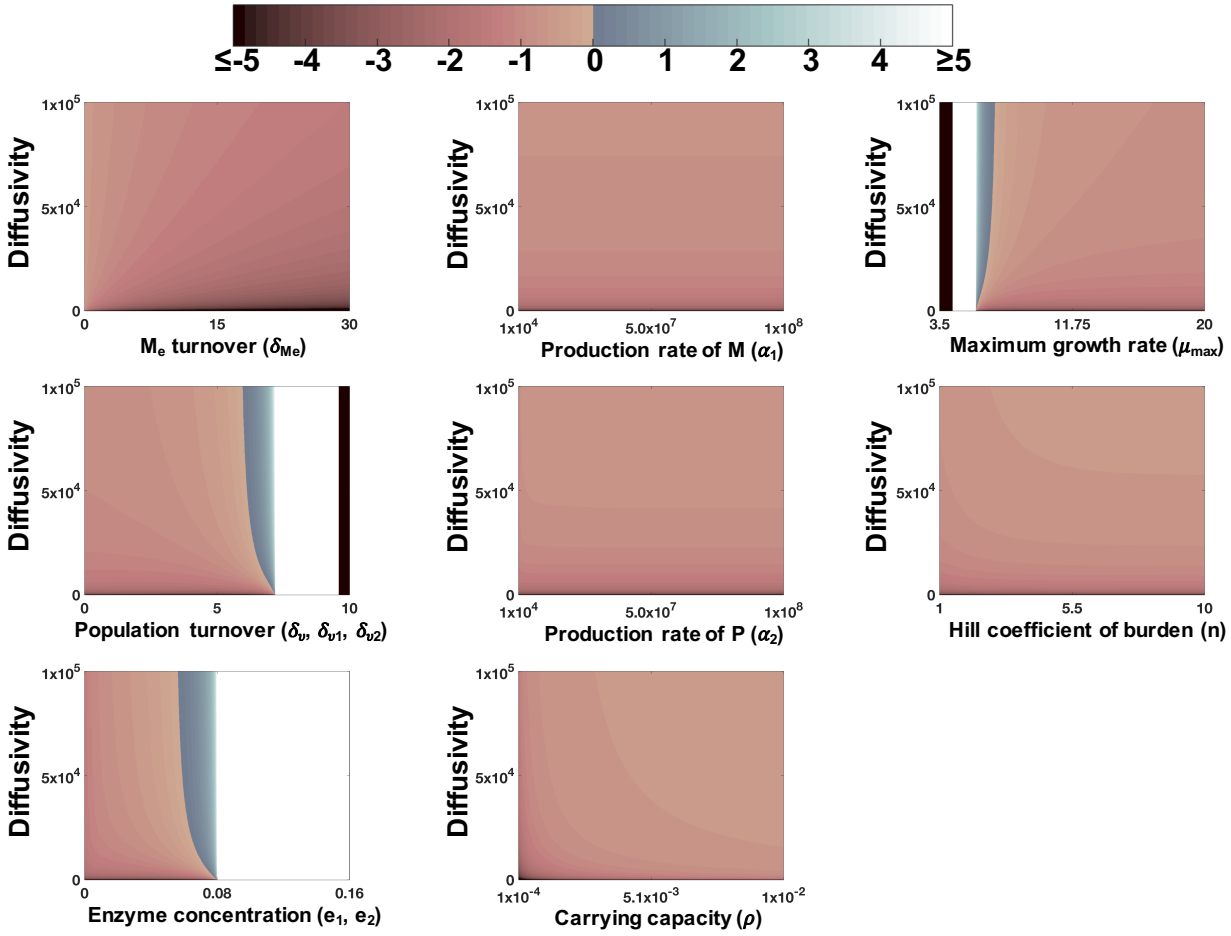

Figure S1. Sensitivity analysis of which parameters influence the criterion (related to Figure 1). The color of the heat map indicates the magnitude of $\ln\left(\frac{p_{DOL}v_2}{p_{SC}v}\right)$. Enzyme concentration ($e$), population turnover ($\delta_v$), and maximum specific growth rate (μ_{max}) determine whether DOL is favored over SC and how much one design strategy outperforms the other because they strongly influence the right side of Eq 16. Increasing e and δ_v increasingly favor DOL, whereas increasing μ_{max} increasingly favors SC. The remaining parameters do not influence which design strategy is favored.

Table S2. Parameters for corresponding growth effects (related to figure 2)

ND variable	ND Value	ND Value Range
$\beta = \frac{K_M}{K_{burd}}$ (first enzyme) $\gamma = \frac{K_P}{K_{burd}}$ (second enzyme)	25 (both enzymes)	$0 - 200$ (both enzymes)
$\theta_m = \frac{K_M}{K_{tox,M}}$	1	$0 - 10$
$\theta_{m_e} = \frac{K_M}{K_{tox,M_e}}$	1	$0 - 10$
$\theta_P = \frac{K_M}{K_{tox,P}}$	1×10^{-5}	$0 - 1 \times 10^{-3}$
$\sigma_m = \frac{K_{ben,M}}{K_M}$	1	$0 - 10$
$\sigma_{m_e} = \frac{K_{ben,M_e}}{K_M}$	1	$0 - 10$
$\sigma_P = \frac{K_{ben,P}}{K_M}$	1×10^4	$0 - 1 \times 10^5$

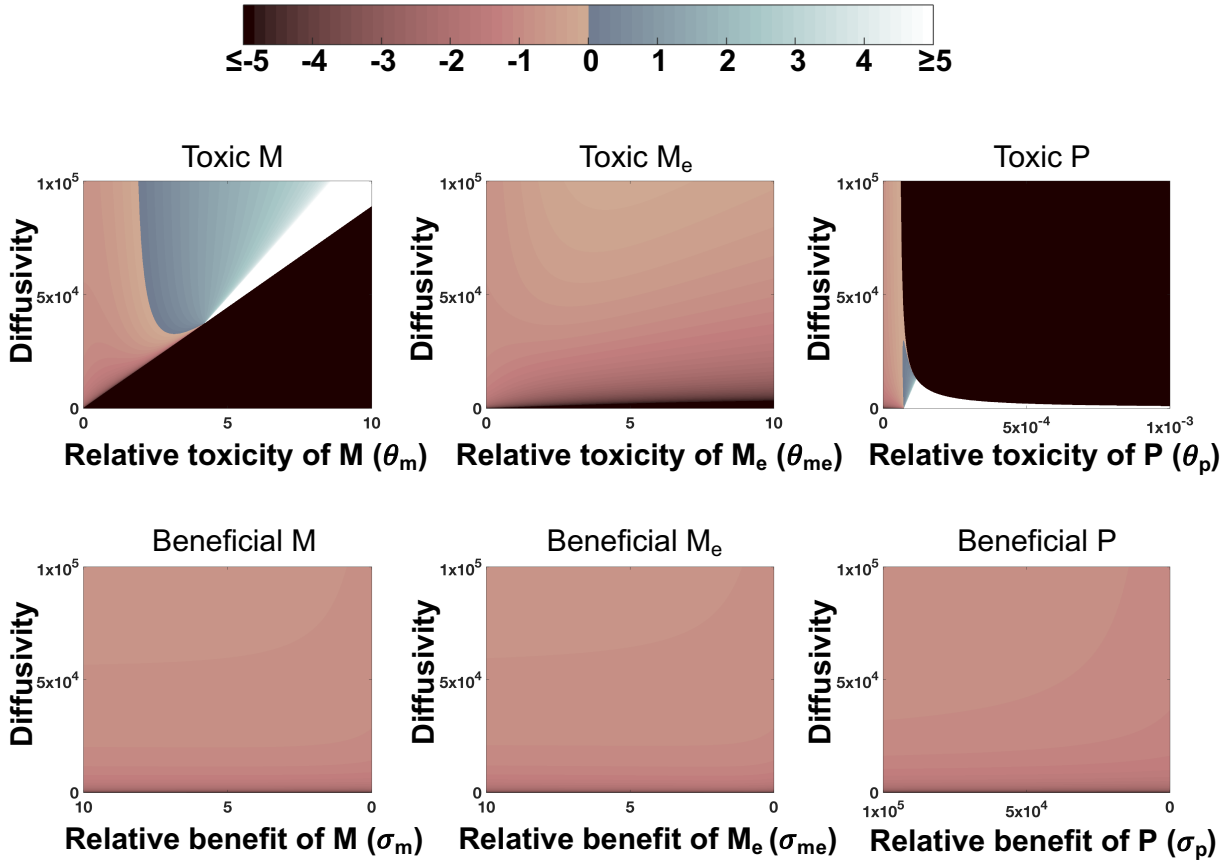

Figure S2. The impact of relative growth effects of metabolites (related to figure 2). The color of the heat map indicates the magnitude of $\ln\left(\frac{p_{DOL}v_2}{p_{SC}v}\right)$. Each heat map corresponds to a single metabolite having either a beneficial or toxic effect on cell growth, and the x-axis corresponds to a varying degree of that growth effect. Increasing toxicity of the intermediate/product favors DOL, while increasing its beneficial effect favors SC.

