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An extended dynamic model of *Lactococcus lactis* metabolism for mannitol and 2,3-butanediol production†

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Biomedical research and biotechnological production are greatly benefiting from the results provided by the development of dynamic models of microbial metabolism. Although several kinetic models of *Lactococcus lactis* (a Lactic Acid Bacterium (LAB) commonly used in the dairy industry) have been developed so far, most of them are simplified and focus only on specific metabolic pathways. Therefore, the application of mathematical models in the design of an engineering strategy for the production of industrially important products by *L. lactis* has been very limited. In this work, we extend the existing kinetic model of *L. lactis* central metabolism to include industrially relevant production pathways such as mannitol and 2,3-butanediol. In this way, we expect to study the dynamics of metabolite production and make predictive simulations in *L. lactis*. We used a system of ordinary differential equations (ODEs) with approximate Michaelis–Menten-like kinetics for each reaction, where the parameters were estimated from multivariate time-series metabolite concentrations obtained by our team through *in vivo* Nuclear Magnetic Resonance (NMR). The results show that the model captures observed transient dynamics when validated under a wide range of experimental conditions. Furthermore, we analyzed the model using global perturbations, which corroborate experimental evidence about metabolic responses upon enzymatic changes. These include that mannitol production is very sensitive to lactate dehydrogenase (LDH) in the wild type (W.T.) strain, and to mannitol phosphoenolpyruvate: a phosphotransferase system (PTS^{Mtl}) in a LDH mutant strain. LDH reduction has also a positive control on 2,3-butanediol levels. Furthermore, it was found that overproduction of mannitol-1-phosphate dehydrogenase (MPD) in a LDH/PTS^{Mtl} deficient strain can increase the mannitol levels. The results show that this model has prediction capability over new experimental conditions and offers promising possibilities to elucidate the effect of alterations in the main metabolism of *L. lactis*, with application in strain optimization.

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Introduction

Lactococcus lactis is a fermentative bacterium, known for its role in the manufacture of dairy products like cheese and buttermilk. The small genome size and a simple metabolism have rendered it an attractive model to implement strain design strategies for the production of added-value compounds, such as the polyols mannitol and 2,3-butanediol (reviewed in ref. 1–3).

While mannitol has a wide range of health-promoting and protective effects,[4,5] 2,3-butanediol is an important chemical feedstock with a wide range of applications.[6,7] Therefore, there is strong interest in the optimization of the industrial production of these compounds.

Mathematical modeling of biochemical networks allows the integration of experimental knowledge into a computational framework to support hypotheses and derive new predictions that can be tested. The predictive capability provided by these models is fundamental to support biomedical research and improve biotechnology production.[8,9] In particular, the field of metabolic engineering[10] takes advantage of mathematical models of cellular mechanisms, in order to discover optimal sets of genetic manipulations for the design of microbial strains that efficiently produce compounds of industrial interest.[11,12] In this regard, a systems biology approach can provide promising tools for LAB metabolic engineering.[13] One of the most common approaches

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for modeling biological networks is based on systems of ordinary differential equations (ODEs). This kind of models represents a powerful tool to understand interesting properties of the cellular mechanisms and enables the design of new biological pathways.[14,15]

In the last years, there have been a growing number of mathematical models in the literature of *L. lactis*, such as *L. lactis* genome-scale model and kinetic models (for a detailed review, see ref. 13).

Although genome-scale models have a clear advantage in terms of scalability when compared to dynamic models, they contain several limitations. Besides disregarding transient behavior to predict dynamic responses to external perturbations, they do not take into account enzymatic regulation effects and metabolite concentration. To date, some kinetic models have been developed, which include the study of the enzymatic regulation[16,17] and metabolic flux analysis.[7,18] In an earlier kinetic model,[7] the authors proposed a dynamic model of *L. lactis* pyruvate branches using available *in vitro* enzyme kinetic data rather than parameter fitting.

Although some useful dynamic models have been built in this way, the kinetic parameters should be used with care, since enzyme characterization is often not performed under physiological conditions or only for few parameters,[19] restricting the *in silico* applicability.[20,21] Furthermore, this earlier model[7] ignores crucial metabolic pathways. Voit and co-workers[16] constructed an approximate power-law kinetics model of *L. lactis* glycolysis with few kinetic parameters identified by *in vivo* metabolite time-series data. Another glycolysis *L. lactis* model using a similar approach but with lin-log kinetics was proposed by del Rosario *et al.*[22] A limitation of these models is that power-law and lin-log kinetics do not reflect clear mapping to biochemistry. Vinga *et al.*[23] reconstructed a dynamic glycolysis model of *L. lactis* based on the dynamic budget theory. One disadvantage of this approach is that no specific biochemical mechanisms are incorporated, and therefore it is at a much higher abstraction level and as such unable to identify targets for strain design. Oh *et al.*[18] constructed a detailed glycolytic model of *L. lactis* using kinetic rate equations from yeast or other species models. However, no time-course simulation and validation were performed. A recent work[24] presented an (ODE)-based model, but some of the reactions in this glycolysis model are represented as lumped versions of some important biosynthetic pathways and aggregate key glycolysis species to reduce complexity.

Despite the fact that this field has attracted considerable attention, most of the available models are very generic and do not take into account industrially important products for other metabolic engineering design studies, *e.g.* mannitol and 2-3-butanediol, thus severely restricting their usage in this context.

In this work, we substantially extend an existing glycolysis kinetic model (Levering *et al.*[24]), by including the 2,3-butanediol and mannitol biosynthetic pathways, and the branches to ethanol and acetate. Enzyme kinetics was modeled with approximate convenience kinetics with product sensitivity and parameterized using more experimental *in vivo* NMR data. The model was then validated for a wider range of conditions to make predictions about the dynamic behavior of *L. lactis*. Finally, our model was applied to finding new potential targets for production of 2,3-butanediol and mannitol in the system.

