Metabolic Capacity of *Bacillus subtilis* for the Production of Purine Nucleosides, Riboflavin, and Folic Acid

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Abstract: We developed a stoichiometric model of *Bacillus subtilis* metabolism for quantitative analysis of theoretical growth and biochemicals production capacity. This work concentrated on biochemicals that are derived from the purine biosynthesis pathway; inosine, guanosine, riboflavin, and folic acid. These are examples of commercially relevant biochemicals for which *Bacillus* species are commonly used production hosts. Two previously unrecognized, but highly desirable properties of good producers of purine pathway-related biochemicals have been identified for optimally engineered product biosynthesis; high capacity for reoxidation of NADPH and high bioenergetic efficiency. Reoxidation of NADPH, through the transhydrogenase or otherwise, appears to be particularly important for growth on glucose, as deduced from the corresponding optimal carbon flux distribution. The importance of cellular energetics on optimal performance was quantitatively assessed by including a bioenergetic efficiency parameter as an unrestricted, ATP dissipating flux in the simulations. An estimate for the bioenergetic efficiency was generated by fitting the model to experimentally determined growth yields. The results show that the maximum theoretical yields of all products studied are limited by pathway stoichiometry at high bioenergetic efficiencies. Simulations with the estimated bioenergetic efficiency of *B. subtilis,* growing under glucose-limiting conditions, indicate that the yield of these biochemicals is primarily limited by energy and thus is very sensitive to the process conditions. The maximum yields that can reasonably be expected with *B. subtilis* on glucose were estimated to be 0.343, 0.160, and 0.161 (mol product/mol glucose) for purine nucleosides, riboflavin, and folic acid, respectively. Potential strategies for improving these maximum yields are discussed. *Biotechnol Bioeng* **59**: 227–238, 1998.
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INTRODUCTION

Driven by economic and environmental considerations, industrial interest in biological routes for production of chemicals has recently become intense. In many such applications, carbon feedstock is a significant component of manufacturing cost. Therefore maximizing the carbon yield of product, relative to carbon consumed for cell and by-product formation, is a priority.

The actual yield achieved depends on complex interactions among the metabolic processes involved in product biosynthesis. The basic framework for these processes is the structure and stoichiometry of the metabolic reactions involved. This stoichiometric framework imposes constraints on achievable yields. Several prior studies have analyzed metabolic stoichiometry to determine stoichiometric limitations and maximum theoretical yields in several bacterial species that are relevant to the industrial manufacture of biochemicals. The first studies concentrated on products of anaerobic fermentations (Papoutsakis, 1984; Papoutsakis and Meyer, 1985). Later, biochemical production capabilities were established for *Escherichia coli* (Forberg et al., 1988; Varma et al., 1993), which plays an important role in the manufacture of aromatic compounds from the shikimat pathway (Berry, 1996). Also the coryneform bacteria, commonly used in amino acid fermentations (Jetten and Sinskey, 1995; Sahm et al., 1995), have been extensively investigated with respect to potential stoichiometric limitations (de Hollander, 1994; Vallino and Stephanopoulos, 1994; Vallino and Stephanopoulos, 1994).

However, only limited information is available for the equally important, gram-positive bacilli, which include a variety of industrially relevant species for protein and biochemical production (Arbige et al., 1993). Several members of this genus, such as *Bacillus amyloliquefaciens, B. licheniformis, B. pumilus,* or *B. subtilis,* have a long tradition as safe and stable producers of the purine nucleosides, inosine and guanosine, in commercial processes (Kuninaka, 1996; Miyagawa and Kanzaki, 1991). These nucleosides are raw material for the synthesis of the flavor enhancers inosine monophosphate (IMP) and guanosine monophosphate (GMP). *Bacillus subtilis* has also proven to be a potent host for the recently commercialized process of riboflavin (vitamin B_2) production (Perkins et al., 1991; van Loon et al.,

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1996). Currently, purine nucleosides and riboflavin are commercially produced in bulk quantities by fermentation with *Bacillus* spp. (Kuninaka, 1996; van Loon et al., 1996), and folic acid is also a promising potential product of *B. subtilis.* Both vitamins, riboflavin and folic acid, are important additives in the animal feed and human food industries, and their biosynthesis proceeds mainly via the purine nucleotide pathway.

Here we report on primary pathway analysis in *B. subtilis* for maximum production of biomass and purine pathway-related biochemicals, including purine nucleosides and the vitamins riboflavin and folic acid, from different carbon substrates. In the developed stoichiometric model, maximum theoretical yields and optimal carbon flux distributions were determined by using a linear constrained optimization technique (van Gulik and Heijnen, 1995; Varma et al., 1993). Alternative scenarios in primary metabolism, with a particular emphasis on cellular energetics, were investigated to derive conclusions on the choice of host organism as well as metabolic engineering (Bailey, 1991) and process strategies.