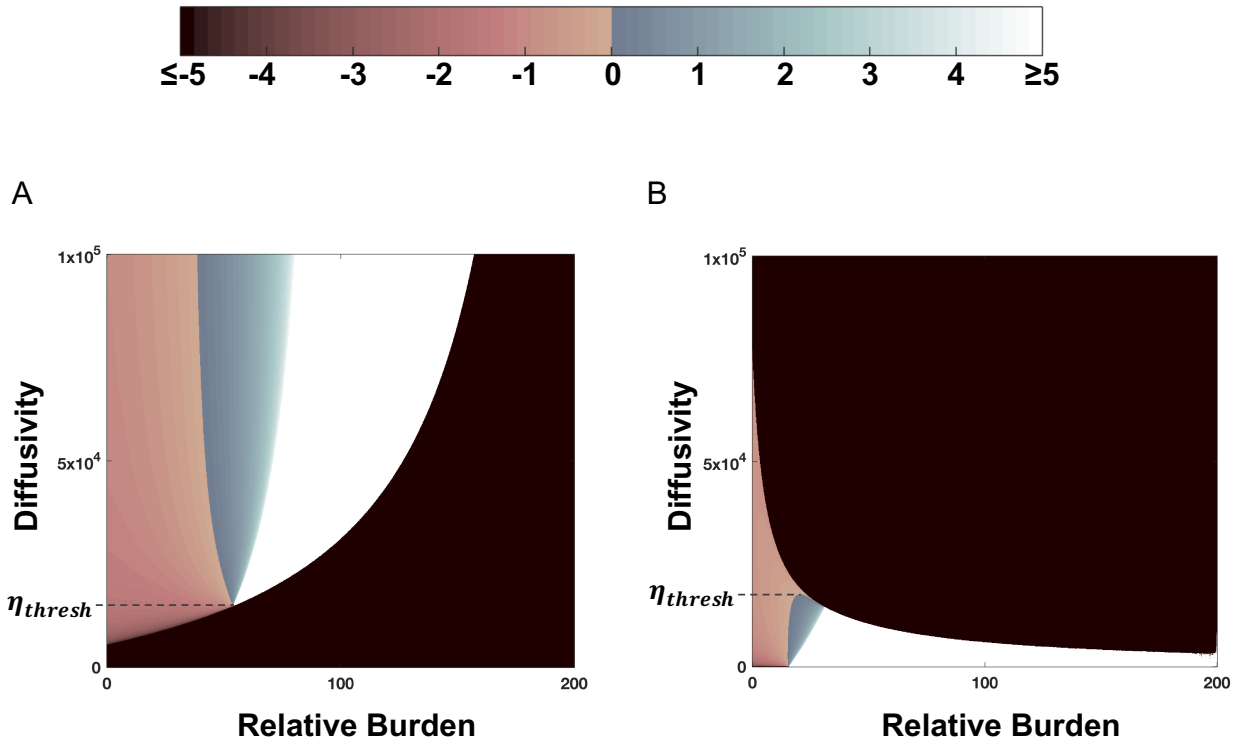

Figure S3. The shape of the criterion changes for high pathway toxicity (related to figure 2). The color of the heat map indicates the magnitude of ln $\left(\frac{p_{DOL}v_2}{p_{SC}v}\right)$.

(A) The intermediate M is highly toxic ($\theta_m = 1$) Unlike the base model, DOL is favored over SC only above a certain diffusivity (η_{thresh}). The region in which all populations die (black) also greatly increases for high toxicity. **(B)** The final product P is highly toxic ($\theta_P = 1 \times 10^{-4}$). DOL is favored over SC only below a certain diffusivity (η_{thresh}) because high product yield causes more cell death. Thus, for sufficiently high toxicity, in contrast to the criterion, increasing η favors SC. The region in which all populations die (black) also greatly increases for high toxicity.

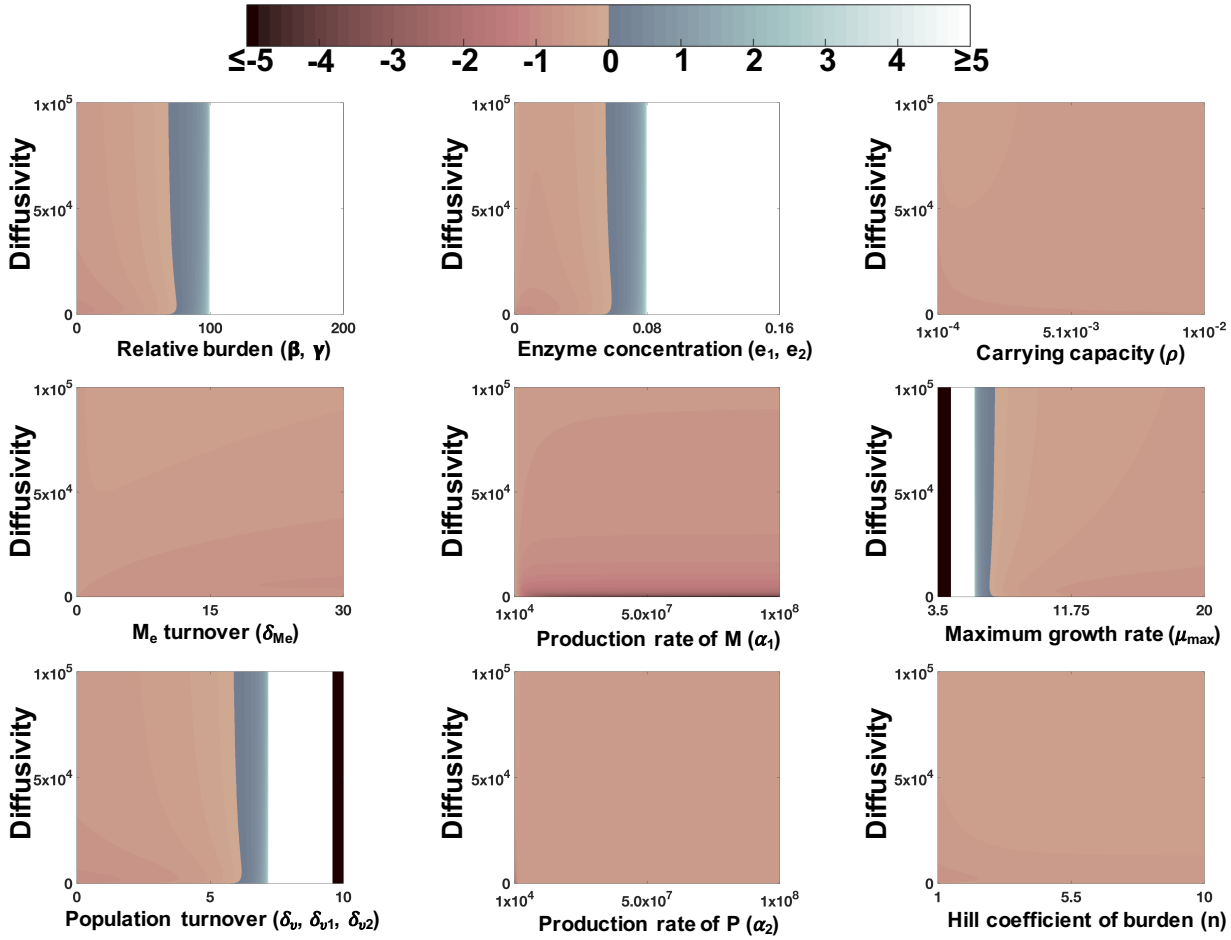

Figure S4. The assumption that $K_P \gg M$ does not change our conclusions from the criterion (related to figure 3). The color of the heat map indicates the magnitude of $\ln\left(\frac{p_{DOL}v_2}{p_{SC}v}\right)$. The same parameters from the base model, namely relative burden, enzyme concentration, population turnover, and maximum specific growth rate, determine which design strategy is favored and to what extent it is favored over the other. The shape of the curve is consistent to the original criterion except for very low η.

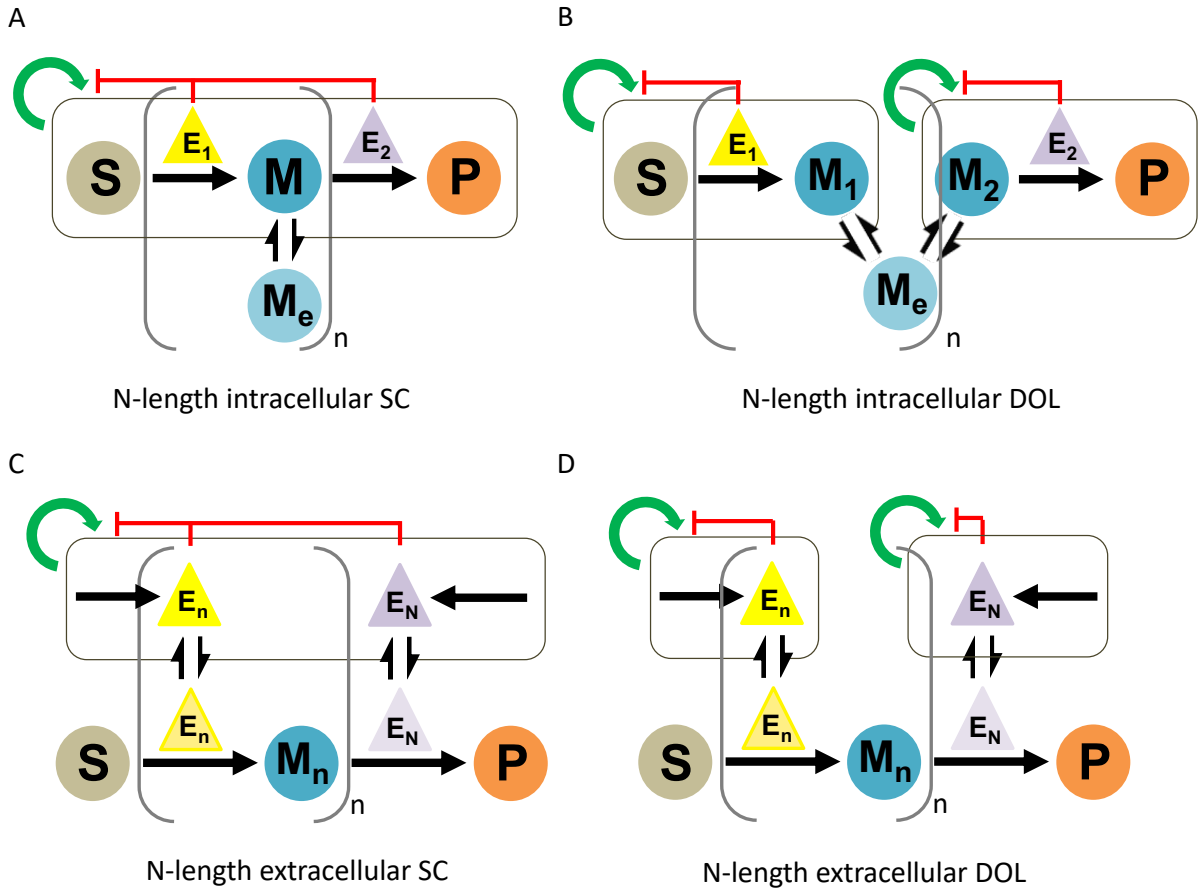

Figure S5. Model frameworks for arbitrary length pathways (related to figure 3).
(A) Model framework for an arbitrary length intracellular pathway within a single population. Substrate (S) is converted into intracellular intermediate (M_n), which diffuses into the extracellular environment ($M_{n,e}$) and is directly converted to the next intermediate. The final intermediate is directly converted to final product (P).
(B) Model framework for an arbitrary length intracellular pathway within multiple populations. Substrate (S) is converted into intracellular intermediate (M_n), which diffuses from one population into extracellular environment ($M_{n,e}$) and then into the next population (M_{n+1}). The final intermediate is converted to P.
(C) Model framework for an arbitrary length extracellular pathway within a single population. A single cell produces all the enzymes necessary to catalyze the pathway. Each enzyme (E_n) diffuses into the extracellular environment ($E_{n,e}$) to catalyze a single step of the N-length conversion of substrate (S) into final product (P).
(D) Model framework for an arbitrary length extracellular pathway within two populations. Multiple populations each produce a single enzyme, all of which catalyze the pathway. Each enzyme (E_n) diffuses into the extracellular environment ($E_{n,e}$) to catalyze a single step of the N-length conversion of substrate (S) into P.

