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Research article

Dynamic modeling of internal and external metabolites with energetic and oxidative agents in hyaluronic acid production by *Streptococcus equi* subsp. *zooepidemicus*

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Supplementary

1. Analysis of the proposed model

1.1. State space representation

The balances given in Eqs 2.1–2.13, together with the specific flow of extracellular glucose into the cells and the reaction rates given in Eqs 2.14–2.24 can be written in compact form as

$$\dot{\mathbf{x}} = f(\mathbf{x}, \boldsymbol{\lambda}) \tag{1.1}$$

where $\mathbf{x} \in \mathbb{R}_+^{13}$ and $\boldsymbol{\lambda} \in \mathbb{R}_+^{19}$ are the state and parameter vectors, respectively, while $f : \mathbb{R}_+^{13} \times \mathbb{R}_+^{19} \to \mathbb{R}^{13}$ is a mapping for the kinetic and rate processes. Particularly, the form of x, $\boldsymbol{\lambda}$, and $f(\mathbf{x}, \boldsymbol{\lambda})$ are

$$\mathbf{x} = \begin{pmatrix} [\text{GLE}] \\ [\text{LA}] \\ [\text{HA}] \\ [\text{X}] \\ \zeta_{\text{GLU}} \\ \zeta_{\text{G6P}} \\ \zeta_{\text{F6P}} \\ \zeta_{\text{PEP}} \\ \zeta_{\text{PYR}} \\ \zeta_{\text{AGL}} \\ \zeta_{\text{NAG}} \\ \zeta_{\text{ATP}} \\ \zeta_{\text{NADH}} \end{pmatrix}, \ \boldsymbol{\lambda} = \begin{pmatrix} k_G \\ k_D \\ m \\ k_1 \\ k_2 \\ k_3 \\ k_4 \\ k_5 \\ k_6 \\ k_7 \\ k_8 \\ k_9 \\ k_{10} \\ \alpha \\ \beta \\ Y_{\text{G6P/PYR}} \\ Y_{\text{PEP/PYR}} \\ Y_{\text{G6P/X}} \\ Y_{\text{PEP/X}} \end{pmatrix}, \text{ and } f(\mathbf{x}, \boldsymbol{\lambda}) = \begin{pmatrix} -J_{\text{GL}} \\ r_5 \\ r_8 \\ Y_{\text{G6P/X}} r_9 + Y_{\text{PEP/X}} r_{10} - k_D [\text{X}] \\ -r_1 + J_{\text{GL}} \\ r_1 - r_2 - r_6 - Y_{\text{G6P/PYR}} r_9 \\ r_2 - r_3 - r_7 \\ 2r_3 - r_4 - Y_{\text{PEP/PYR}} r_{10} \\ r_4 - r_5 - r_9 - r_{10} \\ r_6 - r_8 \\ r_7 - r_8 \\ -r_1 + r_3 + r_4 - r_6 - r_7 \\ 2r_3 - r_5 + r_6 + r_9 + r_{10} \end{pmatrix}.$$

In terms of stoichiometric coefficients and rates $f(\mathbf{x}, \boldsymbol{\lambda})$ can be also expressed as

$$f(\mathbf{x}, \boldsymbol{\lambda}) = M(\boldsymbol{\lambda}) R(\mathbf{x}, \boldsymbol{\lambda}) \tag{1.2}$$

where

$$M(\boldsymbol{\lambda}) = \begin{pmatrix} M_e(\boldsymbol{\lambda}) \\ M_i(\boldsymbol{\lambda}) \end{pmatrix}$$

and

$$R(\mathbf{x}, \boldsymbol{\lambda}) = \begin{pmatrix} J_{\text{GL}} \\ r_1 \\ r_2 \\ r_3 \\ r_4 \\ r_5 \\ r_6 \\ r_7 \\ r_8 \\ r_9 \\ r_{10} \\ k_D [\text{X}] \end{pmatrix},$$

with

$$M_e(\boldsymbol{\lambda}) = \begin{pmatrix} -1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & Y_{\mathrm{G6P/X}} \\ 0 & 0 & 0 & Y_{\mathrm{PEP/X}} \\ 0 & 0 & 0 & -1 \end{pmatrix}^T$$