METHOD

Biochemical Reaction Network

The biochemical reaction network for *B. subtilis,* as given in Figure 1, was constructed on the basis of prior network analysis and experiments (Sauer et al., 1997; Sauer et al.,

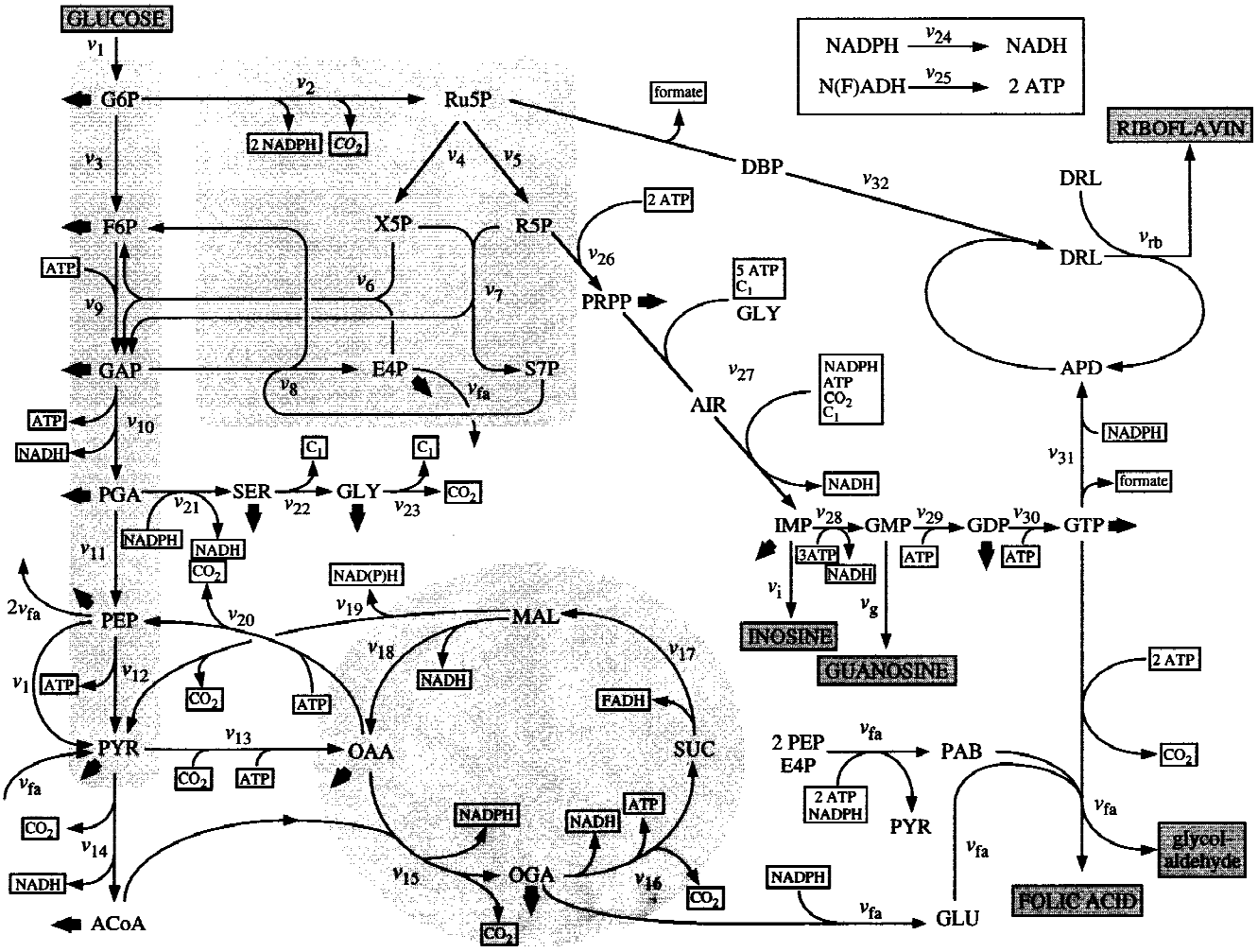

Figure 1. Biochemical reaction network constructed for central carbon metabolism and purine pathway-related biochemicals in *Bacillus subtilis* using glucose as the substrate (alternative substrate pathways are discussed in the text). Glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle reactions are marked with a grey background and cofactors are boxed. Shaded arrows indicate the withdrawal of precursors for biomass synthesis. Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvate; PYR, pyruvate; ACoA, acetyl coenzyme A; OAA, oxaloacetate; OGA, oxoglutarate; SUC, succinate; MAL, malate; SER, serine; GLY, glycine; Ru5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate; S7P, seduheptulose-7-phosphate; PRPP, phosphoribosyl diphosphate; IMP, inosine monophosphate; GMP [GDP, GTP], guanosine mono-[di-, tri-]phosphate; APD, 5-amino-6-(ribitylamino)-2,4(1H, 3H)-pyrimidinedione; DRL, 6,7-dimethyl-8-ribityllumazine. AIR (5′-phosphoribosyl-5-aminoimidazole), DBP (3,4-dihydroxy-2-butanone), GLU (glutamate), and PAB (p-amino benzoic acid) are shown for clarity but were not incorporated as individual metabolites in their pathways for the matrix calculations. Glycolaldehyde is considered to be formed as a byproduct of folic acid with an equal molar ratio.

1996). Published biochemistry was used for the construction of the biosynthetic pathways to purine nucleotides, riboflavin, and folic acid (Gottschalk, 1986; Green et al., 1996; Perkins and Pero, 1993; Zalkin and Nygaard, 1996). The previously determined biomass composition of *B. subtilis* (Sauer et al., 1996) was employed in combination with textbook biochemistry (Neidhardt et al., 1990) to include detailed growth requirements for energy and precursors (Table I). However, minor corrections were necessary to adapt the data published by Neidhardt et al. (1990) for stoichiometric analysis. Specifically, corrections were introduced for the biosynthesis of the amino acids aspartate, asparagine, histidine, methionine, and tryptophan as well as all nucleotides (Gottschalk, 1986). A mole percentage of G + C equal to 43% was taken as the basis for DNA precursor calculations (Priest, 1993). Additionally, the energetic requirements for polymerization reactions of cellular macromolecules were incorporated (Neidhardt et al., 1990). Because only solutions with a non-negative ATP balance are plausible, ATP was defined as a final product in the optimization calculation.