Methods

Network structure modification and extension

The metabolic network was reconstructed for the identification of the main *L. lactis* metabolite dynamics. The previously published model of Levering *et al.*[24] was the starting point for the network reconstruction, which was expanded by using a large number of the pathway reactions taken from the literature.[7,25] The network structure in this work is depicted in Fig. 1. Abbreviations of metabolites and reaction names can be found in the corresponding list. We updated and extended the original model[24] to describe the relevant products pathways by changing or including the following: (i) reactions catalyzed by phosphoglucose isomerase (PGI), pyruvate formate lyase (PFL), α-acetolactate synthase (ALS), 2,3-butanediol dehydrogenase (BDH) and mannitol-1-phosphate dehydrogenase (MPD) have been modeled as reversible reactions and the remaining new equations as irreversible reactions; (ii) allosteric regulation (inhibition) of MPD by fructose 6-phosphate (*f6p*);[26] (iii) division of the mixed-acid branch into acetate and ethanol production pathways – for simplicity acetyl-P and acetaldehyde were not considered as in ref. 25; (iv) inclusion of mannitol metabolism as described in ref. 25 and 27; (v) incorporation of acetoin and 2,3-butanediol production pathways; (vi) the *f6p* intracellular metabolite and the corresponding PGI reaction were added; (vii) allosteric regulation (inhibition) of alcohol dehydrogenase (AE) by *atp*[28] and (viii) allosteric regulation (inhibition) of PFL by *g3p*. The complete ODEs are listed in Additional File 1 (ESI†). It contains a total of 26 metabolites and 15 internal reactions, including enzymatic effectors (7 inhibitors and 3 activators) and 6 exchange reactions.

Model formulation, assumptions and parameterization

For modeling we used a set of ODEs with approximate Michaelis–Menten-like kinetics (convenience kinetic rate expression proposed in ref. 29 with product sensitivity) for each transporter and enzyme-catalyzed reaction (only for ATPase reaction a Hill equation is used), with initial guess parameters from the previous *L. lactis* model[24] and literature search (see Additional File 2, ESI†). This enzyme kinetics requires less kinetic information than a full description of the reaction, accommodates various reaction stoichiometries and describes enzyme regulation. For example, in the case of a regulated reaction A ↔ 2B activated by C and inhibited by D, the convenience rate is formulated with the following structure:

$$r_j = \frac{K_i^D}{[D]+K_i^D}\frac{[C]}{[C]+K_a^C}\frac{V_{max}^j\frac{[A]}{K_m^A}-\frac{V_{max}^j}{K_{eq}^j}\frac{[B]^2}{K_m^A}}{\left(1+\frac{[A]}{K_m^A}\right)+\left(1+\frac{[B]}{K_m^B}+\left(\frac{[B]}{K_m^B}\right)^2\right)-1}$$

where K_m^A and K_m^B are Michaelis–Menten constants, V_{max}^j is the maximal rate constant of reaction r_j, and K_i^D and K_a^C represent inhibition and activation constants, respectively. The convenience kinetic rate law proposed in ref. 29 is based

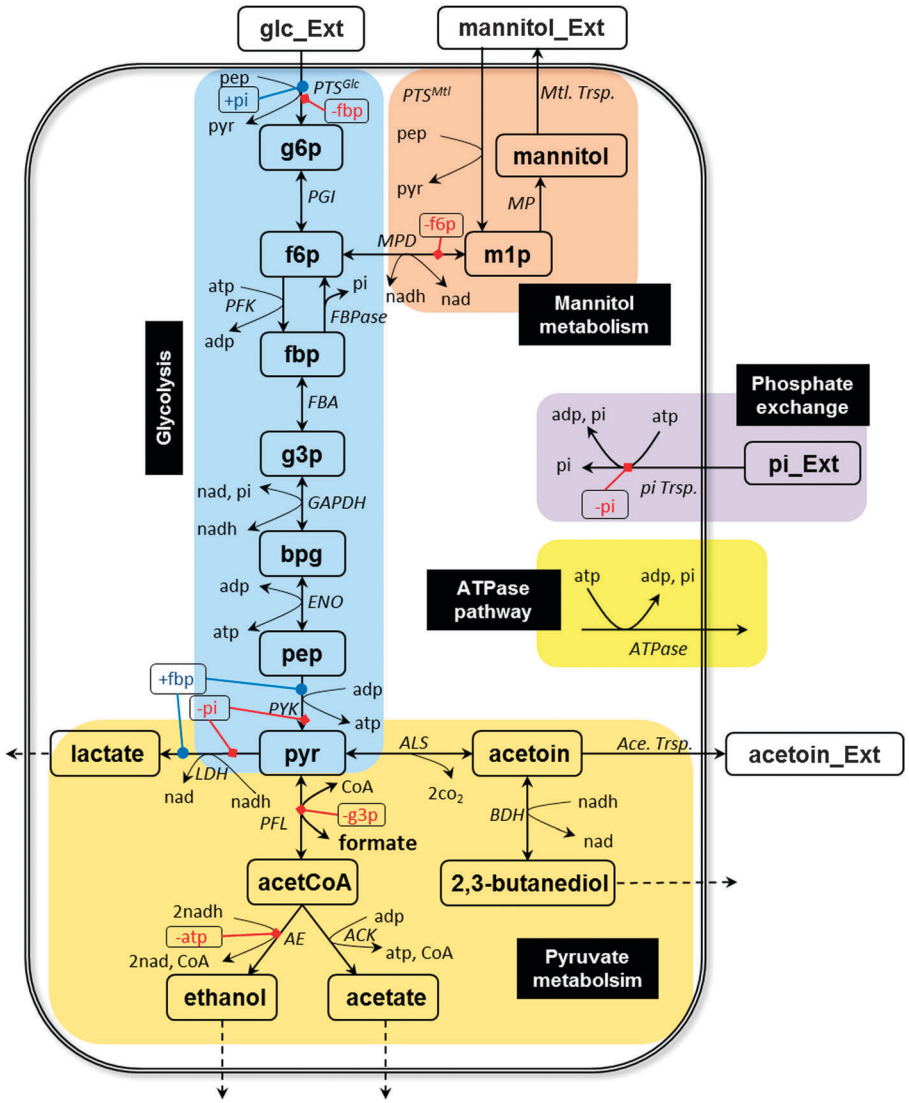

Fig. 1 Schematic network representation of the central metabolism of *L. lactis*. These include the glycolysis (light blue box), mannitol metabolism (pink box), phosphate exchange (light purple box), ATPase pathway (yellow box) and pyruvate metabolism (beige box). Circular nodes represent metabolites and italic names of enzymes/reactions. Red metabolites (close red circle-ends) are inhibitors and blue metabolites (close blue circle-ends) are activators. The arrows indicate the reaction reversibility. Abbreviations of metabolite and reaction names are given in the abbreviations list.

on the random order binding Michaelis–Menten equation and can be applied to any reaction stoichiometry. To take into consideration the enzymatic regulatory effects (inhibition and activation), we adopted the multiplication by a regulatory factor as suggested in ref. 30. The kinetic rate equations for each reaction are given in Additional File 4 (ESI†). Prior to parameter fitting, we specified the initial metabolite and parameter values concentrations as possible based on literature data and experimental measurements (see Additional File 3 and 2 (ESI†), respectively). In all simulations, we assumed that the equilibrium constants (K_{eq}) and Hill coefficient parameters (n) are known, for which previously reported values are used (see Additional File 2, ESI†), which were held constant and thus the number of unknown parameters was reduced from 112 to 102. The model was constructed and simulated in the COPASI software version 4.8[31] using the LSODA ODE solver.