and

$$M_i(\boldsymbol{\lambda}) = \begin{pmatrix} 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ -1 & 1 & 0 & 0 & 0 & 0 & 0 & -1 & 0 \\ 0 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & -1 & 2 & 0 & 0 & 0 & 1 & 2 \\ 0 & 0 & 0 & -1 & 1 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & -1 \\ 0 & -1 & 0 & 0 & 0 & 1 & 0 & -1 & 1 \\ 0 & 0 & -1 & 0 & 0 & 0 & 1 & -1 & 0 \\ 0 & 0 & 0 & 0 & 0 & -1 & -1 & 0 & 0 \\ 0 & -Y_{\mathrm{G6P/PYR}} & 0 & 0 & -1 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & -Y_{\mathrm{PEP/PYR}} & -1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \end{pmatrix}^T$$

as the extra and intra-cellular stoichiometric coefficients.

The measure data are the extracellular molar concentrations of glucose, lactic acid, hyaluronic acid and the mass concentration of biomass, i.e., [GLE], [LA], [HA], and [X], therefore the output vector of measurements can be expressed as

$$\mathbf{y} = C\mathbf{x} \in \mathbb{R}_+^{13} \tag{1.3}$$

where $C = \begin{pmatrix} \mathbf{I}_4 & \mathbf{0}_{4\times 9} \end{pmatrix}$, with $\mathbf{I}_4$ as the identity matrix of dimension 4.

1.2. Quasi-steady state simplification

Under the assumption of quasi-steady state for the intracellular dynamics it holds that

$$\mathbf{0} = M_i(\boldsymbol{\lambda})\, R(\mathbf{x}, \boldsymbol{\lambda}) \tag{1.4}$$

while the dynamical behavior of the extracellular concentration is

$$\dot{\mathbf{y}} = M_e(\boldsymbol{\lambda})\, R(\mathbf{x}, \boldsymbol{\lambda}). \tag{1.5}$$

Equation 1.4 has more unknown reaction rates that equations, thus a solution as a function of r_1 and r_2 is:

$$\begin{pmatrix} J_{\mathrm{GL}} \\ r_3 \\ r_4 \\ r_5 \\ r_6 \\ r_7 \\ r_8 \\ r_9 \\ r_{10} \end{pmatrix} = \begin{pmatrix} 1 & 0 \\ -\frac{\sigma_2}{\sigma_1} & \frac{\sigma_1-\sigma_3}{\sigma_1} \\ \frac{\sigma_1+3\sigma_2}{\sigma_1} & \frac{3\sigma_3-\sigma_1}{\sigma_1} \\ \frac{\frac{\sigma_1}{2}+\sigma_2}{\sigma_1} & \frac{\frac{\sigma_1}{2}+\sigma_3}{\sigma_1} \\ \frac{\sigma_2}{\sigma_1} & \frac{\sigma_3}{\sigma_1} \\ \frac{\sigma_2}{\sigma_1} & \frac{\sigma_3}{\sigma_1} \\ \frac{\sigma_2}{\sigma_1} & \frac{\sigma_3}{\sigma_1} \\ \frac{\sigma_4}{\sigma_1} & \frac{\sigma_5}{\sigma_1} \\ \frac{\sigma_6}{\sigma_1} & \frac{\sigma_7}{\sigma_1} \end{pmatrix} \begin{pmatrix} r_1 \\ r_2 \end{pmatrix} \tag{1.6}$$

where

$$\begin{aligned} \sigma_1 &= 2\left(2 + \frac{1}{Y_{\mathrm{G6P/PYR}}} + \frac{5}{Y_{\mathrm{PEP/PYR}}}\right) \\ \sigma_2 &= \frac{2}{\mathrm{Y}_{\mathrm{G6P/PYR}}} - \frac{2}{Y_{\mathrm{PEP/PYR}}} - 1 \\ \sigma_3 &= 3 - \frac{2}{Y_{\mathrm{G6P/PYR}}} + \frac{6}{Y_{\mathrm{PEP/PYR}}} \\ \sigma_4 &= \frac{5}{4}(3\sigma_2 + \sigma_3) + Y_{\mathrm{G6P/PYR}}(6\sigma_1 - 21\sigma_2 + 15\sigma_3) \\ \sigma_5 &= -32\sigma_1 + 91\sigma_2 + 73\sigma_3 \\ \sigma_6 &= \frac{2\sigma_1 - 7\sigma_2 - 5\sigma_3}{4} - 3Y_{\mathrm{PEP/PYR}}(3\sigma_2 + \sigma_3) \\ \sigma_7 &= -6\sigma_1 + 69\sigma_2 + 31\sigma_3 \end{aligned}$$

are constants that only depend on stoichiometric coefficients.