The cellular monomeric composition can be accurately specified, but the bioenergetic efficiency, as specified by the bioenergetic parameters Y_{ATP} (biomass yield per mole ATP) and P-to-O ratio (P/O) (moles ATP produced per mole reducing equivalents consumed in oxidative phosphoryla-

Table I. Precursor and energy requirements for biomass formation (μmol/g dry weight) of *Bacillus subtilis* 1012, purine nucleotides, riboflavin, and folic acid biosynthesis (mol/mol).[a]

		Stoichiometry															
Precursor	Amount	G6P	F6P	R5P	E4P	GAP	PGA	PEP	PYR	ACoA	OAA	OGA	C_1	CO_2	ATP	NADH	NADPH
Ala[b]	404								1								1
Arg	198											1		1	7	−1	4
Asx[c]	426										1				1		1
Cys	60						1								4	−1	5
Glx[b,d]	544											1			0.5		1
Gly	495						1						−1			−1	1
His	86			1									1		6	−3	2
Ile	288								1		1			−1	2		5
Leu	368								2	1				−2		−1	2
Lys	342								1		1			−1	2		4
Met	118						1		−1		1		1		6	−1	8
Phe	183				1			2						−1	1		2
Pro	175											1			1		3
Ser	239						1									−1	1
Thr	239										1				2		3
Trp	0			1	1	−1	1	2	−1					−1	4	−1	2
Try	125				1			2						−1	1	−1	2
Val	335								2					−1			2
Dap[e]	95								*1*		*1*				*2*		*4*
Protein				86	308		912	616	2322	368	1413	917	−291	−1811	5769	−1861	10388
RNA				630			368				262		368	368	6540	−1366	1163
DNA				100			50				50		79	50	1015	−200	307
Lipids						194	65		335	1574	103			−632	1419	−168	3535
Peptidoglycan			190					95	285	190	95	95			855		760
Glycogen		154													154		
Polyamines												59			118		180
Polymerization															22738		
Total		154	190	816	308	194	1395	711	2942	2132	1923	1071	156	−2025	38608	−3595	16333
Inosine				1			1						1	1	8	−2	2
Guanosine				1			1						1	1	11	−3	2
Riboflavin				3			1						1	−2	13	−3	3
Folic Acid				1	1		1	2	−1			1	1		17	−3	4

[a]For definitions of abbreviations, see legend to Fig. 1; negative values indicate formation.
[b]Murein fraction of alanine and glutamate subtracted and included in peptidogylcan.
[c]Asparagine and aspartate.
[d]Glutamine and glutamate.
[e]Diaminopimelate accounted for in peptidoglycan but not in protein.

tion), is still a matter of debate. Evidence is accumulating that suggests variability in the efficiency of energy generation in oxidative phosphorylation and thus in the operating P/O (Neijssel and Teixeira de Mattos, 1994). Additionally, Y_{ATP} is not constant because it is influenced by the energetic requirements for maintenance as well as all growth-dependent functions that do not directly lead to biomass. In addition to the mass balances required for flux analysis, however, an ATP mass balance is important for optimization studies to evaluate the influence of cellular energetics on product formation. In order to avoid extensive assumptions about details of ATP generation and consumption, a newly defined energetic efficiency parameter was introduced that accounts for variations in the fundamental energetic parameters Y_{ATP} and P/O. This parameter, ξ, was formulated as a separate, ATP dissipating flux. Thus, ξ is defined as a substrate-specific amount of ATP that is consumed in processes other than biomass or product formation:

$$\xi = Y_{ATP/S}^{max} - \nu_{ATP}^{BM} Y_{BM/S}^{obs} - \nu_{ATP}^{P} Y_{P/S}^{obs} \quad (1)$$

where ν_{ATP}^{BM} and ν_{ATP}^{P} are the ATP fluxes for biomass and product formation and $Y_{BM/S}^{obs}$ and $Y_{P/S}^{obs}$ are the observed biomass and product yields on a given substrate. The theoretical range for ξ is between zero and the maximum amount of ATP formed per molecule of substrate ($Y_{ATP/S}^{max}$). Non-zero values represent a lower efficiency of oxidative phosphorylation, maintenance requirements, and/or lower Y_{ATP} values. For the optimal, but practically impossible, energetic efficiency with ξ equal to 0, only the ATP requirements specified in Table I can exist and P/O must equal 2. For the substrate-specific maximum value of ξ, no ATP is available for growth or product formation. Because ξ is not known a priori, it was treated as an unrestricted flux for maximum yield calculations. Hence, the solutions are a function of the energetic efficiency. To obtain flux distributions for a specific organism, the operating ξ of growing cells was estimated by fitting the model to experimental maximum biomass yield data. This value was used as a representative estimate of the energetic efficiency for simulations with non-growing cells.