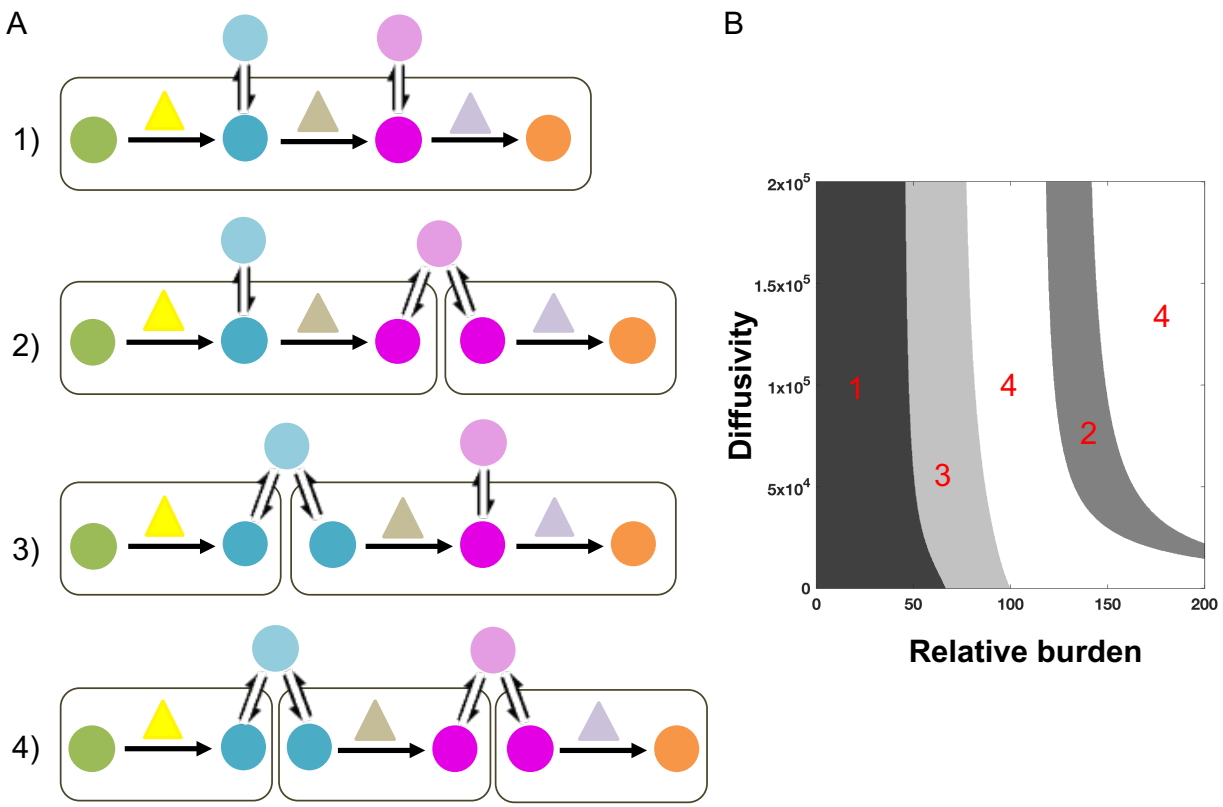

Figure S6. The criterion is consistent for a 3-step pathway (related to figure 3).
(A) A three-enzyme pathway can have four possible configurations. 1 and 4 correspond to the analogous SC and DOL architectures from the base models, respectively. 2 and 3 represent hybrid SC-DOL architectures: one cell is responsible for producing multiple enzymes (representing SC), but multiple populations are required to catalyze the entire pathway (representing DOL).
(B) Comparing the four possible architectures of a 3-step pathway. The color values correspond to which architecture produced the most final product for changing burden and diffusivity. The complete SC architecture was favored most for low values of burden, followed by 1, 4, 3, and 4 again for increasing values of burden. 3 performed the best at moderately high levels of burden (even more than full DOL) because it incorporates design advantages from both SC (less resource sharing and transport) and DOL (reduced enzyme burden) design strategies. However, since 3 still contains multiple enzymes in a single population, even higher burden promoted 4.

Supplemental Section 2. Derivation of SC and DOL base models

2.1 Derivation of Single Cell (SC) and Division of Labor (DOL) models

We make several biologically relevant assumptions to simplify model development and analysis. Relaxing these assumptions (e.g. see Supplemental Sections 3.4.1-3.4.3) do not change the qualitative aspects of our conclusions. These assumptions include:

- The systems for both configurations are well mixed in each compartment (inside a cell or in the extracellular space).
- The inherent degradation of metabolites is significantly slower than kinetic reactions and thus its rate may be set to approximately 0. Including metabolite degradation (see Supplemental Section 3.4.2) leads to a similar criterion.
- Transport of the metabolite across the cell membrane occurs via passive diffusion at a rate proportional to the concentration gradient between two compartments. Assuming active transport (see Supplemental Section 3.4.3) leads to a similar criterion.
- Concentrations of both enzymes are always at steady state

2.1.1 Intracellular Single Cell (SC)

With these assumptions, we derive a system of ODEs detailing intermediate and product concentrations for an intracellular pathway in a single population:

$$\frac{dM}{dt} = \frac{k_1 E_1 S}{K_M + S} - k_{diff}(M - M_e) - \frac{k_2 E_2 M}{K_P + M}, \quad \text{(S2.1)}$$

$$\frac{dM_e}{dt} = \frac{V}{V_e} k_{diff}(M - M_e) - d_{M_e} M_e, \quad \text{(S2.2)}$$

$$\frac{dP}{dt} = \frac{k_2 E_2 M}{K_P + M} - d_P P. \quad \text{(S2.3)}$$

We further assume $S \gg K_M$, $K_P \gg M$, and non-dimensionalize these ODEs to facilitate modeling analysis, which gives us our dimensionless SC model in Eqs 1-3 in the main text. The dimensionless variables are described in Table S1. In Supplemental Sections 3.4.4.-3.4.5, we show that relax these assumptions do not affect our conclusions.

2.1.2 Intracellular Division of Labor (DOL)

We also derive another system of ODEs for the same pathway in two populations

$$\frac{dM}{dt} = \frac{k_1 E_1 S}{K_M + S} - k_{diff}(M - M_e) \quad , \quad \text{(S2.4)}$$

$$\frac{dM_e}{dt} = \frac{V_1}{V_e} k_{diff}(M - M_e) - \frac{V_2}{V_e} k_{diff}(M_e - M_2) - d_{M_e} M_e, \quad \text{(S2.5)}$$

$$\frac{dM_2}{dt} = k_{diff}(M_e - M_2) - \frac{k_2 E_2 M_2}{K_P + M_2}, \quad \text{(S2.6)}$$

$$\frac{dP}{dt} = \frac{k_2 E_2 M_2}{K_P + M_2} - d_P P. \quad \text{(S2.7)}$$

We non-dimensionalize these equations using the same variable relationships and assumptions to obtain the full, dimensionless DOL model (Eqs 4-7 in the main text). Like the SC model, we assume $S \gg K_M$ and $K_P \gg M$ when deriving the dimensionless model. The dimensionless variables are described in Table S1.

2.1.3 Cell growth equations for both SC and DOL

Our models also account for the cell growth dynamics of each population. Population growth in SC is modeled using a general logistic function with first-order cell death:

$$\frac{dN}{dt} = k_{grow} N \left(1 - \frac{N}{N_{max}}\right) - d_N N, \quad \text{(S2.8)}$$

And in DOL

$$\frac{dN_1}{dt} = k_{grow,1} N_1 \left(1 - \frac{N_1 + bN_2}{N_{max,1}}\right) - d_{N_1} N_1 \, , \quad \text{(S2.9)}$$

$$\frac{dN_2}{dt} = k_{grow,2} N_2 \left(1 - \frac{aN_1 + N_2}{N_{max,2}}\right) - d_{N_2} N_2 \, , \quad \text{(S2.10)}$$

where *a* and *b* represent competition between the two populations for the same resources. For a population with sufficiently large cell number, the total biomass is proportional to the total cell volume. Thus, the cell density above can be interpreted as the total cell volume, which we use as the basis to derive the dimensionless models. We further

assume separate carrying capacities for the DOL populations ($N_{max,1} \neq N_{max,2}$) and no competition ($a = b = 0$) for resources to obtain the dimensionless cell growth equations for SC and DOL (Eq 8-10 in the main text). The dimensionless variable relationships and values are detailed in Table S1.

2.2 Definition of dimensionless variables and choice of parameter values

Table S1 lists definition of system variables, their corresponding dimensionless counterparts (when applicable), and choice of parameter values for numerical analyses.

Parameter values are chosen/calculated according to estimated values from literature and are detailed in Table S1 below. Dimensionless (ND) values are calculated based on the variable relationships in Table S1. Parameter ranges are chosen to incorporate at least ~10-fold higher or lower than the estimated value. Ranges of transport rates, growth rates, death rates, and enzyme concentrations are chosen based on sensitivity analysis. Intermediate and cell populations are assumed to turnover at the same rate to represent operation of a chemostat, where turnover rate constant is approximately equal to the chemostat dilution rate constant. Carrying capacity is estimated mathematically using average volume of cell and cell numbers at steady state.

2.3 Derivation of design criterion

We derive the final form of the design criterion that dictates the conditions favoring division of labor in an intracellular pathway by comparing the total product synthesis of SC and DOL (inequality detailed in Results). The most general form of the criterion is given by

$$\frac{\alpha_1 e_1 \eta \upsilon_1 \upsilon_2}{\delta_{m_e} + \frac{\delta_{m_e} \eta}{\alpha_2 e_2} + \eta \upsilon_2} > \frac{\alpha_1 e_1 (\delta_{m_e} + \eta \upsilon) \upsilon}{\delta_{m_e} + \frac{\delta_{m_e} \eta}{\alpha_2 e_2} + \eta \upsilon}. \quad \text{(S2.11)}$$

Through algebraic simplification, we obtain the criterion in Results given by Eq 16. From sensitivity analysis on the criterion for varying diffusivity and burden (Figure 1D) and other parameters (Figure S1), maximum specific growth rate, specific death rate, and enzyme concentration significantly influence which strategy to what extent it is favored because they strongly influence the left-hand side of Eq 16. In contrast, the criterion is not as sensitive to changing production rate constants, turnover rate constants, carrying capacity, or Hill coefficient because they weakly influence the right-hand side of Eq 16.