From the first row of Eq 1.6 it is straightforward to obtain the intracellular glucose correlation as a function of the extracellular glucose concentration and the intracellular ATP correlation: $\zeta_{\mathrm{GLU}} = k_G\,[\mathrm{GLE}]\,/\,(k_G m + k_1\zeta_{\mathrm{ATP}})$. Therefore, the rate of reaction 1 becomes

$$r_1 = k_1 \frac{[\mathrm{GLE}]}{m + \frac{k_1}{k_G}\zeta_{\mathrm{ATP}}}\zeta_{\mathrm{ATP}} = J_{\mathrm{GL}}. \tag{1.7}$$

With the equation for r_6 given in Eq 2.20 and the fifth row of Eq 1.6 it holds that $\zeta_{\mathrm{G6P}} = \sigma_2 r_1\,/\,(\sigma_1 k_6 \zeta_{\mathrm{NAD^+}}\zeta_{\mathrm{ATP}} - \sigma_3 k_2)$, therefore r_2 is proportional to r_1 and so the remaining reaction rates, i.e.,

$$r_i = Y_i r_1 \tag{1.8}$$

where

$$\begin{aligned} Y_2 &= \frac{\sigma_2 k_2}{\sigma_1 k_6 \zeta_{\mathrm{NAD^+}}\zeta_{\mathrm{ATP}} - \sigma_3 k_2}, \\ Y_3 &= \left(1 - \frac{k_6}{k_2}\zeta_{\mathrm{NAD^+}}\zeta_{\mathrm{ATP}}\right) Y_2, \end{aligned}$$

$$
\begin{aligned}
Y_4 &= \left(\frac{k_6}{k_2}\frac{\sigma_1+3\sigma_2}{\sigma_2}\zeta_{\mathrm{NAD^+}}\zeta_{\mathrm{ATP}} - \frac{\sigma_2+\sigma_3}{\sigma_2}\right)Y_2,\\
Y_5 &= \frac{1}{2}\left(\frac{\sigma_2-\sigma_3}{\sigma_2} + \frac{k_6}{k_2}\frac{\sigma_1+2\sigma_2}{\sigma_2}\zeta_{\mathrm{NAD^+}}\zeta_{\mathrm{ATP}}\right)Y_2,\\
Y_6 &= Y_7 = Y_8 = \frac{k_6}{k_2}\zeta_{\mathrm{NAD^+}}\zeta_{\mathrm{ATP}}Y_2,\\
Y_9 &= \left(\frac{\sigma_2\sigma_5-\sigma_3\sigma_4}{\sigma_1\sigma_2} + \frac{k_6}{k_2}\frac{\sigma_4}{\sigma_2}\zeta_{\mathrm{NAD^+}}\zeta_{\mathrm{ATP}}+\right)Y_2, \text{ and}\\
Y_{10} &= \left(\frac{\sigma_2\sigma_7-\sigma_3\sigma_6}{\sigma_1\sigma_2} + \frac{k_6}{k_2}\frac{\sigma_6}{\sigma_2}\zeta_{\mathrm{NAD^+}}\zeta_{\mathrm{ATP}}\right)Y_2.
\end{aligned}
$$

Notice that all the reactions rates $r_2, r_3, \ldots, r_{10}$ are proportional to r_1, but with a variable yield coefficients that depend on the energetic and oxidative state of the microorganisms of the factor $\zeta_{\mathrm{NAD^+}}\zeta_{\mathrm{ATP}}$. Due to the relations given in Eqs 2.25 and 2.26, ζ_{ATP} is proportional to a fraction of the biomass concentration and reflects the energetic state inside the cells. Therefore, Eq 1.7 has the following limit cases for the glucose consumption: 1) when the cells has sufficient energy, in such a way that $\zeta_{\mathrm{ATP}} \gg k_G m/k_1$, the rate of glucose consumption approaches to $r_1 = k_G\,[\mathrm{GLE}]$ and only depends on the rate of glucose absorption, while for small cells' energy, i.e., $\zeta_{\mathrm{ATP}} \ll k_G m/k_1$, it holds that $r_1 = \frac{k_1}{m}\,[\mathrm{GLE}]\,\zeta_{\mathrm{ATP}} \propto [\mathrm{GLE}]\,[\mathrm{X}]$. Finally, the dynamical behavior of the extracellular variables given in Eq 1.5 can be expressed as:

$$
\begin{pmatrix} \frac{d[\mathrm{GLE}]}{dt} \\ \frac{d[\mathrm{LA}]}{dt} \\ \frac{d[\mathrm{HA}]}{dt} \\ \frac{d[\mathrm{X}]}{dt} \end{pmatrix} = \begin{pmatrix} -1 \\ Y_5 \\ Y_8 \\ Y_{\mathrm{G6P/X}}Y_9 + Y_{\mathrm{PEP/X}}Y_{10} \end{pmatrix} k_1 \frac{[\mathrm{GLE}]}{m + \frac{k_1}{k_G}\zeta_{\mathrm{ATP}}}\zeta_{\mathrm{ATP}} - \begin{pmatrix} 0 \\ 0 \\ 0 \\ k_D\,[\mathrm{X}] \end{pmatrix}. \tag{1.9}
$$

Therefore, under the assumption of quasi-steady state for the intracellular dynamics, the proposed model has only one reaction rate and variable biomass, lactic acid, and hyaluronic acid yield coefficients that depend on the energetic and oxidative state of the microorganisms, that according to the model can vary with the initial state of the microorganisms and the external glucose concentration. Obviously without the assumption of quasi-steady state the proposed model has a reacher behavior than that described by Eq 1.9.

2. Parameters estimation and interval of confidence

2.1. Parametric sensitivity matrix

To estimate the confidence intervals of the parameters of the proposed model, the covariance matrix must be calculated, for which an estimate of the output's parametric sensitivity matrix, $\frac{\partial \mathbf{y}}{\partial \boldsymbol{\lambda}} = C\frac{\partial \mathbf{x}}{\partial \boldsymbol{\lambda}} \in \mathbb{R}^{4\times 19}$, is necessary. Thus, to compute the state's parametric sensitivity matrix $\mathbf{S} = \frac{\partial \mathbf{x}}{\partial \boldsymbol{\lambda}} \in \mathbb{R}^{13\times 19}$, Equation 1.1 can be integrated together with the following equation:

$$
\dot{\mathbf{S}} = A\mathbf{S} + B, \qquad \mathbf{S}|_{t=0} = \mathbf{0} \tag{2.1}
$$

where $A = \frac{\partial f}{\partial \mathbf{x}} = M\frac{\partial R}{\partial \mathbf{x}} \in \mathbb{R}^{13\times 13}$ and $B = \frac{\partial f}{\partial \boldsymbol{\lambda}} = M\frac{\partial R}{\partial \boldsymbol{\lambda}} + W \in \mathbb{R}^{13\times 19}$, with $W = \left(\sum_{k=1}^{19}\frac{\partial M}{\partial \lambda_k}\right)R$.

2.2. Parameters estimation

The fitting procedure was performed with a least squares method using the Nealder-Mead minimization algorithm [35], with the following error [36]:

$$E = \sum_{i=1}^{N_e} \sum_{j=1}^{4} \sum_{k=1}^{10} w_{i,j} \left(y_{i,j,k} - \hat{y}_{i,j,k} \right)^2 \tag{2.2}$$

where $y_{i,j,k} = y_{i,j}(t_k)$ and $\hat{y}_{i,j,k} = \hat{y}_{i,j}(t_k)$ represent the data and the prediction at the k-th sampling time of the i-th experiment and the j-th measurement variable. N_e is the total number of experiments. Particularly, the experimental data from experiments 1, 2, 4, and 6 were used to estimate the parameters of the proposed model ($N_e = 4$). Finally, $w_{i,j}$ is the weight of the i-th experiment and the j-th measurement variable defined as:

$$w_{i,j} = \frac{1}{\left[\max_k \left(y_{i,j,k} \right) - \min_k \left(y_{i,j,k} \right) \right]^2}. \tag{2.3}$$