After selecting appropriate reaction kinetics, we calibrated all the parameters system by collective fitting to *in vivo* NMR metabolite data (time series) from *L. lactis* MG1363 resting cells.[32] The binding affinity constants (Michaelis–Menten constants, inhibition and activation constants) and rate constants are estimated by a global fit using the time series metabolite data. Since the experimental data for the initial metabolite concentration of *adp*, *atp*, *nadh*, phoshornolpyruvate (*pep*) and 1,3-diphosphoglycerate (*bpg*) were not measured experimentally for *L. lactis*, we estimated this initial metabolite concentration from the model together with the kinetic parameters. The remaining initial metabolite concentrations were set at their measured values. Upper and lower bounds for the parameters were constrained to values within [0.01, 100] mM for Michaelis–Menten constants (K_m) and [0.001, 1000] mM s^{-1} for maximal rate constants (V_{max}), except for the optimized initial metabolites,

which are assumed to be between [0, 10^9] mM. To estimate the kinetic parameters we used first the algorithm of fitting based on the evolutionary programming (EP) method implemented in COPASI,[31] where the number of generations was set to 2000 and a population size of 100 models. Subsequently, this solution was then used as the starting value and the model was additionally fitted with the local optimization method Hookes and Jeeves (HJ) algorithm with standard settings also from COPASI. In this way, we reduced the probability of missing the global minimum. To make sure that this method does not converge to a suboptimal solution, ten optimization runs were performed.

The experimental data used for model verification purposes were also taken from previous work by members of our team.[32]

Parameter sensitivity control analysis

To assess the importance of various enzymes/reactions on the production of key products (*e.g.* mannitol) we used global sensitivity analysis of control coefficients, describing how each metabolite production changes in response to changes in the maximal activity (V_{max} values) of a reaction in the model.

A sensitivity testing process consists of the analysis of the effect of parameter uncertainty on the model outcome. Common applications of parameter sensitivity analysis include model reduction,[33] robustness analysis[34] and identification of control points.[35] A classical approach of sensitivity analysis for biochemical reaction systems described by ODEs is the so-called metabolic control analysis (MCA).[36] However, this analysis is carried out to assess only systemic steady state properties (*i.e.* influence of the steady state concentrations or fluxes in the models by infinitesimally small changes in individual reactions of the system) and is only valid for small perturbations of one parameter around a specific operating point.

In this work, global sensitivity analysis was performed for the wild type and two mutant strains (LDH and LDH/PTS^{Mtl}) using the SBML-SAT software package[37] for MATLAB. These two mutant strains were simulated by multiplying the optimized values of the appropriate rate equations (V_{max} of LDH and PTS^{Mtl}) by 0.01 (for a 99% decrease in enzyme activity) and by zero, respectively. The method used for this analysis was partial rank correlation coefficient (PRCC) analysis, which is routinely applied to systems with a nonlinear relationship between the systems inputs and outputs.[38] In our work, N equidistant sampling points for each kinetic parameter (V_{max}) subject to sensitivity analysis were varied in the range of 0.01–100× of its reference value (fixing all other parameters at their reference values) with N = 5000 simulations, using an M (number of parameters subject to variation) dimensional Latin hypercube and the model output type *integrated response* (*i.e.* total amount of state variable during the time course) for each analysis was selected. More details and the PRCC method implementation in the SBML-SAT tool could be found in ref. 37.

Results and discussion

The following sections describe the extension of the previously glycolysis *L. lactis* model proposed by Levering *et al.*,[24] enriched with knowledge from published studies[25,27] and *in vivo* time-series data, which was further used for parameterization and validation. We then performed sensitivity analysis of global perturbation to identify which enzymes/reactions had large impacts on the overall mannitol and 2,3-butanediol production.

Analysis of the model

Model parameterisation – comparison of *in vivo versus in silico* dynamics. The reconstructed *L. lactis* network used in this work (illustrated in Fig. 1) has 26 metabolites, 15 internal and 6 exchange reactions. The model includes the glycolysis, pyruvate and mannitol metabolism, phosphate exchange as well as the ATPase pathway.

After this building step, the kinetic model was parameterized *de novo* to fit the experimental time-series data for metabolite concentration after addition of a glucose pulse of 40 mM (see Methods for more information). Since most of the kinetic parameters have been estimated based on *in vitro* studies as given in Additional File 2 (ESI†), the model parameters had to be re-estimated based on the measured *in vivo* metabolites data. A total of eight time-courses of metabolite profiles, of which 2 are extracellular (glucose and lactate) and 6 intracellular (*pep*, *nadh*, *nad*, *pi*, *fbp and atp*), were used for this fitting. These experimental data consist of metabolite profiles from the central carbon metabolism of the bacterium *L. lactis*, obtained using *in vivo* NMR under anaerobic conditions.[32] The experimental time series data with 40 mM extracellular glucose pulse and the corresponding model fittings are shown in Fig. 2, where the results for ten independent runs of the optimization method (see the Methods section for details) are shown.