Stoichiometric Model

The metabolite balances of the stoichiometric matrix were formulated for the reactions defined in Figure 1. Based on thermodynamics and typical metabolite pool sizes, the following reactions were considered to be physiologically irreversible: ν_1, ν_2, ν_9, ν_{12-16}, ν_{19-23}, and ν_{25-32}. For glucose substrate analyses, the reaction catalyzed by the phosphoenolpyruvate carboxykinase (PEP CK) (ν_{20}) was considered to be inactive unless indicated otherwise. Additionally, the following assumptions on *B. subtilis* metabolism are included in the stoichiometric model: (a) conversion of NADPH to NADH via the transhydrogenase is not energy-dependent and reversible; (b) NADH, NADPH, and FADH are energetically equivalent; (c) depending on the actual biosynthetic pathway, regeneration of ammonia (NH_3) donors in biosynthesis was included in the model by three mechanisms with the following stoichiometries (Gottschalk, 1986):

$$NH_3 + \text{glutamate} + ATP = \text{glutamine} \quad (2)$$

$$NH_3 + \text{oxoglutarate} + NADPH = \text{glutamate} \quad (3)$$

$$NH_3 + \text{fumarate} + NADPH = \text{aspartate} + NADH \quad (4);$$

(d) secretion of the products investigated proceeds via passive transport mechanisms; (e) for glycerol and citrate substrate analyses, the reactions from glyceraldehyde-3-phosphate (GAP) to fructose-6-phosphate, involving the fructose-1,6-bisphosphatase, were included without ATP generation rather than as a reversible phosphofructokinase; (f) for glycerol uptake via facilitated diffusion (Saier et al., 1993) the glucose uptake mass balance equation for ν_1 was replaced by

$$\text{glycerol} + ATP = \text{glyceraldehyde-3-phosphate} + NADH \quad (5);$$

(g) citrate uptake was assumed to proceed via a proton symport mechanism (Boorsma et al., 1996) with the following mass balance for ν_1:

$$\text{citrate} + 1/3\ ATP = \text{oxoglutarate} + NADPH + CO_2 \quad (6);$$

(h) for sucrose substrate analyses, the uptake was assumed to require only one molecule of PEP via the sucrose-specific PTS system (Postma et al., 1993). Expenditures for the assimilation of nitrogen and sulphur were calculated for growth on NH_4^+ and SO_4^{2-} salts in all cases. For comparative reasons, in some cases the biochemical network of *E. coli* central carbon metabolism was used. It was assumed to differ from the above-described *B. subtilis* network by employing the PEP carboxylase for the anaplerotic reaction, rather than the pyruvate carboxylase. Therefore, the mass balance equation for ν_{13} changes to

$$PEP + CO_2 \rightarrow \text{oxaloacetate} \quad (7).$$

Solving the Metabolic Network

The general principle of flux balance analysis has been extensively covered in the literature and was recently reviewed (Holms, 1996; Varma and Palsson, 1994). In this study, the problem of how much product could potentially be made has been addressed considering constraints imposed by metabolic stoichiometry. Depending on the actual case studied, the dimensions of the stoichiometric matrices were 28 to 34 reaction equations by 28 to 33 metabolite species. Of the metabolite species, 25 to 29 were key intermediates, leaving 3 to 4 macroscopic substances (CO_2, ATP, biomass, and product). The steady-state flux balance is formulated as a linear optimization problem in which the production of a particular product is maximized subject to

$$\mathbf{S} \cdot \mathbf{v} \geqslant \mathbf{b} \quad (8)$$

where **S** is the stoichiometric matrix of the metabolic network, **v** is the vector of unknown reaction fluxes, and **b** is the production rate vector for all the metabolites in the network. For this purpose, the 1p function from the MATLAB Optimization Toolbox (The MathWorks, Inc., Natick, MA) was used. Bounds were imposed so that irreversible reactions must have non-negative fluxes. The intracellular metabolites were defined as equally constrained species while the variable products CO_2, ATP, and biomass (or biochemicals) were allowed to accumulate.

Maximizing substrate conversion to biomass or metabolites were examined as the objective functions. The computed solution consists of the maximum yield and the corresponding flux distribution. Optimization of the objective function within the defined network is limited by at least one metabolic constraint. In this study, limiting energetic and stoichiometric constraints were identified. Energetically limited (ATP-limited) cases are defined by the lack of surplus ATP formation, which is seen as ATP accumulation in the hypothetical output reaction. When energy limits production, the yield becomes a function of ξ. Stoichiometrically limited (non-ATP-limited) cases are defined by CO_2 production from decarboxylation reactions in the primary pathways *and* accumulation of ATP as a final product. In these cases, the maximum yield is not a function of energetic efficiency and is independent of ξ. However, stoichiometric limitations with a small surplus ATP generation are, in practice, also likely to be energy-limited and so are labeled as limited by stoichiometry *and* energy. The energetic efficiency at the transition from operating energetic to stoichiometric limitations is indicated by the ξ* value. In the calculations, this ξ* value equals the hypothetical ATP production when P/O is assumed to be 2 and only the ATP requirements specified in Table I were used. Redox constraints are not likely to limit formation of the products investigated because *B. subtilis* metabolism has the biochemical potential to convert energy into reducing equivalents and vice versa. For the conversion of reducing equivalents into energy, *B. subtilis* metabolism was shown to be capable of NADPH reoxidation (Sauer et al., 1997), possibly via a transhydrogenase mechanism (Olausson et al., 1995) or an ATP-driven cycle consisting of the NADPH-dependent malic enzyme, the pyruvate carboxylase, and the malate dehydrogenase.

