

# Flux distribution in *Bacillus subtilis*: inspection on plurality of optimal solutions

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## Abstract

Linear programming problems with alternate solutions are challenging due to the choice of multiple strategies resulting in the same optimal value of the objective function. However, searching for these solutions is a tedious task, especially when using mixed integer linear programming (MILP), as previously applied to metabolic models. Therefore, judgment on plurality of optimal metabolic flux distributions (solutions) a priori to applying MILP approach could prevent unnecessary computations. In this work for the first time, the reduced cost coefficients for the non-basic variables in a current solution of a metabolic model were utilized to inspect the possibility of multiple optimal flux distributions. If there exists at least one non-basic variable with zero reduced cost coefficient, multiplicity of optimal solution may occur where MILP can be used to find these solutions. This approach was implemented on a metabolic network of *Bacillus subtilis* aiming to reduce the cell energy requirement. Solving the model at fixed specific growth rate of 0.4 1/h resulted in minimum energy requirement of 12.67 mmol/g-h. Inspection of reduced cost coefficients showed that six non-basic variables had zero reduced cost coefficients at current solution, which shows that there can exist multiple optimal solutions. Subsequently, by applying MILP, five optimal flux distributions at minimized energy requirement were identified, among which one showing no acid production and minimum glucose consumption rate was selected as the superior solution.

**Keywords:** *Bacillus subtilis*; flux balance analysis; metabolic reaction network; multiple optimal flux distribution; reduced cost coefficient; mixed integer linear programming

## INTRODUCTION

Computational (modeling and simulation) investigations on microbial processes have advantages such as requiring less time and cost as compared to experimental studies. Constraint-based modeling is an approach which uses stoichiometric information of a metabolic network to construct a metabolic model with linear constraints (Raman and Chandra, 2009; Llaneras and Pico, 2008; Edwards *et al.*, 2002). The model is commonly under-determined and has a set of solutions or a feasible solution region. This feasible solution region is a polyhedral convex region with some vertices. Flux balance analysis (FBA) selects an objective function and uses linear programming (LP) to find an optimal solution representing a flux distribution within the metabolic network of a microorganism. However, there are often multiple optimal solutions for a metabolic network resulting in the same optimal value of objective function (Reed and Palsson, 2004; Mahadevan and Schilling, 2003; Phalakornkule *et al.*, 2001; Lee *et al.*, 2000). Finding these multiple solutions is challenging due to the choice of multiple strategies, which indicates the flexibilities of a metabolic network. In fact, the existence of these solutions can be an indication of the abilities of a metabolic network to perform a particular task and can represent biologically meaningful diverse exploitation of possible biochemical reactions. Among these optimal solutions, one or more superior solutions could exist which can optimize other objective functions defined based on the scope of the investigation. For example, the solution with less genetic manipulations determined by a genetic engineer or the solution with less substrate consumption and by-product excretion could be selected as a superior flux distribution.

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A few studies have been carried out focusing on multiple optimal flux distributions in metabolic models. Lee *et al.* (2000) used a recursive mixed integer linear programming (MILP) approach to find optimal solutions of an *E. coli* metabolic model including 33 reactions and 16 intracellular metabolites. Phalakornkule *et al.* (2001) distinguished different potential flux patterns in a mutant using MILP approach for designing  $^{13}\text{C}$  NMR experiments. Since application of MILP is computationally complex, expensive and intractable for large scale models (Reed and Palsson, 2004; Mahadevan and Schilling, 2003), flux variability analysis (FVA) was presented by Mahadevan and Schilling (2003) to study multiple optimal solutions of an *E. coli* genome scale metabolic model. Although FVA identifies the flux variability (minimum and maximum flux values) through each reaction, it does not determine all possible optimal solutions (Mahadevan and Schilling, 2003). In addition, for a metabolic model with  $n$  variable fluxes, FVA solves  $2n$  LP problems, which makes it time-consuming. Therefore, judgment on the plurality of optimal metabolic flux distributions (solutions) prior to searching all optimal solutions could be advantageous as this prevents pointless computations where only a unique optimal solution exists.