2.3. Interval of confidence

To estimate the parameters' confidence intervals all experimental runs were used ($N_e = 6$), thus the total number of observations are $4 \times 10 N_e$. The number of fitting parameter are 19 and the initial conditions were also estimated, thus the degrees of freedom are $\nu = 143$ and the total mean square error is $\text{MSE} = E/\nu$. With the aid of the sensitivity matrix, the Fisher matrix is

$$F = \sum_{i=1}^{N_e} \sum_{j=1}^{4} \sum_{k=1}^{10} w_{i,j} \left([SC]_{i,j,k} \right)^T [C\mathbf{S}]_{i,j,k} \tag{2.4}$$

where $[C\mathbf{S}]_{i,j,k} \in \mathbb{R}^{1\times 19}$ is the j-th row of the output's parametric sensitivity matrix at the k-th sampling time of the i-th experiment. The covariance matrix can be computed as $\text{COV} = F^{-1}\text{MSE} \in \mathbb{R}^{19\times 19}$ to estimate the confidence interval for each parameter, λ_i, $i = 1, 2, \ldots, 19$, as $\lambda_i \pm t_{1-\alpha/2,\nu} \sqrt{\text{COV}_{ii}}$, where $t_{1-\alpha,\nu}$ is the $100(1-\alpha)$ percent confidence interval of the Student's t distribution with ν degrees of freedom. Finally, the elements of the correlation matrix are

$$\rho_{i,j} = \frac{\text{COV}_{ij}}{\sqrt{\text{COV}_{ii}\text{COV}_{jj}}}. \tag{2.5}$$

In particular, for the proposed model and the experimental data for the 6 experimental runs the elements of the correlation matrix are shown in Table 1.

	k_G	k_D	m	k_1	k_2	k_3	k_4	k_5	k_6	k_7	k_8	k_9	k_{10}	α	β	$Y_{G6P/PYR}$	$Y_{PEP/PYR}$	$Y_{G6P/X}$	$Y_{PEP/X}$
k_G	1	0.00	0.54	0.01	0.01	-0.01	0.00	-0.00	0.01	0.01	-0.00	0.01	0.00	0.00	0.00	-0.00	-0.00	0.00	-0.00
k_D	0.00	1	0.00	0.35	0.59	-0.46	0.27	0.81	-0.18	-0.21	0.10	0.33	0.33	-0.04	0.61	-0.48	-0.36	-0.25	-0.28
m	0.54	0.00	1	0.00	0.00	-0.00	0.00	0.00	0.00	-0.00	-0.00	0.00	0.00	-0.00	0.00	-0.00	-0.00	-0.00	-0.00
k_1	0.01	0.35	0.00	1	0.81	-0.93	0.34	-0.21	0.72	0.40	-0.57	0.28	0.62	0.01	0.74	-0.70	-0.33	0.10	-0.53
k_2	0.01	0.59	0.00	0.81	1	-0.95	0.75	0.23	0.20	-0.17	-0.56	-0.07	0.90	-0.44	0.99	-0.97	-0.77	-0.42	-0.85
k_3	-0.01	-0.46	-0.00	-0.93	-0.95	1	-0.60	0.01	-0.48	-0.11	0.53	-0.08	-0.83	0.26	-0.91	0.89	0.60	0.17	0.75
k_4	0.00	0.27	0.00	0.34	0.75	-0.60	1	0.25	-0.28	-0.65	-0.57	-0.69	0.94	-0.92	0.81	-0.89	-0.99	-0.86	-0.97
k_5	-0.00	0.81	0.00	-0.21	0.23	0.01	0.25	1	-0.70	-0.62	0.28	0.03	0.11	-0.21	0.31	-0.21	-0.35	-0.48	-0.12
k_6	0.01	-0.18	0.00	0.72	0.20	-0.48	-0.28	-0.70	1	0.89	-0.23	0.51	0.03	0.51	0.08	-0.07	0.33	0.68	0.06
k_7	0.01	-0.21	-0.00	0.40	-0.17	-0.11	-0.65	-0.62	0.89	1	0.17	0.72	-0.38	0.80	-0.29	0.33	0.69	0.92	0.47
k_8	-0.00	0.10	-0.00	-0.57	-0.56	0.53	-0.57	0.28	-0.23	0.17	1	0.39	-0.62	0.46	-0.56	0.61	0.56	0.40	0.62
k_9	0.01	0.33	0.00	0.28	-0.07	-0.08	-0.69	0.03	0.51	0.72	0.39	1	-0.46	0.90	-0.16	0.31	0.65	0.81	0.57
k_{10}	0.00	0.33	0.00	0.62	0.90	-0.83	0.94	0.11	0.03	-0.38	-0.62	-0.46	1	-0.75	0.92	-0.98	-0.93	-0.66	-0.99
α	0.00	-0.04	-0.00	0.01	-0.44	0.26	-0.92	-0.21	0.51	0.80	0.46	0.90	-0.75	1	-0.53	0.65	0.91	0.94	0.83
β	0.00	0.61	0.00	0.74	0.99	-0.91	0.81	0.31	0.08	-0.29	-0.56	-0.16	0.92	-0.53	1	-0.98	-0.84	-0.53	-0.88
$Y_{G6P/PYR}$	-0.00	-0.48	-0.00	-0.70	-0.97	0.89	-0.89	-0.21	-0.07	0.33	0.61	0.31	-0.98	0.65	-0.98	1	0.90	0.60	0.95
$Y_{PEP/PYR}$	-0.00	-0.36	-0.00	-0.33	-0.77	0.60	-0.99	-0.35	0.33	0.69	0.56	0.65	-0.93	0.91	-0.84	0.90	1	0.89	0.96
$Y_{G6P/X}$	0.00	-0.25	-0.00	0.10	-0.42	0.17	-0.86	-0.48	0.68	0.92	0.40	0.81	-0.66	0.94	-0.53	0.60	0.89	1	0.73
$Y_{PEP/X}$	-0.00	-0.28	-0.00	-0.53	-0.85	0.75	-0.97	-0.12	0.06	0.47	0.62	0.57	-0.99	0.83	-0.88	0.95	0.96	0.73	1