RESULTS

Biomass Generation

The objective function of maximum conversion of different carbon substrates into *B. subtilis* biomass was analyzed using the constructed stoichiometric model. Results obtained by using this model may be taken as a representative example for all organisms with similar central carbon metabolism and biomass composition. The overall bioenergetic efficiency of bacterial metabolism was included as the energetic efficiency parameter (ξ), which takes variations in P/O and Y_{ATP} into consideration and thus links energy and stoichiometry. Because the energetic efficiency of bacterial cells is not known a priori, the influence of changes in these factors were quantitatively analyzed by expressing yield as a function of ξ. Maximum theoretical biomass yields during growth on three typical substrates with a high (glycerol), intermediate, (glucose), or low (citrate) degree of reduction are given in Figure 2. For direct comparison, all biomass yield values were reported per six moles of carbon. A linear dependency of calculated biomass yields on ξ was found for glucose and glycerol, indicating that the yields are limited by energy. Similarly, a linear dependency was found for citrate over a wide range of ξ; however, at extremely low ξ (below 2.5) the yield is limited by network stoichiometry, due to the irreversibility of the citrate synthase reaction (ν_{15}). The same linear dependency was found when changing the anaplerotic reaction of *B. subtilis* to the *E. coli* alternative (data not shown), where PEP and CO_2 are converted to oxaloacetate via the PEP carboxylase (Gottschalk, 1986). However, using the *E. coli* network, biomass yields on all substrates were stoichiometrically limited at very low ξ values below 4.7, 1.6, and 2.5 for glucose, glycerol, and citrate, respectively. At these very low ξ values, the biomass yield using the *E. coli* network is limited by the supply of PEP on glucose or glycerol and the irreversibility of the citrate synthase reaction when citrate is the substrate. Generally, these results indicate that the biomass yield of organisms with *B. subtilis*- and *E. coli*-like networks depends largely on the bioenergetic efficiency and the degree of reduction of the substrate. The latter, in turn, determines the fraction of ATP that is generated via oxidative phosphorylation during substrate catabolism.

The macromolecular composition assumed in this study is representative for a standard cell and consists of 50% protein, 21% RNA, and 3% DNA (Neidhardt et al., 1990; Sauer et al., 1996). However, bioprocesses typically utilize slow-growing cells for which the macromolecular compo-

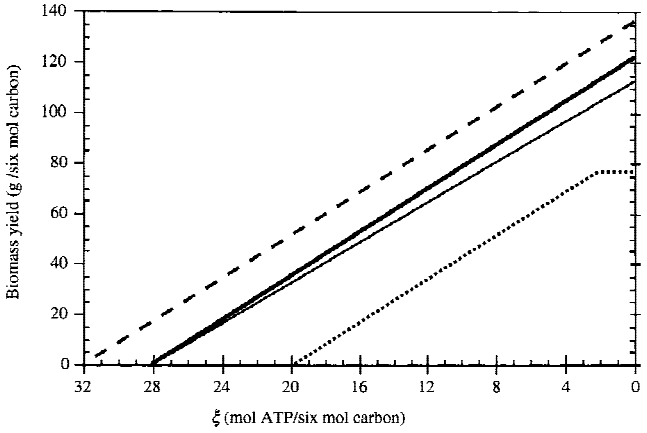

Figure 2. Calculated biomass yield at different values of the energetic efficiency parameter (ξ) on glucose (solid line), glycerol (dashed line), and citrate (dotted line). The thin solid line indicates biomass yields on glucose when a higher protein content than in the standard biomass composition is assumed (see text for details).

sition is likely to deviate from these standard values. The relative protein content of bacterial cells is known to be negatively correlated with the specific growth rate, and thus is highest in slow-growing cells (Bremer and Dennis, 1996). As an extreme case, a relative composition of 72% protein, 10% RNA, 5% DNA, and 13% for all other components was assumed. Detailed energy and precursor requirements for cells with such a macromolecular composition were used for sensitivity analysis. Despite this rather large deviation in macromolecular composition, the maximum yield on glucose was found to be only slightly lower as compared to the standard macromolecular composition (Fig. 2). Largest yield reductions of up to 7% occurred only at unrealistic ξ values close to 0. Thus, the solutions for biomass generation are not overly sensitive to variations in cellular macromolecular composition.

What maximum yield might be achieved in practice on a given substrate will depend on the energetic efficiency of the organism and the process conditions. A fit of the model to experimentally determined maximum biomass yields (Y_{GLC}^{max}) in glucose-limited chemostat cultivations (Sauer et al., 1996) gives a substrate-specific ξ_{GLC} value equal to 9.5 (ATP per molecule glucose) for *B. subtilis.* Experimental values for Y^{max} on glycerol (GOL) are not available for *Bacillus* spp., but can be expected to be higher than on glucose because glycerol is a more reduced substrate. Assuming that Y_{GOL}^{max} of *B. subtilis* is similar to that of *E. coli* (100 g/mol) (Farmer and Jones, 1976), an ξ_{GOL} equal to 4.4 (8.9 per 6 moles carbon) was obtained. The optimal carbon flux distribution for a *B. subtilis* variant, with a realistic ξ_{GLC} of 9.5 and ξ_{GOL} of 4.4, growing on glucose or glycerol is illustrated in Figure 3. The solutions show significant fluxes through the pentose phosphate pathway (PPP), in particular when glycerol is the substrate. This result implies a major contribution of the PPP to NADPH generation, in addition to the isocitrate dehydrogenase reaction of the tricarboxylic acid (TCA) cycle. With glucose as the substrate, almost doubled fluxes were found in the TCA cycle compared with glycerol. Higher bioenergetic efficiency would lead to reduced fluxes in the TCA cycle for both substrates (data not shown). Such a change would be accompanied by higher fluxes through the PPP when glucose is the substrate, and increased fluxes in the pyruvate shunt (ν_{13} and ν_{19}) when the substrate is glycerol. It should be noted that transhydrogenase activity is not required for either solution.