Supplemental Section 3. Different base model configurations

3.1 Derivation of SC and DOL models with enzyme or metabolite growth effects

We maintain the same model assumptions from the base model in these alternative models. We also make an additional biologically relevant assumption to simplify model development and analysis:

- Extracellular metabolites only affect the growth of the population(s) in which they are produced or transported. This is a reasonable assumption if the cells are not tightly packed (modulated by the value of carrying capacity) and the system is well-mixed (a previous assumption).

3.1.1 Metabolic burden affecting cell growth

In line with a previous study [7], we model the metabolic burden of enzyme expression such that growth rate decreases with an increasing enzyme concentration. We separately model three different forms of burden to demonstrate three different configurations of the base model: Hill burden, exponential burden, and linear burden.

Burden	SC	DOL
Hill[1]	$\frac{K_{burd}^n}{K_{burd}^n + (E_1 + E_2)^n}$	$\frac{K_{burd}^n}{K_{burd}^n+E_1^n}$ (population 1) $\frac{K_{burd}^n}{K_{burd}^n+E_2^n}$ (population 2)
Exponential[2]	$e^{-(\frac{E_1+E_2}{K_{burd}})^n}$	$e^{-\left(\frac{E_1}{K_{burd}}\right)^n}$ (population 1) $e^{-\left(\frac{E_2}{K_{burd}}\right)^n}$ (population 2)
Linear[3]	$1-\left(\frac{E_1+E_2}{K_{burd}}\right)^n$	$1-\left(\frac{E_1}{K_{burd}}\right)^n$ (population 1) $1-\left(\frac{E_2}{K_{burd}}\right)^n$ (population 2)

[1] K_{burd} represents the concentration of enzyme at which growth rate is reduced by 50%.
[2] K_{burd} represents the concentration of enzyme at which growth rate is reduced by 63%
[3] K_{burd} represents the concentration of enzyme at which growth rate is reduced by 100%

In all equations, E_1 and E_2 represent the steady state intracellular concentrations of enzymes. In the extracellular models (see Supplemental Section 4.1.1), the dimensionless maximal enzyme concentration is given by $e_{1,\max} = r_{e_1}$ and $e_{2,\max} = r_{e_1} / \delta$. Hill burden can also be represented by independent burdens ($\frac{K_{burd,1}^n K_{burd,2}^n}{(K_{burd,1}^n+{E_1}^n)(K_{burd,2}^n+{E_2}^n)}$) where each enzyme reduces cellular growth rate linearly independent from one another. However, this and any variant of burden expressions does not change the form of the criterion.

We non-dimensionalize these equations to obtain dimensionless equations 11-13 in the main text detailing burden for the base model as well as expressions for alternate forms of burden. The dimensionless variables relationships are detailed in Table S2.

	SC	DOL
Hill	$\frac{1}{1+(\beta e_1+\gamma e_2)^n}$	$\frac{1}{1+(\beta e_1)^n}$ (population 1) $\frac{1}{1+(\gamma e_2)^n}$ (population 2)
Exponential	$e^{-(\beta e_1+\gamma e_2)}$	$e^{-\beta e_1}$ (population 1) $e^{-\gamma e_2}$ (population 2)
Linear	$1-(\beta e_1+\gamma e_2)$	$1-\beta e_1$ (population 1)

		$1-\gamma e_2$ (population 2)

3.1.2 Effects of intermediates or products on cell growth

We can adjust the models to account for potential growth effects by intermediates or products by modifying the expression for growth rate in the cell growth equations. We represent this by G in the SC models and G_1, G_2 in the DOL models, where G represents a multiplication of all the following metabolite growth effects: a toxic intracellular intermediate (TI), a toxic extracellular intermediate (TIE), a toxic intracellular product (TP), a toxic extracellular product (TPE), a beneficial intracellular intermediate (BI), a beneficial extracellular intermediate (BIE), a beneficial intracellular product (BP), and a beneficial extracellular product (BPE). As a result, G differs depending on the architecture and population, where G>1 means that the pathway promotes growth, and G<1 means that the pathway suppresses growth. The expressions for each interaction are given by:

$$TI = \frac{K_{tox,M}^n}{K_{tox,M}^n + M^n}, \quad \text{(S3.1)}$$

$$TIE = \frac{K_{tox,M_e}^n}{K_{tox,M_e}^n + M_e^n}, \quad \text{(S3.2)}$$

$$TP = \frac{K_{tox,P}^n}{K_{tox,P}^n + P^n}, \quad \text{(S3.3)}$$

$$BI = 1 + \frac{M^n}{K_{ben,M}^n + M^n}, \quad \text{(S3.4)}$$

$$BIE = 1 + \frac{M_e^n}{K_{ben,M_e}^n + M_e^n}, \quad \text{(S3.5)}$$

$$BP = 1 + \frac{P^n}{K_{ben,P}^n + P^n}. \quad \text{(S3.6)}$$

K_{tox} represents the metabolite concentration that produces half-maximal toxicity and K_{ben} represents the metabolite concentration that produces half-maximal benefit. In the base case with no interactions, every term is set to 1.

We non-dimensionalize equations S2.1-S2.6 in the same manner as the expression for metabolic burden to fit into our dimensionless model:

$$TI = \frac{1}{1+(\theta_m m)^n}, \quad \text{(S3.7)}$$

$$TIE = \frac{1}{1+(\theta_{m,e} m_e)^n}, \quad \text{(S3.8)}$$

$$TP = \frac{1}{1+(\theta_P p)^n}, \quad \text{(S3.9)}$$

$$BI = 1 + \frac{m^n}{\sigma_m^n + m^n}, \quad \text{(S3.10)}$$

$$BIE = 1 + \frac{m_e^n}{\sigma_{m,e}^n + m_e^n}, \quad \text{(S3.11)}$$

$$BP = 1 + \frac{p^n}{\sigma_p^n + p^n}. \quad \text{(S3.12)}$$

where the dimensionless parameters are detailed in Table S2.

3.2 Definition of dimensionless variables and choice of parameter values

We detail the specific dimensionless variables for growth effects in Table S2. The values for relative enzyme burden are varied between no growth inhibition and complete extinction of the DOL populations. Parameter value ranges for metabolite growth effects are also chosen to establish a wide range of growth promotion/inhibition.

3.3 Visualizing the criterion for changing growth effect

We construct additional heat maps varying diffusivity and growth effect magnitude according to the values listed in Table S2 (Figure S2). From the heat maps, we can see that DOL becomes more favored over SC for increasing toxicity as it is functionally identical to burden. In contrast, SC becomes more favored over SC for increasing benefit as it is analogous to increasing maximum specific growth rate. An analytical border is not seen when increasing benefit because the parameters used in the base case already favor SC.

Notably, high pathway toxicity changes the shape of the analytical border between SC and DOL significantly (Figure S3). For example, in the base model DOL has a change to outperform SC if $\eta > 0$. However, if

M is sufficiently toxic, then η must exceed a certain threshold η_{thresh} before DOL can outperform SC. If P is sufficiently toxic, then the trend reverses: above a certain η_{thresh}, DOL can never be favored over SC. This happens because product toxicity combined with our objective function of maximizing product yield means that maximizing product synthesis will kill the population and thus moderate yield is most successful.

3.4 Different variations of the base model

3.4.1 Production of another byproduct that directly affects cell growth

Dynamics of additional intermediates or products can be accounted for by introducing additional ODEs. So long as the byproduct is not required for production of the final product, they do not change the explicit criterion (Eq 16 in the main text). The effects of these additional molecules can also be captured in the growth rate function similar to growth-affecting products. This results in similar expressions to those in Supplemental Section 3.1.2.

3.4.2 Enzyme expression is beneficial to growth

Similar to growth-affecting intermediates/products, growth-promotion by enzyme expression can be captured in the growth rate function. As a result, the form of the criterion is similarly not affected by this additional interaction, resulting in similar expressions to those in Supplemental Section 3.1.2.

3.4.3 Presence of other populations in the system

Additional species could affect the system in many ways: for example, they could directly affect the kinetics of the metabolic pathway and/or compete with the existing populations for resources. However, these additional interactions are already accounted for in the final form of the criterion. In the first case, the impact on the kinetics would be accounted for in the parameter values without changing the criterion. In the second case, species competition is accounted for on the left-hand side of the criterion, which represents overall biomass increase of DOL over SC. If additional species also participate in carrying out the pathway or affect the accumulation of metabolites in the pathway, we have derived a general criterion for pathways carried out by more than two species (see Supplemental Section 5.1) and shown that its form is identical to Eq 16.

3.4.4 Populations compete for nutrients and resources

If the populations in DOL compete for resources (such as occupying the same niche in an environment), they would share a carrying capacity as in Eqs S2.9 and S2.10. Without any force to stabilize their coexistence, however, the slowest growing of the two populations would crash. In other words, the system would be unable to support and carry out the pathway. If the two populations have identical growth rates, the results are identical to the case of separate but equal carrying capacities (which we assumed in Figure 1D).