In this research, FBA is utilized to investigate multiple optimal flux distributions in the metabolic network of *B. subtilis*. Although there are a lot of experimental investigations on *B. subtilis*, limited studies have been conducted on the modeling of this bacterium especially using constraint-based modeling approaches (Oh *et al.*, 2007; Skolpap *et al.*, 2007; Zamboni and Sauer, 2003; Zhu, 2003; Sauer *et al.*, 1998). These limited studies focus on the prediction of growth rate, yields of commercial biochemical products, activity of pyruvate kinase enzyme, folic acid production and the metabolic impact of electron rerouting in the respiratory chain of *B. subtilis* during batch growth on glucose. Investigations on minimization of energy requirement (ATP) and calculation of multiple optimal flux distributions have not yet been carried out for *B. subtilis* metabolic model. Therefore, minimization of ATP requirement as objective function and existence of multiple optimal flux distributions in *B. subtilis* are studied in this research. To check the existence of multiple optimal solutions in a metabolic model, a criterion based on Simplex algorithm (Taha, 2006; Bazaraa *et al.*, 1990) is utilized. After finding the first optimal flux distribution and checking the existence of multiple optimal solutions, MILP

approach and MATLAB software are used to find all alternate optimal solutions.

**Theory of assessment:** Mass balances on metabolites (compounds) participating in intermediary metabolism can be shown by a set of differential equations (Raman and Chandra, 2009; Llaneras and Pico, 2008; Kauffman *et al.*, 2003; Edwards *et al.*, 2002) as given in Eq. 1:

$$\frac{dy}{dt} = AX - b \quad (1)$$

where  $y$  is the vector of metabolite concentrations,  $X$  and  $b$  are vectors of variable and measured fluxes, respectively, and  $A$  is the  $n \times m$  stoichiometric matrix ( $m$ : No. of equations and  $n$ : No. of variable fluxes). Assuming a pseudo steady state condition (Llaneras and Pico, 2008), the time derivatives of metabolite concentrations become zero. The resulting set of linear equations is commonly under-determined (i.e.,  $n > m$ ) and hence, by considering an objective function, Eq. 1 could be shown in standard LP formulation (Bazaraa *et al.*, 1990) as given in Eqs. 2 and 3.

$$\text{Minimize } Z = CX \quad (2)$$

$$\begin{aligned} \text{Such that } AX &= b \\ X &\geq 0 \end{aligned} \quad (3)$$

where  $Z$  is the objective function and  $C$  is the vector of objective function coefficients. This LP problem can be solved by using optimization algorithms such as Simplex (Taha, 2006; Bazaraa *et al.*, 1990). Simplex starts with dividing the variables into basic and non-basic followed by finding the first feasible solution and substitution of a non-basic with a basic variable, iteratively, to improve the objective function value. Equations 2 and 3 can be rewritten as Eqs. 4 and 5 using non-basic variables:

$$\text{Minimize } Z = C_B B^{-1} b + \sum_{j \in R} (c_j - z_j) x_j, \quad z_j = C_B B^{-1} a_j \quad (4)$$

$$\begin{aligned} \text{Such that } X_B &= \bar{b} - \sum_{j \in R} y_j x_j, \quad \bar{b} = B^{-1} b, \quad y_j = B^{-1} a_j \\ X_B &\geq 0, \quad x_j \geq 0, \quad j \in R \end{aligned} \quad (5)$$

where  $X_B (m \times 1)$ ,  $C_B (1 \times m)$  and  $B (m \times m)$  are basic sub-vectors and sub-matrix of  $X$ ,  $C$  and  $A$ , respectively,  $a_j$  and  $c_j$  are the  $j^{\text{th}}$  column of matrix  $A$  and vector  $C$ , respectively, and  $R$  is the current set of indices of non-basic variables. The term  $(c_j - z_j)$  in Eq. 4 is referred to

as reduced cost coefficient for non-basic variable with index  $j$ . Any feasible solution having negative reduced cost coefficient will not be optimal since increasing the value of that non-basic variable reduces the objective function (see Eq. 4). In other words, the feasible solution will be optimal if the reduced cost coefficients are greater than or equal to zero for all  $j \in R$ .