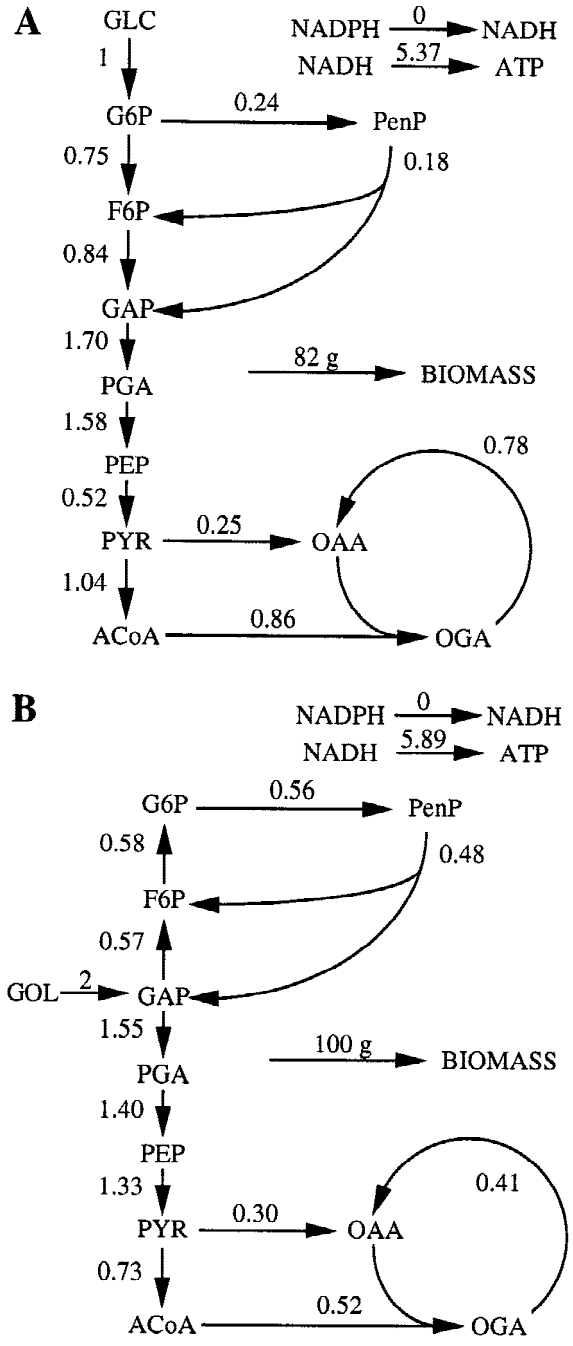

Figure 3. Flux distribution for the maximum biomass production of *Bacillus subtilis*, with ξ_{GLC} and ξ_{GOL} assumed to be equal to 9.5 and 4.4, respectively, growing on glucose (GLC) **(A)** or glycerol (GOL) **(B)** as substrate. The glycerol solution is presented for the uptake of two molecules (6 moles carbon) for direct comparison with the glucose solution.

Biochemicals Production from Glucose

The use of non-growing cells was assumed for biochemical production, because such a system has the potential to give maximum possible yield. The theoretical maximum yield of biochemicals was determined by setting the objective to maximize product formation within the defined stoichiometric model. The product yield is a linear function of ξ_{GLC}, for as long as product formation is energetically limited. However, the yield becomes independent of ξ_{GLC} when product formation is stoichiometrically limited. In this case, the yield becomes a horizontal line when plotted vs. ξ_{GLC} and the energetic efficiency at the transition between energetic and stoichiometric limitations is referred to as ξ_{GLC}^{*}. Maximum conversion of glucose into inosine, guanosine, riboflavin, and folic acid within the stoichiometric network is given in Table II. Computed biochemical yields on glucose were largely independent of the assumed ξ_{GLC} and thus appear to be mainly limited by stoichiometry, as indicated by the relatively high ξ_{GLC}^{*} values. Generally, the metabolic

Table II. Maximum theoretical yield of biochemicals on glucose in the absence of growth.

Product	Maximum yield (mol/mol)	ξ^*_{GLC} [a] (ATP/glucose)
Inosine	0.316	12.1
Guanosine	0.316	11.8
Riboflavin	0.154	12.0
Folic acid	0.154	10.6

[a]The bioenergetic efficiency at which the transition from operating energetic to stoichiometric limitations occurs.

bottleneck for the maximum yield of all products considered is the supply of PEP when glucose is the substrate. Although identical yields were calculated for riboflavin and folic acid, stoichiometric constraints appear to limit only at higher energetic efficiency for the latter product because the yield is a function of energetics down to a lower ξ^*_{GLC} value. Thus, energetic efficiency of a host organism with a *B. subtilis*-like network appears to be more important for maximum folic acid formation, which is reasonable because its formation is energetically more costly and its precursor molecules derive from several positions in central metabolism. However, when changing the network to the *E. coli* alternative, with PEP carboxylase instead of pyruvate carboxylase, the maximum folic acid yield on glucose was reduced to 0.133 (mol/mol) with a ξ^*_{GLC} value of 12.9. This result can be explained by the higher PEP demands for folic acid biosynthesis compared to the other products, for which the maximum yields were not affected by using the alternative anaplerotic reaction.