3.4.5 Intracellular degradation of intermediate metabolites

Consider the case where the intermediate can also degrade within the cell. Here, the dimensionless quantities are defined differently to facilitate solving an analytical solution; otherwise, the pathway kinetics are identical to the base model. In SC, the dimensionless rates of change intracellular and extracellular products are given by

$$\frac{dm}{d\tau} = \alpha_1 e_1 - m - \eta(m - m_e) - \alpha_2 e_2 m, \quad \text{(S3.13)}$$

$$\frac{dm_e}{d\tau} = \upsilon\eta(m - m_e) - \delta_{m_e} m_e, \quad \text{(S3.14)}$$

$$\frac{dp}{d\tau} = \alpha_2 e_2 m - \delta_P, \quad \text{(S3.15)}$$

where $\tau = td_I$. The variables are defined identically to the base models except for $\eta, \alpha_1, \alpha_2, \delta_{I_e}, and\ \delta_P$. These variables are analogous to the corresponding base model parameters except that they are non-dimensionalized by the intracellular degradation rate constant of the intermediate. Similarly, for DOL, the corresponding dimensionless rates of changes of intracellular and extracellular products are given by

$$\frac{dm_1}{d\tau} = \alpha_1 e_1 - m_1 - \eta(m_1 - m_e), \quad \text{(S3.16)}$$

$$\frac{dm_e}{d\tau} = \upsilon_1\eta(m_1 - m_e) - \upsilon_2\eta(m_e - m_2) - \delta_{m_e} m_e, \quad \text{(S3.17)}$$

$$\frac{dm_2}{d\tau} = \eta(m_e - m_2) - m_2 - \alpha_2 e_2 m_2, \quad \text{(S3.18)}$$

$$\frac{dp}{d\tau} = \alpha_2 e_2 m_2 - \delta_P p. \quad \text{(S3.19)}$$

Again, the corresponding base model parameters are identical, and time-related variables are non-dimensionalized by the intracellular degradation rate constant of the intermediate. Additionally, the equations for cell growth are identical to the base case; however, the dimensionless variable relationships change. Specifically, $\mu = \frac{k_{grow}}{d_{P_2}}$, $\mu_1 = \frac{k_{grow,1}}{d_{P_2}}$, and $\mu_2 = \frac{k_{grow,2}}{d_{P_2}}$.

We derive the criterion for this specific configuration using the same methods as for the base model. The resulting criterion is given by

$$\frac{\overline{v}_{DOL}^2}{v^2+\varepsilon v} > \theta_I \quad \text{(S3.20)}$$

where $\theta_I = \frac{(v_1+v_2)\left(\eta^2+\eta(1+\alpha_2 e_2)\right)+\alpha_2 e_2\left(\delta_{m_e}+\delta_{m_e}\eta+\eta^2 v_2\right)+\delta_{m_e}(1+2\eta+\eta^2)}{\delta_{m_e}+\alpha_2 e_2 \delta_{m_e}+\delta_{m_e}\eta+\eta v+\alpha_2 e_2 \eta v}$ in this case.

3.4.6 One-dimensional active transport of the intermediate

Transport of the intermediate can also occur via active transport, relying on transport proteins to export the metabolite out of the cell against the concentration gradient. In such cases, the additional burden of expressing transport enzymes must also be included in the expression for burden-affected growth rate. The system of dimensionless ODEs corresponding to kinetics of the pathway in the SC configuration is thus given by:

$$\frac{dm}{d\tau} = \alpha_1 e_1 - \eta m - \alpha_2 e_2 m, \quad \text{(S3.21)}$$

$$\frac{dm_e}{d\tau} = v\eta m - \delta_{m_e} m_e, \quad \text{(S3.22)}$$

$$\frac{dp}{d\tau} = \alpha_2 e_2 m - \delta_P p, \quad \text{(S3.23)}$$

and in the DOL configuration is given by

$$\frac{dm_1}{d\tau} = \alpha_1 e_1 - \eta m_1, \quad \text{(S3.24)}$$

$$\frac{dm_e}{d\tau} = v_1 \eta m_1 - v_2 \eta m_e - \delta_{m_e} m_e, \quad \text{(S3.25)}$$

$$\frac{dm_2}{d\tau} = \eta m_e - \alpha_2 e_2 m_2, \quad \text{(S3.26)}$$

$$\frac{dp}{d\tau} = \alpha_2 e_2 m_2 - p. \quad \text{(S3.27)}$$

We derive the criterion for this specific configuration using the same methods as the base model. The resulting criterion is given by

$$\frac{v_1 v_2}{v(\varepsilon+v_2)} > \frac{1}{1+\frac{\eta}{\alpha_2 e_2}}, \quad \text{(S3.28)}$$

which has a distinct form from the base model criterion but has a similar interpretation. The left-hand side still represents an overall gain in cell density by DOL over SC weighted by ε, while the right-hand side is a different expression for θ_I specific to 1-D transport.

3.4.7 Intermediate concentration is comparable to Michaelis-Menten constant

We relax our previous assumption that the concentration of the intermediate is significantly smaller than the Michaelis-Menten constant for product synthesis. In this case, the system of dimensionless ODEs corresponding to the SC configuration is thus given by

$$\frac{dm}{d\tau} = \alpha_1 e_1 - \eta(m - m_e) - \frac{\alpha_2 e_2 m}{\varphi+m}, \quad \text{(S3.29)}$$

$$\frac{dm_e}{d\tau} = v\eta(m - m_e) - \delta_{m_e} m_e, \quad \text{(S3.30)}$$

$$\frac{dp}{d\tau} = \frac{\alpha_2 e_2 m}{\varphi+m} - p, \quad \text{(S3.31)}$$

and in DOL are given by

$$\frac{dm_1}{d\tau} = \alpha_1 e_1 - \eta(m_1 - m_e), \quad \text{(S3.32)}$$

$$\frac{dm_e}{d\tau} = v_1 \eta(m_1 - m_e) - v_2 \eta(m_e - m_2) - \delta_{m_e} m_e, \quad \text{(S3.33)}$$

$$\frac{dm_2}{d\tau} = \eta(m_e - m_2) - \frac{\alpha_2 e_2 m_2}{\varphi+m_2}, \quad \text{(S3.34)}$$

$$\frac{dp}{d\tau} = \frac{\alpha_2 e_2 m_2}{\varphi+m_2} - p, \quad \text{(S3.35)}$$

where $\varphi = \frac{K_P}{K_M}$ and $e_2 = \frac{E_2}{K_M}$. Using the same derivation methods as the base model, we cannot derive a simple criterion because of the nonlinearity of the system (resulting in two steady state solutions for p). However, the parameter spaces determining which design strategy is favored can be obtained by numerical simulation. The general trends and conclusions from the original criterion are still consistent after relaxing this assumption, only changing at very low η (see Figure S4).

Supplemental Section 4. Other two-step pathway architectures

4.1 Derivation of SC and DOL models and corresponding criteria for different pathway architectures

4.1.1 An extracellular pathway

We derive a system of dimensionless ODEs detailing intermediate, product, and enzyme concentrations in an extracellular pathway catalyzed by either a single population or two populations (Figure 3, #1). In the single cell architecture, the ODEs are given by

$$\frac{dm_e}{d\tau} = \alpha_1 e_{1,e} - \alpha_2 e_{2,e} m_e - \delta_{m_e} m_e, \quad \text{(S4.1)}$$

$$\frac{dp}{d\tau} = \alpha_2 e_{2,e} m_e - \delta_{p_e} p, \quad \text{(S4.2)}$$

$$\frac{de_1}{d\tau} = r_{e_1} - e_1 - \eta_1 (e_1 - e_{1,e}), \quad \text{(S4.3)}$$

$$\frac{de_2}{d\tau} = r_{e_2} - \delta_e e_2 - \eta_2 (e_2 - e_{2,e}), \quad \text{(S4.4)}$$

$$\frac{de_{1,e}}{d\tau} = \upsilon\eta_1 (e_1 - e_{1,e}) - \delta_{e_{1,e}} e_{1,e}, \quad \text{(S4.5)}$$

$$\frac{de_{2,e}}{d\tau} = \upsilon\eta_2 (e_2 - e_{2,e}) - \delta_{e_{2,e}} e_{2,e}, \quad \text{(S4.6)}$$

where $\tau = t d_{E_1}$ thus all time-related variables are non-dimensionalized by the degradation rate constant of the first enzyme. In the two-population architecture, the ODEs are given by

$$\frac{dm_e}{d\tau} = \alpha_1 e_{1,e} - \alpha_2 e_{2,e} m_e - \delta_{m_e} m_e, \quad \text{(S4.7)}$$

$$\frac{dp}{d\tau} = \alpha_2 e_{2,e} m_e - \delta_{p_e} p, \quad \text{(S4.8)}$$

$$\frac{de_1}{d\tau} = r_{e_1} - e_1 - \eta_1 (e_1 - e_{1,e}), \quad \text{(S4.9)}$$

$$\frac{de_2}{d\tau} = r_{e_2} - \delta_e e_2 - \eta_2 (e_2 - e_{2,e}), \quad \text{(S4.10)}$$

$$\frac{de_{1,e}}{d\tau} = \upsilon_1\eta_1 (e_1 - e_{1,e}) - \delta_{e_{1,e}} e_{1,e}, \quad \text{(S4.11)}$$

$$\frac{de_{2,e}}{d\tau} = \upsilon_2\eta_2 (e_2 - e_{2,e}) - \delta_{e_{2,e}} e_{2,e}. \quad \text{(S4.12)}$$

We derive the final form of the steady-state design criterion for an extracellular pathway in a similar manner to the intracellular model. The criterion for when DOL outperforms SC is given by the following expression:

$$\frac{\alpha_1\alpha_2 e_{1,e}^{DOL} e_{2,e}^{DOL} \upsilon_2}{\delta_{p_e}(\delta_{m_e} + \alpha_2 e_{2,e}^{DOL})} > \frac{\alpha_1\alpha_2 e_{1,e}^{SC} e_{2,e}^{SC} \upsilon}{\delta_{p_e}(\delta_{m_e} + \alpha_2 e_{2,e}^{SC})}. \quad \text{(S4.13)}$$

Through rearranging and simplifying the above expression and assuming that $\alpha_2 e_{2,e} \gg \delta_{m_e}$ (which is true for most biological parameters), we obtain the general form of the criterion for an extracellular pathway in Figure 3C where $\theta_E = \frac{\delta_{e_{1,e}} + \delta_{e_{1,e}}\eta_1 + \eta_1\upsilon_1}{\delta_{e_{1,e}} + \delta_{e_{1,e}}\eta_1 + \eta_1\upsilon}$.

4.1.2 Two independent pathways inside the cell

The remaining models, unless otherwise noted, use the same dimensionless quantities as those in the base model and the extracellular pathway. Thus, we will only show the final dimensionless ODEs.