It should however be mentioned that this optimal solution will be unique if all non-basic variables have positive reduced cost coefficients. In cases where there is at least one non-basic variable with zero reduced cost coefficient, it is possible to increase the value of that non-basic variable with no change in the value of objective function. This may result in multiple optimal solutions with a unique value of objective function. Consider the following LP problem:

$$\begin{aligned} \text{Min } Z_1 &= -x_2 \\ x_1 + x_2 + x_3 &= 6 \\ x_2 + x_4 &= 3 \end{aligned}$$

Solving this problem by using simplex algorithm results in  $Z_{1opt} = -3$  and  $X = \{x_1: 3, x_2: 3, x_3: 0, x_4: 0\}$ . The current basic variables are  $x_1, x_2$  and  $R = \{3, 4\}$ . Having specified  $B, C_B, a_3$  and  $a_4$ , the reduced cost coefficients for the two non basic variables can be calculated as given below:

$$\begin{aligned} c_3 - z_3 &= c_3 - C_B B^{-1} a_3 = 0 \\ c_4 - z_4 &= c_4 - C_B B^{-1} a_4 = 1 \end{aligned}$$

Reduced cost coefficient for  $x_3$  is zero and hence, the problem may have multiple optimal solutions. Figure 1 shows feasible solution region and the two optimal

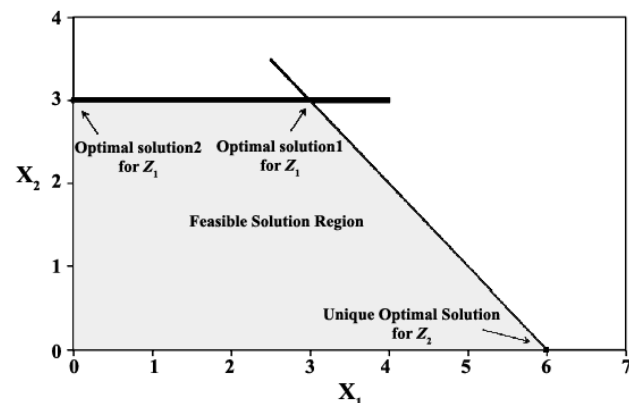


Figure 1. Graphical representation of an LP problem with four variables and two constraints. Shaded area shows the feasible solution region. There are two optimal solutions for  $Z_1$  and a unique optimal solution for  $Z_2$ .

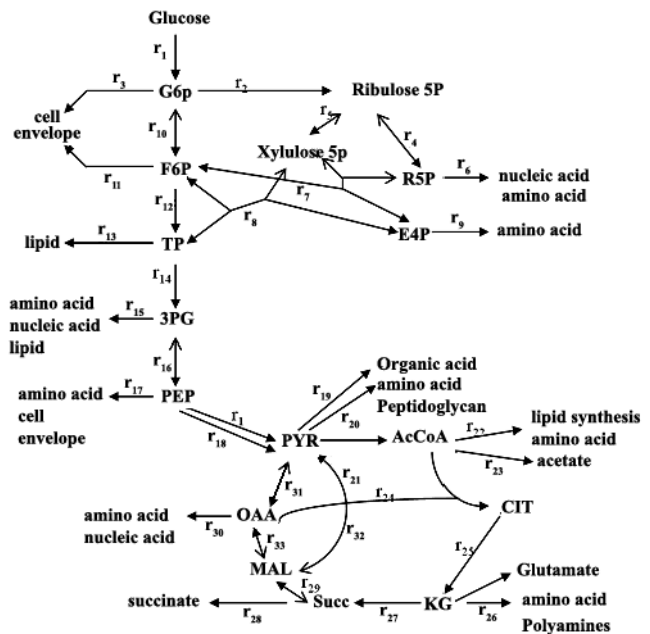


Figure 2. Biochemical reaction network for *Bacillus subtilis* using glucose as the substrate (Zhu, 2003). Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; TP, glyceraldehyde-3-phosphate; 3PG, Glycerate 3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl coenzyme A; OAA, oxaloacetate; KG,  $\alpha$ -ketoglutarate; Succ, succinate; MAL, malate; Ribulose 5P, ribulose-5-phosphate; Xylulose 5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate; CIT, citrate.

solutions for  $Z_1$ .