In organisms with the appropriate energetic efficiency, potentially limiting stoichiometric constraints (supply of PEP) could in principle be overcome by metabolic engineering of central metabolism. Promising strategies would be the engineering of strains either capable of an unrestricted flux through the PEP CK or with glucose permease and hexokinase replacing the phosphotransferase (PTS) glucose uptake system. The absolute highest yield for all products considered is found for a hypothetical metabolic variant with a non-PTS uptake system (Table III). In this case stoichiometric constraints are not limiting and the product yield is limited by energy only. The possibility of improving the yield by such strategies, however, has to be weighed against the likelihood of finding organisms and process conditions that provide the necessary energetic efficiency.

To obtain maximum yields that may be realized with *B. subtilis*, we included information on reported bioenergetic efficiency and preferential pathway usage in the model. The optimal flux distributions predict that pentose phosphates are generated via the non-oxidative branch of the PPP (data not shown). In *B. subtilis*, however, the oxidative PPP was reported to be a major pathway in glucose catabolism and thus the prime physiologically employed route for pentose phosphate supply (Sauer et al., 1997). To simulate this physiologically occurring flux distribution in the model, the fluxes through the reaction ν_4 were constrained to be non-negative. This is, of course, physiologically unrealistic because ν_4 is catalyzed by a reversible epimerase, but it introduces the fewest artificial perturbations to the system while forcing the solution to mimic experimental observations. Yields of all products were reduced by about 10% in such a metabolic variant, when compared to the non-modified variant (Table III). The gluconeogenic PEP CK reaction is normally considered to be inactive during growth on glucose (Diesterhaft and Freese, 1973; Neidhardt et al., 1990). However, a small but significant flux of up to 20% of the initial glucose uptake rate was reported for this reaction in *B. subtilis* (Sauer et al., 1997). Including the constraint of 0 to 20% flux through the PEP CK results in a higher yield increase for guanosine and folic acid than for riboflavin. This increase is achieved at the expense of lower ξ^*_{GLC} in a metabolic variant also using the oxidative PPP for pentose phosphate supply (Table III). Using the *E. coli* network for guanosine and riboflavin does not change the maximum yield of the metabolic variants with the non-PTS glucose uptake system or the oxidative PPP for pentose phosphate generation. However, relaxing the constraints on PEP CK does not improve the yields, as was found with the *B. subtilis* network. For folic acid, the *E. coli* network allows only for a maximum yield of 0.125 (mol/mol) with a ξ^*_{GLC} of 13.8 in the case of the oxidative PPP variant, independent of PEP CK constraints.

What might be considered as the maximum capacity of *B. subtilis* using glucose was obtained from the metabolic variant with an upper bound of 20% for the PEP CK, utilization of the oxidative PPP, and ξ_{GLC} equal to 9.5 as estimated for growing cells (Fig. 4). The resulting maximum yields (mol/mol) of 0.343 for guanosine, 0.160 for riboflavin, and 0.161 for folic acid are limited by energy (folic acid) or a combination of energy and stoichiometry (surplus ATP formation

Table III. Maximum theoretical yield of biochemicals for different metabolic variants using glucose in the absence of growth.

Metabolic variant	Maximum yield (mol/mol)	ξ^*_{GLC} (ATP/glucose)
Guanosine		
PTS^- [a]	0.537	0
PPP[b]	0.286	13.1
PPP, PEP CK[c]	0.343	10.0
Riboflavin		
PTS^-	0.267	0
PPP	0.133	13.9
PPP, PEP CK	0.160	10.8
Folic acid		
PTS^-	0.245	0
PPP	0.143	11.7
PPP, PEP CK	0.171	8.3

[a]Glucose uptake was mediated by a glucose permease and an ATP-requiring hexokinase instead of the phosphotransferase system.

[b]Generation of pentose phosphates via the oxidative branch of the PPP by constraining the flux through reaction ν_4 to be non-negative.

[c]The flux through the PEP carboxykinase (ν_{20}) was constrained to a maximum of 20% of the glucose uptake rate.