We derive a system of dimensionless ODEs detailing intermediate, product, and enzyme concentrations for two independent pathways catalyzed intracellularly by either a single population or two populations (Figure 3, #2). In the SC architecture, the ODEs are given by

$$\frac{dm}{d\tau} = \alpha_1 e_1 - \eta_1 (m - m_e), \quad \text{(S4.14)}$$

$$\frac{dm_e}{d\tau} = \upsilon\eta_1 (m - m_e) - \delta_{m_e} m_e, \quad \text{(S4.15)}$$

$$\frac{dp}{d\tau} = \alpha_2 e_2 - \eta_2 (p - p_e), \quad \text{(S4.16)}$$

$$\frac{dp_e}{d\tau} = \upsilon\eta_2 (p - p_e) - p_e, \quad \text{(S4.17)}$$

and in DOL are

$$\frac{dm}{d\tau} = \alpha_1 e_1 - \eta_1 (m - m_e), \quad \text{(S4.18)}$$

$$\frac{dm_e}{d\tau} = \upsilon_1\eta_1 (m - m_e) - \delta_{m_e} m_e, \quad \text{(S4.19)}$$

$$\frac{dp}{d\tau} = \alpha_2 e_2 - \eta_2(p - p_e), \quad \text{(S4.20)}$$

$$\frac{dp_e}{d\tau} = \upsilon_2 \eta_2 (p - p_e) - p_e. \quad \text{(S4.21)}$$

In order to compare the systems directly, we assume that the final objective is to produce some final product P_{final} that obeys the following ODE:

$$\frac{dp_{final}}{d\tau} = \alpha_{P_f} m_e p_e - \delta_{p_f} p_{final}. \quad \text{(S4.22)}$$

Modeling the objective function in this manner instead of including consumption terms in the ODEs reduces the nonlinearity of the steady-state solutions. This simplifies modeling analysis without significantly changing the parametric spaces where DOL is favored over SC. Using this function, the system that performs the best is the one that produces more total P_{final}. Introducing the steady state solutions for M and P, the criterion becomes

$$\frac{\alpha_{P_f}(\alpha_1 e_1 \upsilon_1^2)(\alpha_2 e_2 \upsilon_2^2)}{\delta_{m_e}\delta_{p_f}} > \frac{\alpha_{P_f}(\alpha_1 e_1 \upsilon^2)(\alpha_2 e_2 \upsilon^2)}{\delta_{m_e}\delta_{p_f}} \quad \text{(S4.23)}$$

which, when simplified, results in the corresponding criterion in Figure 3C.

4.1.3 Two independent pathways outside the cell

We derive a system of dimensionless ODEs detailing intermediate, product, and enzyme concentrations for two independent pathways catalyzed outside the cell by either a single population or two populations (Figure 3, #3). In the SC architecture, the ODEs are given by

$$\frac{dm_e}{d\tau} = \alpha_1 e_{1,e} - \delta_{m_e} m_e, \quad \text{(S4.24)}$$

$$\frac{dp_e}{d\tau} = \alpha_2 e_{2,e} - \delta_{p_e} p_e, \quad \text{(S4.25)}$$

$$\frac{de_1}{d\tau} = r_{e_1} - e_1 - \eta_1(e_1 - e_{1,e}), \quad \text{(S4.26)}$$

$$\frac{de_2}{d\tau} = r_{e_2} - \delta_e e_2 - \eta_2(e_2 - e_{2,e}), \quad \text{(S4.27)}$$

$$\frac{de_{1,e}}{d\tau} = \upsilon\eta_1\left(e_1 - e_{1,e}\right) - \delta_{e_{1,e}} e_{1,e}, \quad \text{(S4.28)}$$

$$\frac{de_{2,e}}{d\tau} = \upsilon\eta_2\left(e_2 - e_{2,e}\right) - \delta_{e_{2,e}} e_{2,e}, \quad \text{(S4.29)}$$

and in DOL are

$$\frac{dm_e}{d\tau} = \alpha_1 e_{1,e} - \delta_{m_e} m_e, \quad \text{(S4.30)}$$

$$\frac{dp_e}{d\tau} = \alpha_2 e_{2,e} - \delta_{p_e} p_e, \quad \text{(S4.31)}$$

$$\frac{de_1}{d\tau} = r_{e_1} - e_1 - \eta_1(e_1 - e_{1,e}), \quad \text{(S4.32)}$$

$$\frac{de_2}{d\tau} = r_{e_2} - \delta_e e_2 - \eta_2(e_2 - e_{2,e}), \quad \text{(S4.33)}$$

$$\frac{de_{1,e}}{d\tau} = \upsilon_1\eta_1\left(e_1 - e_{1,e}\right) - \delta_{e_{1,e}} e_{1,e}, \quad \text{(S4.34)}$$

$$\frac{de_{2,e}}{d\tau} = \upsilon_2\eta_2\left(e_2 - e_{2,e}\right) - \delta_{e_{2,e}} e_{2,e}. \quad \text{(S4.35)}$$

Like the case of independent pathways inside the cell, we assume that the final objective is to produce some final product P_{final} which obeys the following ODE:

$$\frac{dp_{final}}{d\tau} = \alpha_{P_f} m_e p_e - \delta_{p_f} p_{final}. \quad \text{(S4.36)}$$

Introducing the steady state solutions for M and P, the criterion becomes

$$\frac{\alpha_{P_f}(\alpha_1 e_{1,e}\upsilon_1)(\alpha_2 e_{2,e}\upsilon_2)}{\delta_{m_e}\delta_{p_e}\delta_{p_f}} > \frac{\alpha_{P_f}(\alpha_1 e_{1,e}\upsilon)(\alpha_2 e_{2,e}\upsilon)}{\delta_{m_e}\delta_{p_e}\delta_{p_f}}. \quad \text{(S4.37)}$$

Through rearranging and simplifying the above expression, we obtain the general form of the criterion for two independent pathways occurring outside the cell in Figure 3C where $\theta_{EI} = \sqrt{\left(\frac{\delta_{e_{1,e}} + \delta_{e_{1,e}}\eta_1 + \eta_1\upsilon_1}{\delta_{e_{1,e}} + \delta_{e_{1,e}}\eta_1 + \eta_1\upsilon}\right)\left(\frac{\delta_e\delta_{e_{2,e}} + \delta_{e_{2,e}}\eta_2 + \delta_e\eta_2\upsilon_2}{\delta_e\delta_{e_{2,e}} + \delta_{e_{2,e}}\eta_2 + \delta_e\eta_2\upsilon}\right)}$.

4.1.4 Exchange of metabolites between populations

In certain examples of division of labor, two populations each produce a single metabolite, which they exchange with one another in a phenomenon known as cross-feeding. In contrast, the equivalent single population can produce both metabolites for itself. The set of dimensionless ODEs describing the SC system is identical to S4.14-S4.17. However, the set of ODEs describing the DOL system is given by

$$\frac{dm_1}{d\tau} = \alpha_1 e_1 - \eta_1(m_1 - m_e), \quad \text{(S4.38)}$$
$$\frac{dm_e}{d\tau} = \upsilon_1\eta_1(m_1 - m_e) - \upsilon_2\eta_1(m_e - m_2) - \delta_{m_e}m_e \ , \quad \text{(S4.39)}$$
$$\frac{dm_2}{d\tau} = \eta_1(m_e - m_2) - \delta_m m_2, \quad \text{(S4.40)}$$
$$\frac{dp_2}{d\tau} = \alpha_2 e_2 - \eta_2(p_2 - p_e), \quad \text{(S4.41)}$$
$$\frac{dp_e}{d\tau} = \upsilon_1\eta_2(p_2 - p_e) - \upsilon_2\eta_2(p_e - p_1) - \delta_{p_e}p_e, \quad \text{(S4.42)}$$
$$\frac{dp_1}{d\tau} = \eta_2(p_e - p_1) - p_1. \quad \text{(S4.43)}$$

Since each metabolite ends up in a different cell, we cannot use the same objective function of producing a third product. As a result, we use two criteria to compare which system has the highest yield. Specifically, DOL will outperform SC in this architecture if it produces more of both metabolites. Thus, the general forms of the inequalities for both M and P, respectively, are

$$\frac{\alpha_1 e_1\eta_1\upsilon_1\upsilon_2}{\delta_m\delta_{m_e}+\delta_{m_e}\eta_1+\delta_m\eta_1\upsilon_2} > \frac{\alpha_1 e_1(\delta_{m_e}+\eta_1\upsilon)\upsilon}{\delta_{m_e}\eta_1}, \quad \text{(S4.44)}$$
$$\frac{\alpha_2 e_2\eta_2\upsilon_1\upsilon_2}{\delta_{p_e}+\delta_{p_e}\eta_2+\eta_2\upsilon_1} > \frac{\alpha_2 e_2(1+\eta_2\upsilon)\upsilon}{\eta_2}, \quad \text{(S4.45)}$$

which, when simplified, result in two separate criteria, one for each participating population. These criteria are of the same form as the criterion in the base model, except $\theta_{CF1} = \frac{\delta_m\delta_{m_e}+\delta_{m_e}\eta_1+\delta_m\eta_1\upsilon_2}{\delta_{m_e}\eta_1}$ and $\theta_{CF2} = \frac{\delta_{p_e}+\delta_{p_e}\eta_2+\eta_2\upsilon_1}{\eta_2}$.

4.1.5 Hybrid pathway 1 – intracellular first step and extracellular second step

A hybrid intracellular and extracellular two-step, two-enzyme pathway can occur in two possible configurations (Figure 3, #4). We start with the example of a hybrid pathway where the first step occurs inside the cell(s) and the second step occurs outside the cell(s). The set of dimensionless ODEs describing the equivalent SC system is the following:

$$\frac{dm}{d\tau} = \alpha_1 e_1 - \eta_1(m - m_e), \quad \text{(S4.46)}$$
$$\frac{dm_e}{d\tau} = \upsilon\eta_1(m - m_e) - \delta_{m_e}m_e - \alpha_2 e_{2,e}m_e, \quad \text{(S4.47)}$$
$$\frac{dp_e}{d\tau} = \alpha_2 e_{2,e}m_e - \delta_{p_e}p_e, \quad \text{(S4.48)}$$
$$\frac{de_2}{d\tau} = r_{e_2} - \delta_e e_2 - \eta_2(e_2 - e_{2,e}), \quad \text{(S4.49)}$$
$$\frac{de_{2,e}}{d\tau} = \upsilon\eta_2\left(e_2 - e_{2,e}\right) - \delta_{e_{2,e}}e_{2,e}, \quad \text{(S4.50)}$$

where δ_e is the relative degradation rate of the second enzyme to the intermediate. The set of dimensionless ODEs describing the equivalent DOL system is the following:

$$\frac{dm}{d\tau} = \alpha_1 e_1 - \eta_1(m - m_e), \quad \text{(S4.51)}$$
$$\frac{dm_e}{d\tau} = \upsilon_1\eta_1(m - m_e) - \delta_{m_e}m_e - \alpha_2 e_{2,e}m_e, \quad \text{(S4.52)}$$
$$\frac{dp_e}{d\tau} = \alpha_2 e_{2,e}m_e - \delta_{p_e}p_e, \quad \text{(S4.53)}$$
$$\frac{de_2}{d\tau} = r_{e_2} - \delta_e e_2 - \eta_2(e_2 - e_{2,e}), \quad \text{(S4.54)}$$
$$\frac{de_{2,e}}{d\tau} = \upsilon_2\eta_2\left(e_2 - e_{2,e}\right) - \delta_{e_{2,e}}e_{2,e}. \quad \text{(S4.55)}$$

Using the same objective function of highest yield, we can write the general form of the criterion as

$$\frac{\alpha_1\alpha_2 e_1 e_{2,e}^{DOL}\upsilon_1\upsilon_2}{\delta_{p_e}(\delta_{m_e}+\alpha_2 e_{2,e}^{DOL})} > \frac{\alpha_1\alpha_2 e_1 e_{2,e}^{SC}\upsilon^2}{\delta_{p_e}(\delta_{m_e}+\alpha_2 e_{2,e}^{SC})} \quad \text{(S4.56)}$$

where, by implementing the assumption that $\alpha_2 e_{2,e} \gg \delta_{m_e}$, we obtain the design criterion in Figure 3C.