Now if we consider minimization of another objective function, i.e.  $Z_2 = -x_1$ , we will have  $Z_{2opt} = -6, X = \{x_1: 6, x_2: 0, x_3: 0, x_4: 3\}$  and  $R = \{2, 3\}$  resulting in the following reduced cost coefficients for the two non-basic variables  $x_2$  and  $x_3$ :

$$\begin{aligned} c_2 - z_2 &= c_2 - C_B B^{-1} a_2 = 1 \\ c_3 - z_3 &= c_3 - C_B B^{-1} a_3 = 1 \end{aligned}$$

Since both reduced cost coefficients are positive, the optimal solution is unique (see Fig. 1) and one can avoid unnecessary searches for finding non-existing multiple optimal solutions. Therefore by using this criterion, it is possible to inspect the solution plurality without any further computations while MILP and FVA approaches need to construct new mathematical problems which in turn should be solved before verifying the existence of multiple solutions.

**B. subtilis metabolic model:** We used *B. subtilis* metabolic network presented by Zhu (2003) as given in Figure 2. Glycolysis and pentose phosphate pathways have been considered in this network to direct

glucose into formation of pyruvate, free energy ATP (Adenosine Triphosphate), NADPH (Nicotinamide Adenine Dinucleotide Phosphate-Oxidase) and pentoses (5-carbon sugars). The network also includes tri-carboxylic acid (TCA) cycle for synthesis of amino acids, nucleic and fatty acids as well as an anaplerotic reaction that converts pyruvate to oxaloacetate. Reactions involved in synthesis of precursors required in biomass formation are also considered in this network.

Applying FBA, a linear standard metabolic model was constructed including 30 equality constraints and 37 variables (Appendix A). The molar balances for the 16 intracellular metabolites are given in Eqs. 1-16 of Appendix A. Two reactions within this network are reversible based on their zero standard free energy changes (Lee *et al.*, 2000) and hence, the index  $r$  in reactions 10 and 33 shows the reverse reaction fluxes. Assuming constant biomass composition, Zhu (2003) has presented the synthesis of precursors for biomass formation as a linear function of specific growth rate ( $\mu$ ) (see Eqs. 17-27 of Appendix A). In fact, these precursors are used in other pathways such as amino acids, fatty acids and nucleic acids to construct biomass and omission of these pathways results in lumped reactions which directly connect precursors to biomass. The other three constraints were derived from molar balance on ATP and NADPH (see Eqs. 28-30 of Appendix A). These constraints supply connectivity between individual reaction rates. All fluxes were bound between 0 and 20 mmol/g-h, except  $r_{ATP}$  and  $r_{34}$  with unbounded upper limit and lower limit of zero. A fixed specific growth rate ( $\mu$ ) of 0.4 1/h was used in all cases.

**Choice of objective function:** Flux distribution within a cell is highly dependent on the choice of objective function. Based on the scope of study, various linear objective functions have been exploited such as growth rate, ATP production and product formation (Feist and Palsson, 2010; Kauffman *et al.*, 2003; Varma and Palsson, 1994). In this work, ATP requirement which has not been previously used for *B. subtilis*, was selected as the objective function to be minimized. This reaction is commonly referred to as the hydrolysis of ATP into ADP and phosphate. Minimization of ATP requirement for cell metabolism is one of the important objective functions, which has been investigated by some researchers (Kauffman *et al.*, 2003; Varma and Palsson, 1994). It helps to find metabolic pathways with greater yield and hence, it enhances cell performance.

**Flux distribution in *B. subtilis*:** FBA was applied to find the optimal flux distribution within *B. subtilis* metabolic network using Simplex algorithm in MATLAB software. The optimal value of the objective function was found to be 12.68 mmol/g-h. The corresponding flux values for some selected reactions are shown in Table 1. At the optimal solution, fluxes  $r_{10r}$ ,  $r_{19}$ ,  $r_{23}$ ,  $r_{28}$ ,  $r_{29}$ ,  $r_{33}$  and  $r_{34}$  were found to be the current non-basic variables. Apart from  $r_{34}$ , all non-basic variables had zero reduced cost coefficients. Existence of non-basic variables with zero reduced cost coefficients necessitates the search for obtaining other possible optimal flux distributions.