Although each model describes the experimental data relatively well, they differ from each other in the kinetic parameter set. On the basis of previous studies with similar complexity,[39,40] this illustrates an eventual identifiability issue. In fact, the estimated kinetic parameters in complex biochemical models are often not unique given different sets of parameter values that can fit to time series data equally well with identical dynamic behavior.[41] In addition, the objective function contains multiple local minima.[42] Therefore, to enable an exploration of the search space, the optimization procedure was performed ten times with the optimization method (EP + HJ). We have considered the global and local optimization methods sequentially because this type of hybrid approach has proven to have key advantages in non-linear biochemical complex models.[43,44]

In general, all these model fits exhibit, upon simulation, a very good agreement with the measured time course data, showing that the combined global and local optimization approach is an effective means to optimize parameters. It is also possible to observe an instantaneous decrease in *pi* concentration and an instantaneous increase in *fbp* concentration which the kinetic model is able to reproduce remarkably well. This phenomenon (*fbp* accumulation) was also reported in the literature before.[45] The optimized metabolite concentrations and the fitted kinetic parameter values obtained from one of the best optimization runs (red line in Fig. 2) can be found in Additional Files 3 and 2 (ESI†), respectively. Although some of

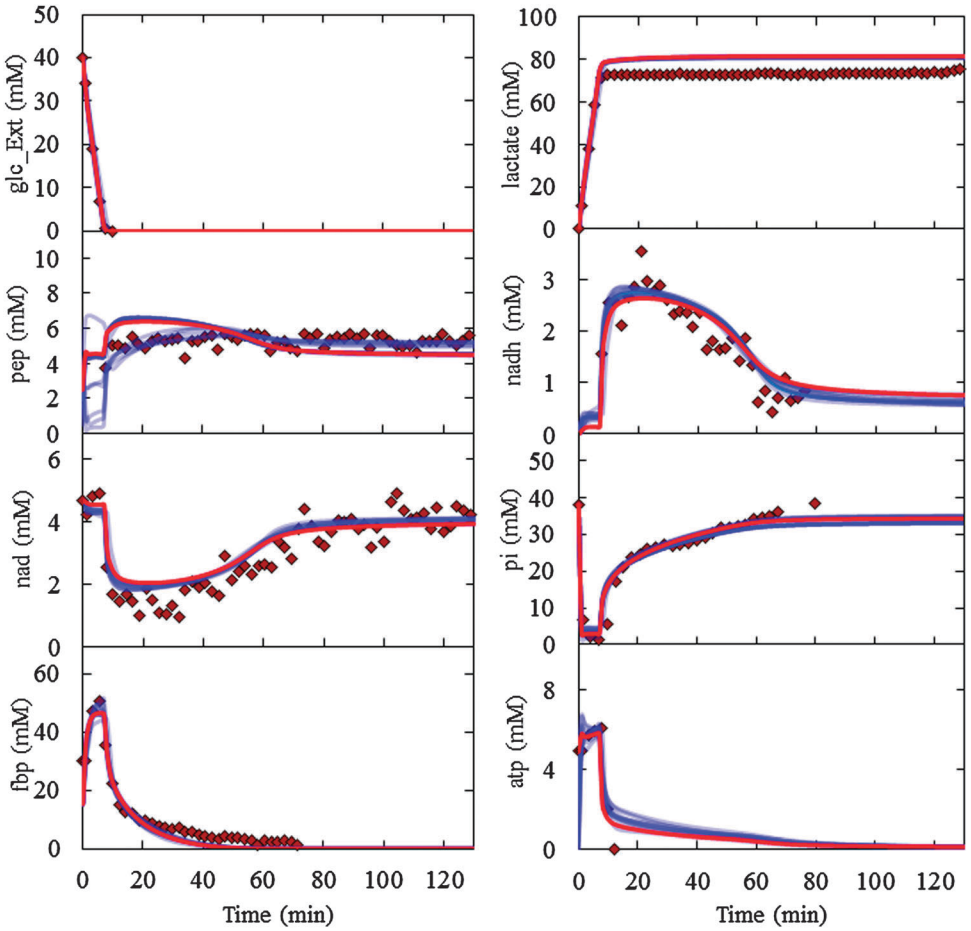

Fig. 2 Time courses of experimental data used for model parameterization and corresponding model fits with 40 mM extracellular glucose pulse for eight variables in *L. lactis* MG1363. Ten fits for each metabolite are shown. In all cases the dotted lines indicate the experimental data and the continuous lines are the simulated dynamics. The red line refers to the simulation for the initial metabolite concentration and estimated kinetic parameter values listed in Additional File 3 and 2 (ESI†), respectively. Only the simulation results for the metabolite concentrations experimentally available are shown for comparison. The remaining metabolite simulation results are given in Additional File 6 (ESI†).

the kinetic parameter values are maybe unrealistic, the main purpose of the model is to help understand what is not yet known about the behavior of the system.

The analysis of the variance of the estimated kinetic parameter sets over the ten simulations performed (shown in Additional Files 5, ESI†) shows that the ranges of some parameters values are significantly high. This means that some model parameters are non-identifiable, probably due to the "*sloppy*" nature of the system,[46] which is a typical feature of complex models with time-series collective fits, even when a complete set of time-resolved measurements of all species are available. However, it is legitimate to draw conclusions based on this type of model,[46] because this problem is mitigated by the fact that the structure of mechanistic models already tightly constrains the possible dynamic behaviors. We therefore considered all fits in the following analysis in order to ensure that some properties of the model are conservative to these uncertainties.

Model verification: dynamic response to different stimuli. To ascertain whether biologically meaningful information can be obtained, we validated the model against independent data sets (*i.e.* different glucose pulse conditions), which were not used before in the parameter fitting procedure. In other words, we tested the predictive properties of our *L. lactis* model by determining whether different conditions could be predicted without any re-fitting procedure. We started with model validation by changing the level of glucose pulse to 20 mM and 80 mM and carried out simulations (Fig. 3 and 4, respectively).

The results show the experimental and simulated dynamics after a 20 mM (only four metabolites were measured) and 80 mM extracellular glucose pulse, obtained with the ten independent runs. As shown in Fig. 3 and 4, the simulation results based on our models are highly consistent with the experimental data (without further fittings of any parameters), namely for the prediction of *fbp* accumulation around 50 mM, a value that corresponds to a saturation phenomenon for the wild type strain. Only the time course of the simulated *g6p* metabolite for the glucose pulse with 20 mM (Fig. 3) and *atp* for a 80 mM bolus glucose (Fig. 4) deviated from their experimentally values, but the dynamic behaviors are qualitatively similar. The remaining simulations describing the experimental data enable the prediction of robust dynamic behavior