Table 1. Parameter's correlation matrix.

2.4. Change of variables to have positive concentrations in the fitting procedure

From Eqs 2.25 and 2.26, it is possible to compute ζ_{ADP} and $\zeta_{\mathrm{NAD^+}}$ as follows:

$$\zeta_{\mathrm{ADP}} = \alpha\,[\mathrm{X}] - \zeta_{\mathrm{ATP}} - \zeta_{\mathrm{AGL}} - \zeta_{\mathrm{NAG}} \geq 0 \tag{2.6}$$

$$\zeta_{\mathrm{NAD^+}} = \beta\,[\mathrm{X}] - \zeta_{\mathrm{NADH}} \geq 0 \tag{2.7}$$

To guarantee that these concentrations are in a physically feasible range (positive), we propose to replace the dynamic for concentrations [AGL], [NAG], [ATP], and [NADH] with the auxiliary variables w_{ATP}, w_{AGL}, w_{NAG} y w_{NADH} correlated with the fraction of the biomass concentration for each species. Therefore, we propose the following change of variables:

$$\zeta_{\mathrm{AGL}} = \alpha\,(1-\phi\,(w_{\mathrm{ATP}}))\,\phi\,(w_{\mathrm{AGL}})\,[\mathrm{X}] \tag{2.8}$$

$$\zeta_{\mathrm{NAG}} = \alpha\,(1-\phi\,(w_{\mathrm{ATP}}))\,(1-\phi\,(w_{\mathrm{AGL}}))\,\phi\,(w_{\mathrm{NAG}})\,[\mathrm{X}] \tag{2.9}$$

$$\zeta_{\mathrm{ATP}} = \alpha\phi\,(w_{\mathrm{ATP}})\,[\mathrm{X}] \tag{2.10}$$

$$\zeta_{\mathrm{NADH}} = \beta\phi\,(w_{\mathrm{NADH}})\,[\mathrm{X}] \tag{2.11}$$

where

$$\phi\,(w) = \frac{1+\tanh\,(w)}{2}. \tag{2.12}$$

Notice that for $w \in (-\infty, \infty)$ it holds that $0 < \phi\,(w) < 1$ and $0 < 1-\phi\,(w) < 1$. Replacing Eqs 2.8–2.11 in Eqs 2.6 and 2.7 it holds that

$$\begin{aligned}\zeta_{\mathrm{ADP}} &= \alpha\,(1-\phi\,(w_{\mathrm{ATP}}))\,(1-\phi\,(w_{\mathrm{AGL}}))\,(1-\phi\,(w_{\mathrm{NAG}}))\,[\mathrm{X}] \geq 0,\\ \zeta_{\mathrm{NAD^+}} &= \beta\,(1-\phi\,(w_{\mathrm{NADH}}))\,[\mathrm{X}] \geq 0.\end{aligned}$$