**Finding multiple optimal solutions:** To find alternate optimal solutions, MILP approach was utilized which converts the LP metabolic model (Eqs. 2 and 3) to a MILP problem by using a recursive method (Lee *et al.*, 2000; Phalakornkule *et al.*, 2001). Letting  $K$  as iteration number and considering  $K=1$  for the first optimal solution, MILP approach for  $K \geq 2$  can be presented as follows:

$$\begin{aligned} \text{Min } Z &= CX \\ AX &= b, X \geq 0 \\ \sum_{i \in NZ^{K-1}} y_i &\geq 1 \\ \sum_{i \in NZ^k} w_i &\leq |NZ^k| - 1, k = 1, 2, \dots, K-1 \\ 0 &\leq x_i \leq U w_i, i \in I \\ y_i + w_i &\leq 1, i \in NZ^{K-1} \end{aligned}$$

where  $NZ^{K-1}$  and  $NZ^K$  are non-zero variables at iterations  $K-1$  and  $K$ , respectively.  $I$  is the set of all variables and  $U$  is a valid upper bound for all variables.  $y_i$  is a binary variable for each non-zero basic variable  $x_i$  and it is used to select the next set of non-zero vari-

**Table 1.** Optimal flux values for some selected reactions.

| Selected Reactions |                                    |                 |
|--------------------|------------------------------------|-----------------|
| Name               | Description                        | Flux (mmol/g-h) |
| $r_1$              | Glucose Uptake                     | 3.04            |
| $r_{18}$           | Phosphoenolpyruvate to Pyruvate    | 0.61            |
| $r_{19}$           | Organic Acid Secretion             | 0               |
| $r_{23}$           | Acetate Secretion                  | 0               |
| $r_{25}$           | Citrate to $\alpha$ -ketoglutarate | 0.43            |
| $r_{28}$           | Succinate Secretion                | 0               |
| $r_{31}$           | Pyruvate to Oxaloacetate           | 4.26            |
| $r_{32}$           | Malate to Pyruvate                 | 3.07            |
| $r_{ATP}$          | Energy Requirement                 | 12.68           |

ables.  $w_i$  is another binary variable for non-zero variable  $x_i$ , which confirms searching all possible states of variables for finding optimal solutions. Iterations are terminated when the value of objective function ( $Z^k$ ) is greater than the value of objective function derived from solving the LP metabolic model ( $Z^l$ ). In order to solve MILP problem, a code in MATLAB software is utilized which is based on branch and bound algorithm.

Five distinct optimal solutions were calculated when ATP requirement was minimized to the same value of  $r_{ATP} = 12.67$  mmol/g-h in *B. subtilis* metabolic network. The optimal fluxes of some selected reactions are given in Table 2. Pentose phosphate and glycolysis pathways in central metabolism of *B. subtilis* were functional in all optimal solutions ( $r_2$  and  $r_{10} \neq 0$ ). For all solutions, the flux value of pentose phosphate pathway ( $r_2$ ) equals to 0.57 mmol/g-h, except solution 1 giving a flux value of 1.59 mmol/g-h. Although the first reaction of TCA cycle ( $r_{24}$ ) was functional in all solutions, TCA cycle was incomplete in some solutions. In solutions 1, 2 and 3, only  $\alpha$ -ketoglutarate was produced in TCA cycle while in solution 4,  $\alpha$ -ketoglutarate and succinate and in solution 5,  $\alpha$ -ketoglutarate and malate were produced. In all solutions, malate was produced by conversion of oxaloacetate to malate via reaction  $r_{33r}$ . Additionally in solution 5,  $r_{29}$  was utilized to convert succinate to malate. Malic enzyme which converts malate to pyruvate ( $r_{32}$ ) is active in all solutions.

Reactions 19, 23 and 28 indicate organic acid, acetate and succinate production, respectively, and flux of reaction 18 demonstrates the activity of pyruvate kinase enzyme. Relationship between total acid production flux ( $r_{19}+r_{23}+r_{28}$ ) and pyruvate kinase flux for five optimal solutions is presented in Figure 3.

The Figure shows a relatively direct relation between acid production and pyruvate kinase activity. Solution 2 with total acid production of 2.04 mmol/g-h has maximum value of  $r_{18}$  since solution 1 and 5

with no-acid production has pyruvate kinase activity of 0.61 and 0.81 mmol/g-h. Hence, it could be concluded that activation until 0.81 mmol/g-h does not result in production of acidic by-products. Solutions 1 and 5 with no-acid production are more appropriate compared to other solutions. In a previous study, Goel *et al.* (1995) proposed inactivation of the pyruvate kinase enzyme as a strategy for elimination of acidic by-products. However, inactivation of pyruvate kinase in *B. subtilis* was reported to be infeasible by Zhu (2003) where a minimum value of 0.3 mmol/g-h was obtained. These results are in good agreement with the results obtained in this work.