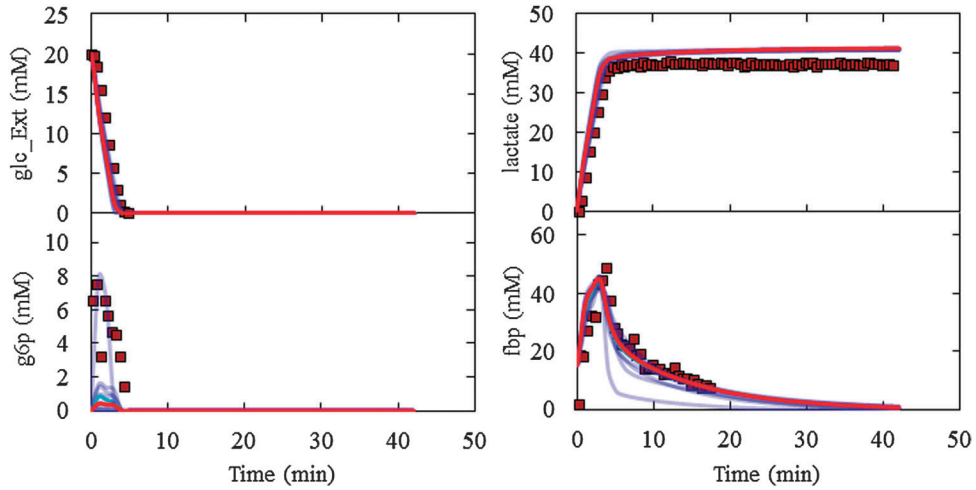

Fig. 3 Time courses of experimental data (squares) and corresponding simulated dynamics (lines) for 10 independent runs with 20 mM extracellular glucose pulse for the wild type strain. The simulation having one of the best optimization runs is indicated by the red line.

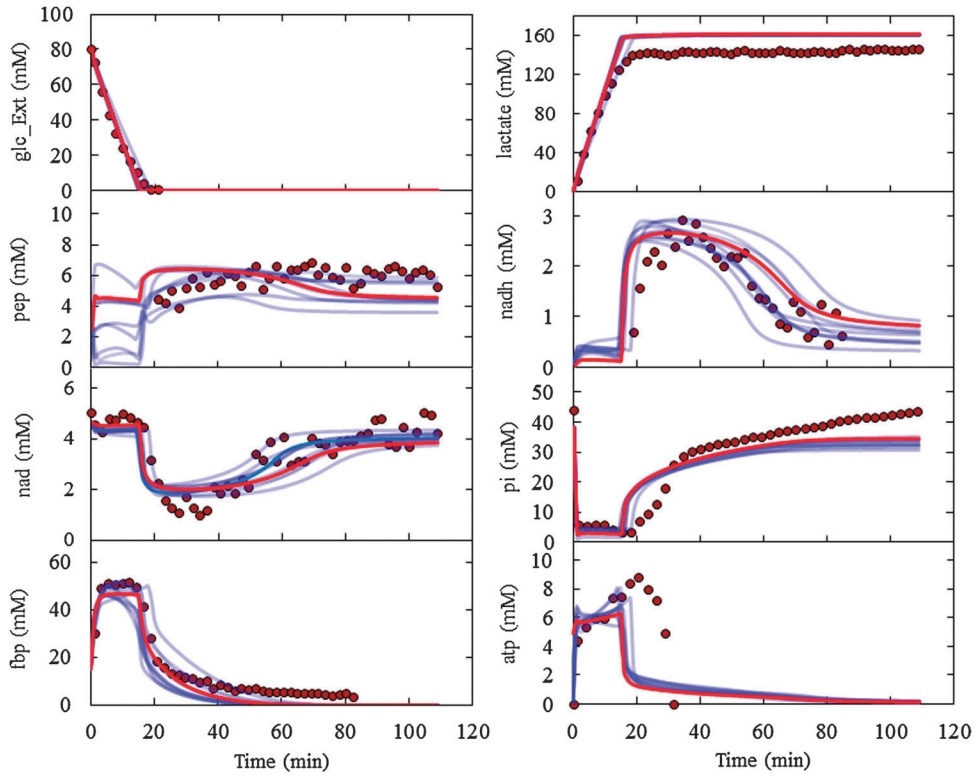

Fig. 4 Experimental time-course data (circles) and simulation results (lines) for 10 independent runs with 80 mM extracellular glucose pulse for the wild type strain. The simulation having one of the best optimization runs is indicated by the red line.

independent of the parameter set. These results demonstrate that our kinetic model captures well the main metabolism in *L. lactis* and gives us confidence for the generation of further predictions even under different conditions.

Perturbation analysis of the model. One major advantage of kinetic models is their capability to assist in developing *in silico* strategies, thus increasing the success rate of metabolic engineering design. In this manner, the overexpression or underexpression of an enzyme can be simulated by increasing or decreasing, respectively, the maximal activity (V_{max}) of the enzyme in question. In this work, after construction and a first validation of the model we have simulated perturbed conditions by sensitivity analysis to detect potential targets that have large influences on the valuable 2,3-butanediol and mannitol products.

In order to address the effects of changes of the parameter (V_{max}) values for every reaction upon the transient dynamics of