4.1.6 Hybrid pathway 2 – extracellular first step and intracellular second step

We now model the other example of a hybrid pathway, in which the first step now occurs extracellularly and the second step occurs intracellularly (Figure 3, #5). The set of dimensionless ODEs describing the equivalent SC system is the following:

$$\frac{dm_e}{d\tau} = \alpha_1 e_{1,e} - \delta_{m_e}m_e - \upsilon\eta_1(m_e - m), \quad \text{(S4.57)}$$
$$\frac{dm}{d\tau} = \eta_1(m_e - m) - \alpha_2 e_2 m, \quad \text{(S4.58)}$$
$$\frac{dp}{d\tau} = \alpha_2 e_2 m - \delta_p p, \quad \text{(S4.59)}$$

$$\frac{de_1}{d\tau} = r_{e_1} - \delta_e e_1 - \eta_2(e_1 - e_{1,e}), \quad \text{(S4.60)}$$

$$\frac{de_{1,e}}{d\tau} = \upsilon\eta_2\left(e_1 - e_{1,e}\right) - \delta_{e_{1,e}} e_{1,e}, \quad \text{(S4.61)}$$

where δ_e is the relative degradation rate of the first enzyme to the intermediate. The corresponding dimensionless ODEs describing the equivalent DOL system are given by

$$\frac{dm_e}{d\tau} = \alpha_1 e_{1,e} - \delta_{m_e} m_e - \upsilon_2\eta_1(m_e - m), \quad \text{(S4.62)}$$

$$\frac{dm}{d\tau} = \eta_1(m_e - m) - \alpha_2 e_2 m, \quad \text{(S4.63)}$$

$$\frac{dp}{d\tau} = \alpha_2 e_2 m - \delta_p p, \quad \text{(S4.64)}$$

$$\frac{de_1}{d\tau} = r_{e_1} - \delta_e e_1 - \eta_2(e_1 - e_{1,e}), \quad \text{(S4.65)}$$

$$\frac{de_{1,e}}{d\tau} = \upsilon_1\eta_2\left(e_1 - e_{1,e}\right) - \delta_{e_{1,e}} e_{1,e}. \quad \text{(S4.66)}$$

From these equations, the general form of the criterion dictating when DOL is favored over SC is given by

$$\frac{\alpha_1 e_{1,e}^{DOL}\eta_1\upsilon_2}{\delta_p\left(\delta_{m_e} + \frac{\delta_{m_e}\eta_1}{\alpha_2 e_2} + \eta_1\upsilon_2\right)} > \frac{\alpha_1 e_{1,e}^{SC}\eta_1\upsilon}{\delta_p\left(\delta_{m_e} + \frac{\delta_{m_e}\eta_1}{\alpha_2 e_2} + \eta_1\upsilon\right)}. \quad \text{(S4.67)}$$

The above criterion is then reduced via algebra to the criterion in Figure 3C where $\theta_{H_2} = \left(\frac{\delta_{m_e} + \frac{\delta_{m_e}\eta_1}{\alpha_2 e_2} + \eta_1\upsilon_2}{\delta_{m_e} + \frac{\delta_{m_e}\eta_1}{\alpha_2 e_2} + \eta_1\upsilon}\right)\left(\frac{\delta_{e_{1,e}} + \delta_{e_{1,e}}\eta_2 + \eta_2\upsilon_1}{\delta_{e_{1,e}} + \delta_{e_{1,e}}\eta_2 + \eta_2\upsilon}\right)$.

Supplemental Section 5. Pathways longer than two steps

5.1 Derivation of SC and DOL models and corresponding design criteria for pathways longer than two steps

5.1.1 3-step intracellular pathway and arbitrary length intracellular pathway

A 3-step intracellular pathway can have up to 4 different possible configurations. However, our inequalities specifically compare only two systems. As a result, we look to derive a criterion comparing a full SC pathway (one population) and a full DOL pathway (3 populations) for this system. Maintaining the same assumptions as the base model, the dimensionless rates of change of intracellular and extracellular products in the 3-step SC model is given by

$$\frac{dm_1}{d\tau} = \alpha_1 e_1 - \eta_1(m_1 - m_{1,e}) - \alpha_2 e_2 m_1, \quad \text{(S5.1)}$$
$$\frac{dm_{1,e}}{d\tau} = \upsilon\eta_1(m_1 - m_{1,e}) - \delta_{m_{1,e}} m_{1,e}, \quad \text{(S5.2)}$$
$$\frac{dm_2}{d\tau} = \alpha_2 e_2 m_1 - \eta_2(m_2 - m_{2,e}) - \alpha_3 e_3 m_2, \quad \text{(S5.3)}$$
$$\frac{dm_{2,e}}{d\tau} = \upsilon\eta_2(m_2 - m_{2,e}) - \delta_{m_{2,e}} m_{2,e}, \quad \text{(S5.4)}$$
$$\frac{dp}{d\tau} = \alpha_3 e_3 m_2 - p, \quad \text{(S5.5)}$$

where δ_i is the relative degradation of the ith (i=1,2,3) metabolite to the first metabolite. In the 3-step DOL model, the rates of change are given by

$$\frac{dm_1}{d\tau} = \alpha_1 e_1 - \eta_1(m_1 - m_{1,e}), \quad \text{(S5.6)}$$
$$\frac{dm_{1,e}}{d\tau} = \upsilon_1\eta_1(m_1 - m_{1,e}) - \upsilon_2\eta_1(m_{1,e} - m_{1,2}) - \delta_{m_{1,e}} m_{1,e}, \quad \text{(S5.7)}$$
$$\frac{dm_{1,2}}{d\tau} = \eta_1(m_{1,e} - m_{1,2}) - \alpha_2 e_2 m_{1,2}, \quad \text{(S5.8)}$$
$$\frac{dm_2}{d\tau} = \alpha_2 e_2 m_{1,2} - \eta_2(m_2 - m_{2,e}), \quad \text{(S5.9)}$$
$$\frac{dm_{2,e}}{d\tau} = \upsilon_2\eta_2(m_2 - m_{2,e}) - \upsilon_3\eta_2(m_{2,e} - m_{2,3}) - \delta_{m_{2,e}} m_{2,e}, \quad \text{(S5.10)}$$
$$\frac{dm_{2,3}}{d\tau} = \eta_2(m_{2,e} - m_{2,3}) - \alpha_3 e_3 m_{2,3}, \quad \text{(S5.11)}$$
$$\frac{dp}{d\tau} = \alpha_3 e_3 m_{2,3} - p. \quad \text{(S5.12)}$$

We derive the general form of the criterion using the same methods as the base model. This criterion can be simplified using algebra into the following expression

$$\frac{\upsilon_1\upsilon_2\upsilon_3}{\upsilon^3 + \varepsilon\upsilon} > \theta_{I,3}\ , \quad \text{(S5.13)}$$

where $\varepsilon = \frac{(\delta_{m_{1,e}} + \eta_1\upsilon)(\delta_{m_{2,e}} + \eta_2\upsilon)}{\eta_1\eta_2}$ and $\theta_{I,3} = \left(\frac{\alpha_2 e_2 \delta_{m_{1,e}} + \delta_{m_1}\eta_1 + \alpha_2 e_2 \eta_1 \upsilon_2}{\alpha_2 e_2 \delta_{m_{1,e}} + \delta_{m_1}\eta_1 + \alpha_2 e_2 \eta_1 \upsilon}\right)\left(\frac{\alpha_3 e_3 \delta_{m_{2,e}} + \delta_{m_2}\eta_2 + \alpha_3 e_3 \eta_2 \upsilon_2}{\alpha_3 e_3 \delta_{m_2} + \delta_{m_2}\eta_2 + \alpha_3 e_3 \eta_2 \upsilon}\right)$. From this criterion, we derive the following general criterion for an intracellular pathway of N steps (Figure S5A and Figure S5B):

$$\frac{\bar{\upsilon}_{DOL}^N}{\upsilon^N + \varepsilon\upsilon} > \theta_{I,N}, \quad \text{(S5.14)}$$

where $\bar{\upsilon}_{DOL}^N = \prod_i^N \upsilon_i$. ε and $\theta_{I,N}$ will have a unique expression depending on the number of steps in the pathway.