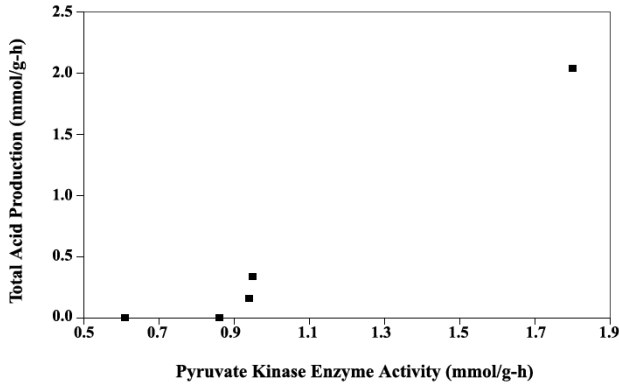
To select a final appropriate strategy, glucose consumption rate was used. Since glucose uptake rates of 3.04 and 2.96 mmol/g-h were obtained in solutions 1 and 5, respectively, solution 5 was found to be superior to solution 1. Solution 1 shows a minimum glycolysis alongside maximum pentose phosphate activations. This is in contrast to the results obtained in solution 5. Therefore, solution 5 is selected as the superior flux distribution with minimum ATP requirement, minimum glucose consumption and no-acid production. Feasibility of this solution in reality needs to be determined by a genetic engineer. Figure 4 shows the optimal fluxes in *B. subtilis* metabolic network for solution 5.

## CONCLUSIONS

In this research, FBA was exploited to obtain the multiple optimal flux distributions at minimized cellular energy requirement (ATP) for *B. subtilis* by using a linear metabolic model including 30 equations and 37 variable fluxes. To check the existence of multiple optimal solutions, a criterion based on the values of reduced cost coefficients was first used. In general, using this criterion avoids pointless searches for finding non-existing multiple optimal solutions while previously proposed approaches need to solve new math-

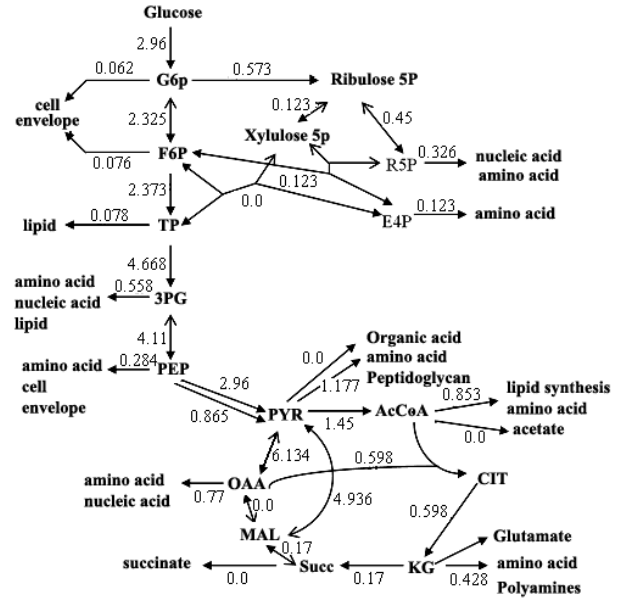
**Table 2.** Optimal fluxes for some selected reactions at the five multiple optimal solutions.

| Solution No. | Reaction Fluxes (mmol/g-h) |       |          |          |          |          |          |          |          |          |          |           |
|--------------|----------------------------|-------|----------|----------|----------|----------|----------|----------|----------|----------|----------|-----------|
|              | $r_1$                      | $r_2$ | $r_{10}$ | $r_{18}$ | $r_{19}$ | $r_{23}$ | $r_{24}$ | $r_{27}$ | $r_{28}$ | $r_{29}$ | $r_{32}$ | $r_{33r}$ |
| 1            | 3.04                       | 1.59  | 1.39     | 0.61     | 0        | 0        | 0.43     | 0        | 0        | 0        | 3.07     | 3.07      |
| 2            | 3.9                        | 0.57  | 3.26     | 1.8      | 2.04     | 0        | 0.43     | 0        | 0        | 0        | 5.11     | 5.11      |
| 3            | 3.04                       | 0.57  | 2.41     | 0.95     | 0        | 0.34     | 0.43     | 0        | 0        | 0        | 5.11     | 5.11      |
| 4            | 3.03                       | 0.57  | 2.4      | 0.94     | 0        | 0        | 0.58     | 0.16     | 0.16     | 0        | 4.95     | 4.95      |
| 5            | 2.96                       | 0.57  | 2.32     | 0.86     | 0        | 0        | 0.6      | 0.17     | 0        | 0.17     | 4.94     | 4.77      |