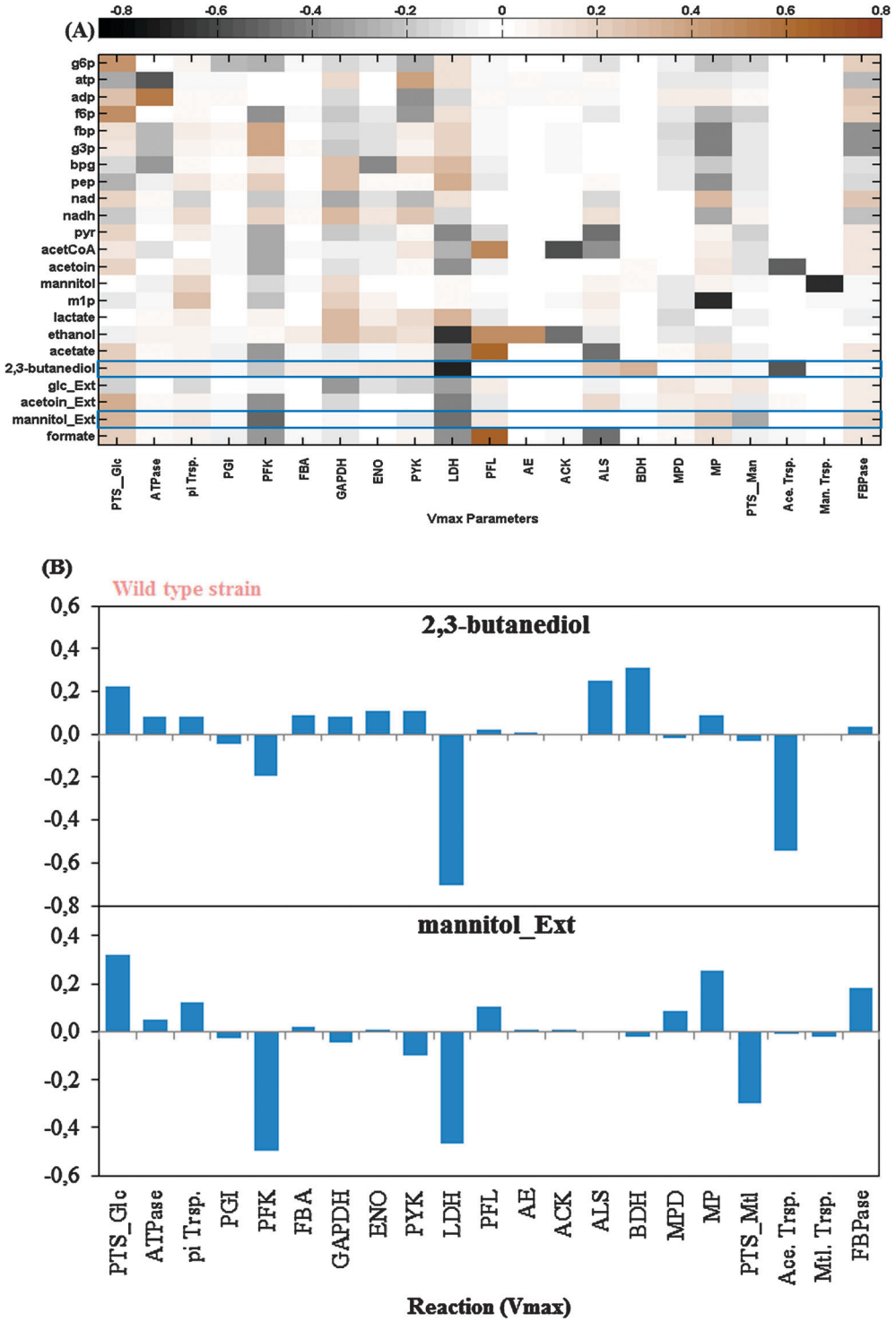

Fig. 5 Global sensitivity analyses of concentration integrated response coefficients (IRCs) to variations in V_{max} for the wild type strain. (A) Effects of changes in V_{max} values of the enzymes/reactions on the main state variable under the given conditions. The colour of a matrix element S_{ij} defines the type of impacts of an enzyme/reaction i on a state variable (metabolite) j. (B) The bar graphs correspond to two of these state variables (2,3-butanediol and mannitol_Ext).

our system, we obtained the sensitivity of time integrated response coefficients (IRCs) by global sensitivity analysis on the model, as described in the Methods section. This approach allowed us to explore the effect of simultaneously changing the kinetic parameters and considered the full trajectory rather than just steady-state conditions. These properties are especially important in the case of glycolysis in *L. lactis*, as they provide pointers to identify enzyme targets for

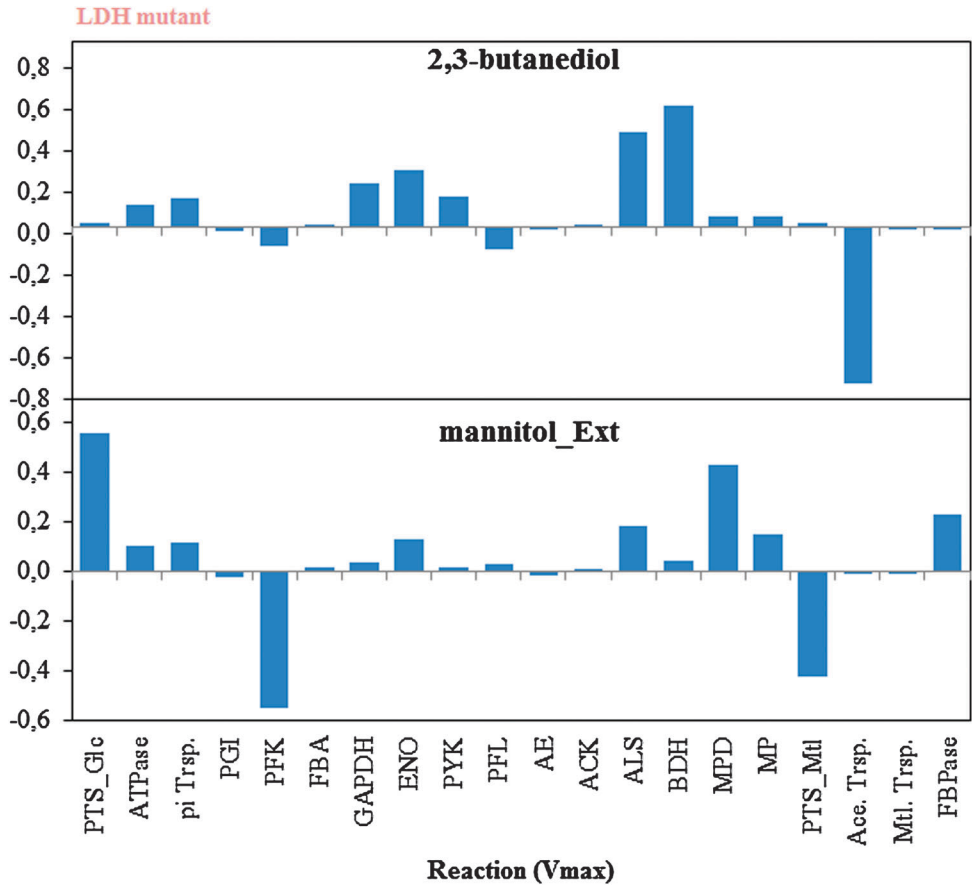

Fig. 6 Concentration response coefficients from global sensitivity analyses to variations in V_{max} for an LDH deficient strain. The response coefficients in Fig. 5B are recalculated as described in the Methods section. The effects of changes in V_{max} values of the enzymes on two state variables (2,3-butanediol and mannitol_Ext) were analyzed in detail. The bar graphs correspond to two of these state variables (2,3-butanediol and mannitol_Ext).

strain optimization that have large impacts on mannitol and 2,3-butanediol production.