Given the properties of function $\phi\,(w)$, it holds that $0 < \zeta_{\mathrm{ADP}} < \alpha\,[\mathrm{X}]$, $0 < \zeta_{\mathrm{ATP}} < \alpha\,[\mathrm{X}]$, $0 < \zeta_{\mathrm{AGL}} < \alpha\,[\mathrm{X}]$, and $0 < \zeta_{\mathrm{NADH}} < \alpha\,[\mathrm{X}]$ for any value of w_{ATP}, w_{AGL}, and w_{NAG}, fulfilling Eq (2.25), while $0 < \zeta_{\mathrm{NAD^+}} < \beta\,[\mathrm{X}]$ and $0 < \zeta_{\mathrm{NADH}} < \beta\,[\mathrm{X}]$, fulfilling Eq 2.26 for any value of w_{NADH}.

The dynamical behavior of the auxiliary variables: w_{ATP}, w_{AGL}, w_{NAG}, and w_{NADH} can be obtained by deriving Eqs (2.8)–(2.11) to obtain:

$$\frac{dw_{\mathrm{AGL}}}{dt} = \frac{\phi\,(w_{\mathrm{AGL}})}{\phi'\,(w_{\mathrm{AGL}})}\left[\frac{\frac{d\zeta_{\mathrm{AGL}}}{dt} + \phi\,(w_{\mathrm{AGL}})\left(\frac{d\zeta_{\mathrm{ATP}}}{dt} - A\frac{d[\mathrm{X}]}{dt}\right)}{\zeta_{\mathrm{AGL}}}\right] \tag{2.13}$$

$$\frac{dw_{\mathrm{NAG}}}{dt} = \frac{\phi\,(w_{\mathrm{NAG}})}{\phi'\,(w_{\mathrm{NAG}})}\left[\frac{\frac{d\zeta_{\mathrm{NAG}}}{dt} + \phi\,(w_{\mathrm{NAG}})\left(\frac{d\zeta_{\mathrm{AGL}}}{dt} + \frac{d\zeta_{\mathrm{ATP}}}{dt} - A\frac{d[\mathrm{X}]}{dt}\right)}{\zeta_{\mathrm{NAG}}}\right] \tag{2.14}$$

$$\frac{dw_{\mathrm{ATP}}}{dt} = \frac{\phi\,(w_{\mathrm{ATP}})}{\phi'\,(w_{\mathrm{ATP}})}\left[\frac{1}{\zeta_{\mathrm{ATP}}}\frac{d\zeta_{\mathrm{ATP}}}{dt} - \frac{1}{[\mathrm{X}]}\frac{d\,[\mathrm{X}]}{dt}\right] \tag{2.15}$$

$$\frac{dw_{\mathrm{NADH}}}{dt} = \frac{\phi\,(w_{\mathrm{NADH}})}{\phi'\,(w_{\mathrm{NADH}})}\left[\frac{1}{\zeta_{\mathrm{NADH}}}\frac{d\zeta_{\mathrm{NADH}}}{dt} - \frac{1}{[\mathrm{X}]}\frac{d\,[\mathrm{X}]}{dt}\right] \tag{2.16}$$

where

$$\phi'\,(w) = \frac{1}{2\cosh^2\,(w)}$$

is the derivative of function $\phi(w)$, while the expressions $\frac{d[\mathrm{X}]}{dt}$, $\frac{d\zeta_{\mathrm{AGL}}}{dt}$, $\frac{d\zeta_{\mathrm{NAG}}}{dt}$, $\frac{d\zeta_{\mathrm{ATP}}}{dt}$, and $\frac{d\zeta_{\mathrm{NADH}}}{dt}$ necessary in Eqs 2.13–2.16 are given in Eqs 2.10–2.13. It is important to mention that the Eqs 2.13–2.16 were only used during the fitting procedure to avoid negative concentrations for arbitrary values of the parameters, but once the parameters are estimated correctly, the original model given in Eqs 2.1–2.26 can be used without problems of negative concentrations.

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