**Figure 3.** Total acid production in *B. subtilis* versus pyruvate kinase activity at the five optimal solutions.

emational problems even when the optimal solution is unique. By solving *B. subtilis* model at fixed specific growth rate of 0.4 1/h via Simplex algorithm, minimum energy requirement of 12.67 mmol/g-h was obtained. Computations revealed six non-basic variables with zero reduced cost coefficients for the current optimal solution and therefore, further investigations were carried out to examine the plurality of optimal solution. By applying mixed integer linear programming (MILP), a total of five optimal flux distributions with the same minimum cellular energy requirement were enumerated, among which one showing no acid production and lowest glucose consumption rate was identified as the superior flux distribution. The multiple optimal solutions can hence be used to select the superior strategies for improving cell performance based on other desired objective functions. In addition, investigations on the practical feasibility of multiple



**Figure 4.** Optimal fluxes (mmol/g-h) for solution 5 showing minimum required energy as well as minimum glucose consumption and by-product secretion in metabolic network of *B. subtilis*.

optimal solutions can result in selection of a solution which is experimentally possible.

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## Appendix A. Metabolic model of *B. subtilis* (30 equations and 37 variable fluxes) in standard LP form ( $\mu$ : specific growth rate).

|   |      |   |      |
|---|------|---|------|
| $r_1 - r_2 - r_3 - r_{10} + r_{10r} = 0$                        | (1)  | $r_{29} - r_{33} + r_{33r} - r_{32} = 0$  | (16) |
| $r_{10} - r_{10r} - r_{12} - r_{11} + r_7 + r_8 = 0$            | (2)  | $r_3 = 0.154\mu$  | (17) |
| $r_{14} - r_{15} - r_{16} = 0$                                  | (3)  | $r_{11} = 0.019\mu$   | (18) |
| $-r_1 + r_{16} - r_{18} - r_{17} = 0$                           | (4)  | $r_{13} = 0.194\mu$   | (19) |
| $r_1 + r_{18} - r_{21} - r_{19} - r_{20} - r_{31} + r_{32} = 0$ | (5)  | $r_{15} = 1.395\mu$   | (20) |
| $-r_{31} + r_{24} + r_{30} - r_{33} + r_{33r} = 0$              | (6)  | $r_{17} = 0.711\mu$   | (21) |
| $r_{21} - r_{24} - r_{23} - r_{22} = 0$                         | (7)  | $r_6 = 0.816\mu$  | (22) |
| $r_{24} - r_{25} = 0$   | (8)  | $r_9 = 0.308\mu$  | (23) |
| $r_{25} - r_{27} - r_{26} = 0$                                  | (9)  | $r_{20} = 2.942\mu$   | (24) |
| $-r_{29} + r_{27} - r_{28} = 0$                                 | (10) | $r_{22} = 2.132\mu$   | (25) |
| $r_2 - r_4 - r_5 = 0$   | (11) | $r_{26} = 1.071\mu$   | (26) |
| $-r_7 + r_4 - r_6 = 0$  | (12) | $r_{30} = 1.923\mu$   | (27) |
| $-r_7 - r_8 + r_5 = 0$  | (13) | $2r_2 + r_{25} + r_{32} = 16.7\mu$  | (28) |
| $r_7 - r_8 - r_9 = 0$   | (14) | $r_{ATP} - r_{34} = 31.7\mu$  | (29) |
| $2r_{12} + r_8 - r_{14} - r_{13} = 0$                           | (15) | $r_{ATP} + r_{12} - 3r_{14} - r_{18} - r_{23} - 3r_{27} - 2r_{21} - 2r_{33} + 2r_{33r} - r_{29} - r_{31} + 2r_{19} = 0$ | (30) |

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