Disruption of LDH in *L. lactis* resulted in production of mannitol.[47,48] Accumulation of mannitol as an end-product of glucose fermentation was achieved by combining the LDH deletion with inactivation of the mannitol PTS.[25] Overexpression of MPD in an LDH deficient strain led to an increase in mannitol production.[49] The major breakthrough in mannitol production originated from combining overproduction of MP with MPD in a LDH/PTS^{Mtl}.[27,50]

To make a second validation of the model, we implemented sensitivity analysis. The matrix of sensitivity control coefficients that shows the response of all the metabolites by simultaneously varying all the reactions/enzymes in this system is shown in Fig. 5A. The sensitivity values (S_{ij}) are in the range of −1 and 1. Here we focus on sensitivity analysis to analyze potential targets (control parameters) of the metabolites which are reported in the literature; the same analysis could be performed for any metabolite in the model (see Fig. 5A). For the sake of simplicity, we restrict this analysis to one of the best fits based on the objective function (red line in Fig. 2), but consistent results (distribution of control in a negative or positive way) were obtained for the other fits (see Additional file 8, ESI†), which illustrates the robustness of the model. This is in agreement with an exploratory study performed by Nikerel *et al.*[51] that concludes that the underlying control structures behavior was robust against non-identifiable parameters used. The LDH enzyme exerted a substantial negative sensitivity over the acetoin production, *i.e.*, decreasing its V_{max} increased acetoin quantity. This result was in accordance with experimental observations and *in silico* modeling predictions performed by Hoefnagel *et al.*[7] The glycolytic enzymes had positive control over lactate production. As expected, the ACK reaction had a significantly negative control over acetyl-CoA (*acetCoA*) production. Acetate production in *L. lactis* was diminished with an excessive PFK overexpression, a result indicating that the *fbp* concentration is increased over the control of PFK enzyme. Furthermore, the overexpression of PFK exerted a positive control over lactate production, as previously described in ref. 52. The formate level was found to be very sensitive to the LDH reaction in *L. lactis* in agreement with ref. 53. Apart from these, the *atp* component responded substantially and negatively to changes in the ATPase consumption rate. It is not surprising that the control coefficients for the *adp* level were complementary to those for *atp*.

Mannitol and 2,3-butanediol are economically valuable metabolites and, given their potential in health and industrial applications, were the main focus of the analysis to find potential targets in *L. lactis*. Sensitivity analysis is dependent

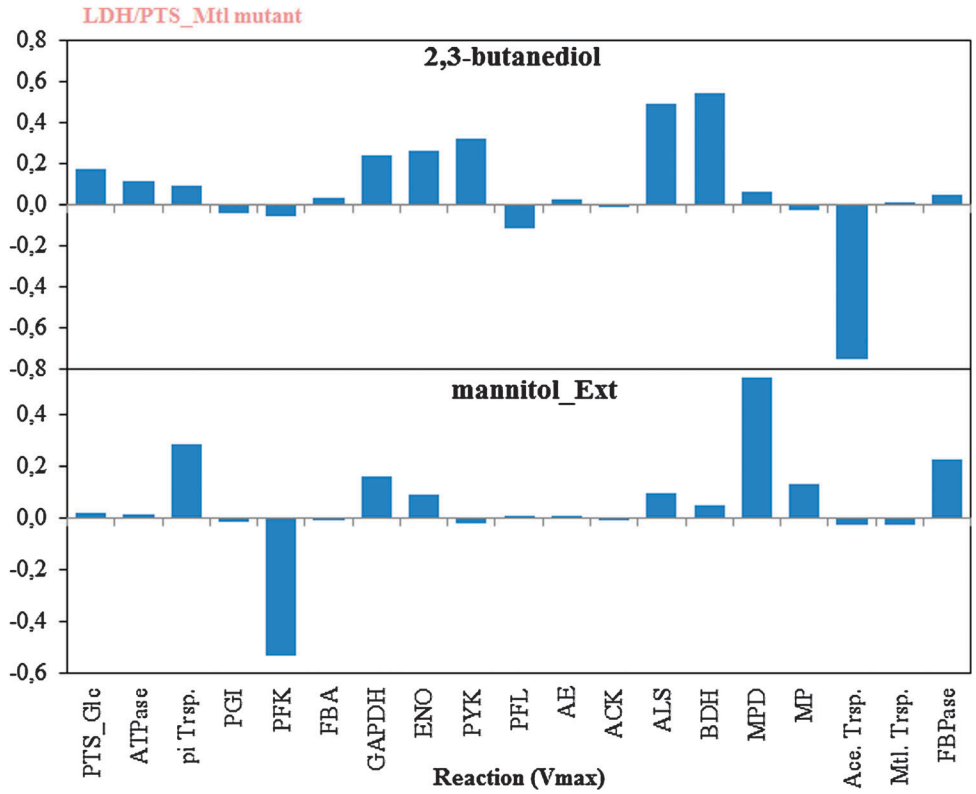

Fig. 7 Concentration response coefficients from global sensitivity analyses to variations in V_{max} for an LDH/PTS^{Mtl} deficient strain. The response coefficients in Fig. 6 are recalculated as described in the Methods section. The effects of changes in V_{max} values of the enzymes on two state variables (2,3-butanediol and mannitol_Ext) were analyzed in detail. The bar graphs correspond to two of these state variables (2,3-butanediol and mannitol_Ext).

Table 1 Summary of relevant physiological outputs obtained using perturbation analysis of the model, including targets experimentally described in the literature and novel targets for intervention not yet tested experimentally

	Target (s)	Observation/candidate target	Ref.
Experimental evidence	LDH	LDH reduction results in a large and positive influence on mannitol and 2,3-butanediol levels.	47–49
	LDH & MPD	LDH deficiency and MPD overexpression increase mannitol levels.	49
	LDH & PTS^{Mtl}	PTS^{Mtl} inactivation in LDH deficiency increases mannitol production.	25
	LDH/PTS^{Mtl} & MP	LDH/PTS^{Mtl} deficiency and MP overexpression increase mannitol.	27
In silico target predictions	PFK	PFK underexpression exerted a large positive control over the mannitol production.	—
	MP	Overproduction of MP increases levels of mannitol production in W.T. larger than the MPD overexpression.	—
	LDH & MP and MPD[a]	LDH deficiency and combination of MP and MPD overexpression increase mannitol levels.	—
	LDH/PTS^{Mtl} & MPD	LDH/PTS^{Mtl} deficiency and MPD overexpression increase mannitol levels.	—

— not described in the literature. [a] This approach has been published only for growing cells.[50]

on the parameter space which varies from fit to fit. Using our collection of fits, we calculated the control coefficients for every model and every strain (W.T., LDH and LDH/PTS^{Mtl}). A significant similarity in sensitivities across the ten fits (correlation coefficient values, R^2 = 0.6 to R^2 = 0.97 between all pairs of fits) for mannitol and 2,3-butanediol dynamics were observed (results can be found in Additional File 7, ESI†). So, even in the absence of knowing the exact parameter values, these results seem to be relatively conserved by parametric uncertainty.