5.1.2 3-step extracellular pathway and arbitrary length extracellular pathway

A 3-step extracellular pathway can also have up to 4 different possible configurations. Like the intracellular case, we derive a design criterion comparing only the full SC and DOL extracellular pathway architectures (Figure S5C and Figure S5D). Thus, the dimensionless ODEs describing a 3-step extracellular SC pathway are given by

$$\frac{dm_{1,e}}{d\tau} = \alpha_1 e_{1,e} - \alpha_2 e_{2,e} m_{1,e} - \delta_{m_{1,e}} m_{1,e}, \quad \text{(S5.15)}$$
$$\frac{dm_{2,e}}{d\tau} = \alpha_2 e_{2,e} m_{1,e} - \alpha_3 e_{3,e} m_{2,e} - \delta_{m_{2,e}} m_{2,e}, \quad \text{(S5.16)}$$
$$\frac{dp_e}{d\tau} = \alpha_3 e_{3,e} m_{2,e} - \delta_p p_e, \quad \text{(S5.17)}$$
$$\frac{de_1}{d\tau} = r_{e_1} - e_1 - \eta_1(e_1 - e_{1,e}), \quad \text{(S5.18)}$$

$$\frac{de_2}{d\tau} = r_{e_2} - \delta_2 e_2 - \eta_2(e_2 - e_{2,e}), \quad \text{(S5.19)}$$
$$\frac{de_3}{d\tau} = r_{e_3} - \delta_3 e_3 - \eta_3(e_3 - e_{3,e}), \quad \text{(S5.20)}$$
$$\frac{de_{1,e}}{d\tau} = \upsilon\eta_1(e_1 - e_{1,e}) - \delta_{e_{1,e}} e_{1,e}, \quad \text{(S5.21)}$$
$$\frac{de_{2,e}}{d\tau} = \upsilon\eta_2(e_2 - e_{2,e}) - \delta_{e_{2,e}} e_{2,e}, \quad \text{(S5.22)}$$
$$\frac{de_{3,e}}{d\tau} = \upsilon\eta_3(e_3 - e_{3,e}) - \delta_{e_{3,e}} e_{3,e}. \quad \text{(S5.23)}$$

The corresponding dimensionless ODEs describing the equivalent 3-step extracellular DOL pathway are given by

$$\frac{dm_{1,e}}{d\tau} = \alpha_1 e_{1,e} - \alpha_2 e_{2,e} m_{1,e} - \delta_{m_{1,e}} m_{1,e}, \quad \text{(S5.24)}$$
$$\frac{dm_{2,e}}{d\tau} = \alpha_2 e_{2,e} m_{1,e} - \alpha_3 e_{3,e} m_{2,e} - \delta_{m_{2,e}} m_{2,e}, \quad \text{(S5.25)}$$
$$\frac{dp_e}{d\tau} = \alpha_3 e_{3,e} m_{2,e} - \delta_p p_e, \quad \text{(S5.26)}$$
$$\frac{de_1}{d\tau} = r_{e_1} - e_1 - \eta_1(e_1 - e_{1,e}), \quad \text{(S5.27)}$$
$$\frac{de_2}{d\tau} = r_{e_2} - \delta_2 e_2 - \eta_2(e_2 - e_{2,e}), \quad \text{(S5.28)}$$
$$\frac{de_3}{d\tau} = r_{e_3} - \delta_3 e_3 - \eta_3(e_3 - e_{3,e}), \quad \text{(S5.29)}$$
$$\frac{de_{1,e}}{d\tau} = \upsilon_1\eta_1(e_1 - e_{1,e}) - \delta_{e_{1,e}} e_{1,e}, \quad \text{(S5.30)}$$
$$\frac{de_{2,e}}{d\tau} = \upsilon_2\eta_2(e_2 - e_{2,e}) - \delta_{e_{2,e}} e_{2,e}, \quad \text{(S5.31)}$$
$$\frac{de_{3,e}}{d\tau} = \upsilon_3\eta_3(e_3 - e_{3,e}) - \delta_{e_{3,e}} e_{3,e}. \quad \text{(S5.32)}$$

Again, we derive the general form of the criterion using the same methods as the base model:

$$\frac{\alpha_1\alpha_2\alpha_3 e_{1,e}^{DOL} e_{2,e}^{DOL} e_{3,e}^{DOL} \upsilon_2}{\delta_p(\delta_{m_{1,e}} + \alpha_2 e_{2,e}^{DOL})(\delta_{m_{2,e}} + \alpha_3 e_{3,e}^{DOL})} > \frac{\alpha_1\alpha_2\alpha_3 e_{1,e}^{SC} e_{2,e}^{SC} e_{3,e}^{SC} \upsilon}{\delta_p(\delta_{m_{1,e}} + \alpha_2 e_{2,e}^{SC})(\delta_{m_{2,e}} + \alpha_3 e_{3,e}^{SC})}. \quad \text{(S5.33)}$$

We further simplify this expression and assume $\alpha_2 \gg \delta_{m_1}$ and $\alpha_3 \gg \delta_{m_2}$ to obtain the following form:

$$\frac{\upsilon_1\upsilon_3}{\upsilon^2} > \theta_{E,3} \quad \text{(S5.34)}$$

where $\theta_{E,3} = \frac{\delta_{e_{1,e}} + \delta_{e_{1,e}}\eta_1 + \eta_1\upsilon_1}{\delta_{e_{1,e}} + \delta_{e_{1,e}}\eta_1 + \eta_1\upsilon}$. Unlike the intracellular case, the only two populations that matter are the first and last population, and the threshold is constant regardless of the pathway length. From this, we determine the following general criterion for an arbitrary length extracellular pathway:

$$\frac{\upsilon_1\upsilon_N}{\upsilon^2} > \theta_{E,N}. \quad \text{(S5.35)}$$

$\theta_{E,N}$ changes depending on the length of the pathway.

5.2 Comparing all configurations of a three-step intracellular pathway

Pathways longer than two steps can have other architectures than complete SC or DOL. For example, a three-step, three-enzyme pathway can have two hybrid architectures that incorporate both SC and DOL design elements. As a result, using the three-step pathway as an example, we compare all different variations to verify our conclusions from the design criterion (Figure S6).

5.2.1 Two steps in first population

In the first hybrid case (2), the first population contains two enzymes catalyzing the first two steps of the pathway, and the second population contains the last enzyme producing the final product (Figure S6A, #2). The set of dimensionless ODEs for product concentrations in this system is thus

$$\frac{dm_1}{d\tau} = \alpha_1 e_1 - \eta_1(m_1 - m_{1,e}) - \alpha_2 e_2 m_1, \quad \text{(S5.36)}$$
$$\frac{dm_{1,e}}{d\tau} = \upsilon\eta_1(m_1 - m_{1,e}) - \delta_{m_{1,e}} m_{1,e}, \quad \text{(S5.37)}$$
$$\frac{dm_2}{d\tau} = \alpha_2 e_2 m_1 - \eta_2(m_2 - m_{2,e}), \quad \text{(S5.38)}$$
$$\frac{dm_{2,e}}{d\tau} = \upsilon_1\eta_2(m_2 - m_{2,e}) - \upsilon_2\eta_2(m_{2,e} - m_{2,2}) - \delta_{m_{2,e}} m_{2,e}, \quad \text{(S5.39)}$$
$$\frac{dm_{2,2}}{d\tau} = \eta_2(m_{2,e} - m_{2,2}) - e_3 m_{2,2}, \quad \text{(S5.40)}$$
$$\frac{dp}{d\tau} = \alpha_3 e_3 m_{2,2} - p. \quad \text{(S5.41)}$$

The per cell productivity, or the steady state concentration of P, is then calculated to be

$$p = \frac{\alpha_1 e_1 (\delta_{m_{1,e}} + \eta_1 \upsilon_1) \eta_2 \upsilon_2}{\left(\delta_{m_{1,e}} + \frac{\delta_{m_{1,e}} \eta_1}{\alpha_2 e_2} + \eta_1 \upsilon_1\right)\left(\delta_{m_{2,e}} + \frac{\delta_{m_{2,e}} \eta_2}{\alpha_3 e_3} + \eta_2 \upsilon_2\right)}. \quad \text{(S5.42)}$$

5.2.2 Two steps in second population

In the second two-population case (3), the roles of the first and second populations are switched, such that the second population contains the last two steps of the pathway (Figure S6A, #3). While the two hybrids look similar, this hybrid will have a lower cell density since the second population contains two enzymes (and thus twice the metabolic burden of the first hybrid). The set of dimensionless ODEs is given by

$$\frac{dm_1}{d\tau} = \alpha_1 e_1 - \eta_1 (m_1 - m_{1,e}), \quad \text{(S5.43)}$$

$$\frac{dm_{1,e}}{d\tau} = \upsilon_1 \eta_1 (m_1 - m_{1,e}) - \upsilon_2 \eta_1 (m_{1,e} - m_{1,2}) - \delta_{m_{1,e}} m_{1,e}, \quad \text{(S5.44)}$$

$$\frac{dm_{1,2}}{d\tau} = \eta_1 (m_{1,e} - m_{1,2}) - \alpha_2 e_2 m_{1,2}, \quad \text{(S5.45)}$$

$$\frac{dm_2}{d\tau} = \alpha_2 e_2 m_{1,2} - \eta_2 (m_2 - m_{2,e}) - \alpha_3 e_3 m_2, \quad \text{(S5.46)}$$

$$\frac{dm_{2,e}}{d\tau} = \upsilon_2 \eta_2 (m_2 - m_{2,e}) - \delta_{m_{2,e}} m_{2,e}, \quad \text{(S5.47)}$$

$$\frac{dp}{d\tau} = \alpha_3 e_3 m_{2,2} - p. \quad \text{(S5.48)}$$

The per cell productivity is then calculated to be

$$p = \frac{\alpha_1 e_1 \eta_1 \upsilon_1 (\delta_{m_{2,e}} + \eta_2 \upsilon_2)}{\left(\delta_{m_{1,e}} + \frac{\delta_{m_{1,e}} \eta_1}{\alpha_2 e_2} + \eta_1 \upsilon_1\right)\left(\delta_{m_{2,e}} + \frac{\delta_{m_{2,e}} \eta_2}{\alpha_3 e_3} + \eta_2 \upsilon_2\right)}. \quad \text{(S5.49)}$$

5.2.3 Comparison of all the possible configurations via numerical simulation

Like our previous analysis, we construct a heat map comparing the final product yield of each system for varying β and η (labeled **1**-**4** in Figure S6B) and other core parameters. We compare this to our analysis of the base model to verify our design principles. Like the base models, **1** maintains the highest efficiency, and **4** has the lowest efficiency. As a result, high η still reduces the overall efficiency of a pathway, and additional transport steps reduce it further. Second, **1** still crashes at the lowest threshold burden, meaning that dividing this larger pathway reduces the burden per population, and **4** is still the most favored for high burden. Thus, our design principles remain consistent from the base model's criterion.

Interestingly, our analysis reveals that complete division of labor is also favored for intermediate levels of burden. By comparison, increasing burden beyond intermediate levels favors **2**, whereas decreasing burden below intermediate levels favors **3**. This is because the two population systems represent a "best of both worlds" middle ground, maintaining similar cell density to **4** while having higher efficiency from one less transport step. Thus, the ideal architecture depends on the tradeoff between cell density and efficiency, which is more pronounced for moderate levels of burden (the extremes stay the same). This demonstrates the robustness of our conclusions for any pathway length or configuration.

Supplemental Section 6. Supplemental References

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