The sensitivity response coefficients for the wild type over mannitol and 2,3-butanediol are shown in Fig. 5B. The sensitive analysis of the model showed that both mannitol and 2,3-butanediol are highly sensitive to perturbation in LDH. This confirmed loss of LDH as an important step in a strategy to improve mannitol and 2,3-butanediol production levels. A strong negative control of LDH over the mannitol and 2,3-butanediol levels has been reported for *L. lactis*.[48,49] Furthermore, the 2,3-butanediol production has the highest positive sensitivity to variations of BDH. The ALS has also a positive effect, but to a lesser extent. Our model also suggests that reduction of PFK activity would lead to increased *f6p*, and consequently higher mannitol production. Accumulation of *f6p*

due to a constriction of the level of PFK in *L. lactis* has been described by others.[54] It is also interesting to note that the PTS^{Mtl} had a negative control over mannitol accumulation. MP reaction was found to have a high positive control to increase mannitol amounts, a result fully in line with the experimental data.[50] Besides, MPD also has a positive effect on mannitol production, but with a smaller magnitude (Fig. 5B).

The sensitivity response coefficients for the LDH mutant strain and the double mutant strain LDH/PTS^{Mtl} are shown in Fig. 6 and 7, respectively. For these strains and based on the sensitivity analysis the following results were found: (i) MPD (an important reaction involved in NAD^+ regeneration) overexpression in the LDH-deficient strain exerted a substantial positive influence over the mannitol concentration as observed in ref. 49; (ii) PTS^{Mtl} disruption in the LDH-deficient background increases mannitol production, as confirmed experimentally;[25] (iii) as expected, up-regulation of BDH, and down-regulation of the acetoin export rate in the LDH-deficient strain were suggested by the sensitivity analysis to increase 2,3-butanediol production; (iv) the underexpression of AE enzyme in the LDH/PTS^{Mtl} double mutant leads to decreased mannitol production. This conclusion is in agreement with a recent observation as reported in ref. 27; and (v) the MP overexpression in the LDH/PTS^{Mtl} mutant had a positive control over mannitol. This prediction is in agreement with experimental data.[27] Also, our computational results showed that both MP and MPD overexpression in the LDH mutant strain have a positive sensitivity over mannitol production. Thus, the combination of MP and MPD appears as one of the most promising strategies to elevate the mannitol level, which is in line with previous findings in *L. lactis*.[50]

One interesting remark is that these sensitive control coefficients are reasonably accurate and in line with experimental evidence as described elsewhere in the literature. For instance, the control patterns found for the wild type strains of *L. lactis* (Fig. 5B) support that the LDH underexpression is an important target for mannitol and 2,3-butanediol production. Moreover, from the results of the present work, promising targets for further hypotheses testing in our laboratory were also identified such as, the overexpression of MP in the wild-type strain. The entire analyzed key phenotypes outputs are summarized in Table 1.

Thus, this improved model of *L. lactis* metabolism is able to capture the main kinetic properties of the *L. lactis* metabolic network, anticipating its application as a tool in hypothesis-driven experimental studies and metabolic engineering. In other words, it is possible to make predictions under experimental conditions differing from the one that was used to calibrate the model, proving its generalization capability.

Conclusions

We have extended a previous kinetic model of *L. lactis*, with the 2,3-butanediol and mannitol production pathways, and the branches to ethanol and acetate. This extended model was consistent with a wide range of experimental data. The simulation discrepancies between experimentally measured metabolite concentrations and simulated values were very small. The model was also able to accurately predict time-courses under new initial conditions, which illustrates its generalization capability.

Through sensitivity analysis of control coefficients, underexpression of PFK and overexpression of MP in a wild type strain are suggested as promising targets to increase the mannitol production in *L. lactis*. Furthermore, the sensitivity analysis identified the combination of MP and MPD overexpression in a LDH deficient strain as key to increase the mannitol level, as experimentally observed for *L. lactis* growing cells.[50] Moreover, it was predicted that MPD overexpression for a LDH/PTS^{Mtl} deficient strain could be a good target for mannitol production. In addition, our other tests are compatible with previously published studies.

We believe that the present model could be used to support a rational exploration of efficient metabolic engineering strategies in *L. lactis*.

Abbreviations

glc_Ext	Glucose extracellular
g6p	Glucose-6-phosphate
f6p	Fructose-6-phosphate
fbp	Fructose-1,6-bisphosphate
g3p	Glyceraldehyde-3-phosphate
bpg	1,3-Diphosphoglycerate
pep	Phoshornolpyruvate
pyr	Pyruvate
lactate	Lactate extracellular
acetoin_Ext	Acetoin extracellular
acetCoA	Acetyl-coenzymeA
m1p	Mannitol-1-phosphate
mannitol_Ext	Mannitol extracellular
atp	Adenosinetriphosphate
adp	Adenosinediphospahte
nad	Nicotinamide adenine dinucleotide
nadh	Dihydronicotinamide adenine dinucleotide
pi	Phosphate intracellular
pi_Ext	Inorganic phosphate extracellular
PTS_Glc and PTS^{Glc}	Phosphotransferase system
ATPase	ATP phosphatase
pi Trsp.	Phosphate transport
PGI	Phosphoglucose isomerase
PFK	Phosphofructokinase
FBA	Fructose-1,6-bisphosphate aldolase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ENO	Enolase
PYK	Pyruvate kinase
LDH	Lactate dehydrogenase
PFL	Pyruvate-dehydrogenase
AE	Alcohol dehydrogenase
ACK	Acetate kinase
ALS	α-Acetolactate synthase
BDH	2,3-Butanediol dehydrogenase
MPD	Mannitol-1-phosphate dehydrogenase
MP	Mannitol 1-phosphatase

PTS_Mtl and PTS^{Mtl}	Mannitol phosphotransferase system
Ace. Trsp.	Acetoin efflux
Mtl. Trsp.	Mannitol efflux
FBPase	Fructose-1,6-bisphosphate phosphatase

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