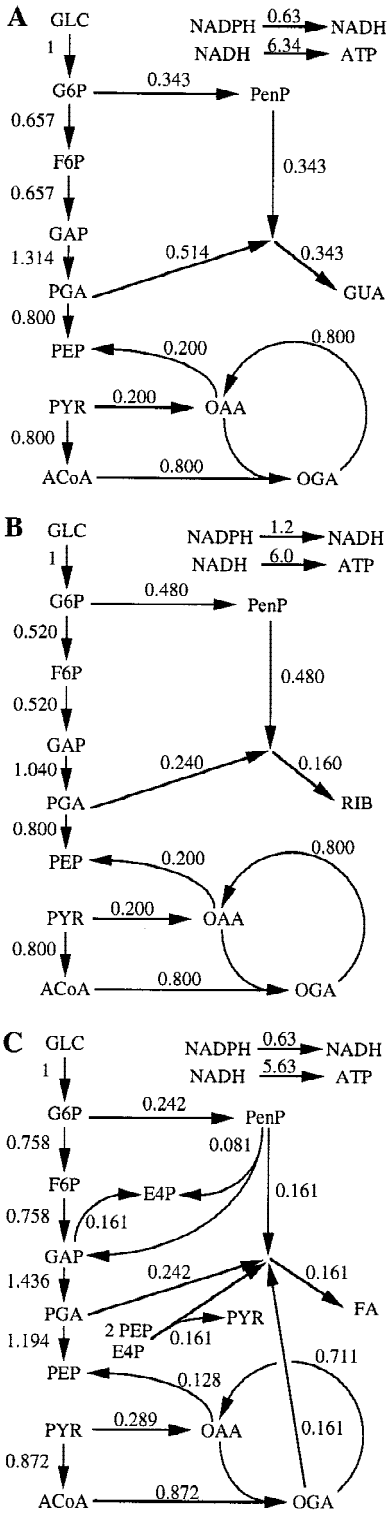

Figure 4. Flux distribution for maximum guanosine (GUA), riboflavin (RIB), and folic acid (FA) production **(A, B,** and **C,** respectively) in *Bacillus subtilis* using glucose as the carbon source. The solutions were computed for a metabolic variant in which pentose phosphates are generated via the oxidative branch of the PPP (non-negative fluxes through v_4), PEP CK is constrained to a maximum of 0.2, and ξ_{GLC} is equal to 9.5.

of 0.47 and 1.34 per glucose for guanosine and riboflavin, respectively). Inosine data were not included because the solutions were essentially identical to those for guanosine, with a slightly higher surplus ATP formation. Optimally engineered product biosynthesis could potentially achieve these yields in practice. One obvious observation of the flux distribution is the complete redundancy of pyruvate kinase for riboflavin formation. Furthermore, high product yield on glucose requires reoxidation of NADPH formed during catabolism back to $NADP^+$, e.g., a transhydrogenase-like activity (Fig. 4), even for metabolic variants in which the non-oxidative PPP is the sole supply route for pentose phosphates (data not shown). Optimal formation of riboflavin necessitates the highest flux through the transhydrogenase because three pentose precursors are required compared to one for the other products.

Biochemicals Production Using Alternative Substrates

Alternative substrates such as glycerol or sucrose might be attractive for commercial production processes because of the stoichiometric limitations associated with glucose utilization at higher bioenergetic efficiency, namely the supply of PEP. Glycerol is taken up via facilitated diffusion and enters at the level of GAP while the glucose-fructose disaccharide sucrose is taken up via a PTS-mechanism; however, only one molecule of PEP is converted to PYR per molecule of sucrose (Postma et al., 1993). Generally, model simulations using glycerol or sucrose as the sole carbon source indicate that the yields are limited either fully or down to unrealistically low ξ^* values by energy (Table IV). Consequently, the maximum yield depends solely on the energetic efficiency of the employed host organism and not on stoichiometric limitations of the metabolic network.

For glycerol, the maximum energetic efficiency (ξ_{GOL}) of growing *B. subtilis* was estimated to be equal to 8.9 (per 6 moles carbon). Maximum yields on glycerol (mole per 6 moles carbon) that may be achieved with *B. subtilis* in practice were obtained using this ξ_{GOL} value and are 0.447 for inosine, 0.439 for guanosine, 0.217 for riboflavin, and 0.202 for folic acid. The flux distribution for the optimal solutions on glycerol with ξ_{GOL} at 8.9 indicate a significant contri-

Table IV. Maximum theoretical yields of biochemicals on glycerol or sucrose in the absence of growth.

Product	Maximum yield on glycerol (mol/2 mol)[b]	ξ^*_{GOL} (ATP/2 glycerol)	Maximum yield on sucrose[a] (mol/0.5 mol)[b]	ξ^*_{SUC} (ATP/0.5 sucrose)
Inosine	0.600	0.2	0.533	0
Guanosine	0.594	0	0.425	0
Riboflavin	0.295	0	0.261	0
Folic acid	0.267	0.7	0.243	0.1

[a]Sucrose solutions were obtained by constraining the maximum flux through PEP CK (v_{20}) to 20% of the initial glucose uptake, based on the rationale given in the Results section for glucose conversion.

[b]The given maximum yield values are normalized to six moles carbon.

bution of the oxidative PPP to the generation of pentose precursors and an apparent lack of transhydrogenase requirement (Fig. 5). When comparing these yields with the *B. subtilis* glucose solutions, an improvement was found on glycerol by 28%, 36%, and 25% for purine nucleosides, riboflavin, and folic acid, respectively. Thus, a substantial maximum yield improvement may be expected for *B. subtilis* when glycerol is used as the carbon source.

For sucrose, the efficiency parameter ξ_{SUC} was assumed to be identical to the glucose values (per 6 moles carbon) of *B. subtilis*, because sucrose is a dimer of glucose and fructose. The resulting maximum yields on sucrose (mol/mol) are 0.734 for inosine, 0.720 for guanosine, 0.356 for riboflavin, and 0.328 for folic acid. Compared to the glucose solution, the sucrose yields are improved by only 5%, 9%, and 2% for purine nucleosides, riboflavin, and folic acid, respectively. The realization of potential further yield improvements on sucrose would require a host with higher energetic efficiency than *B. subtilis.*

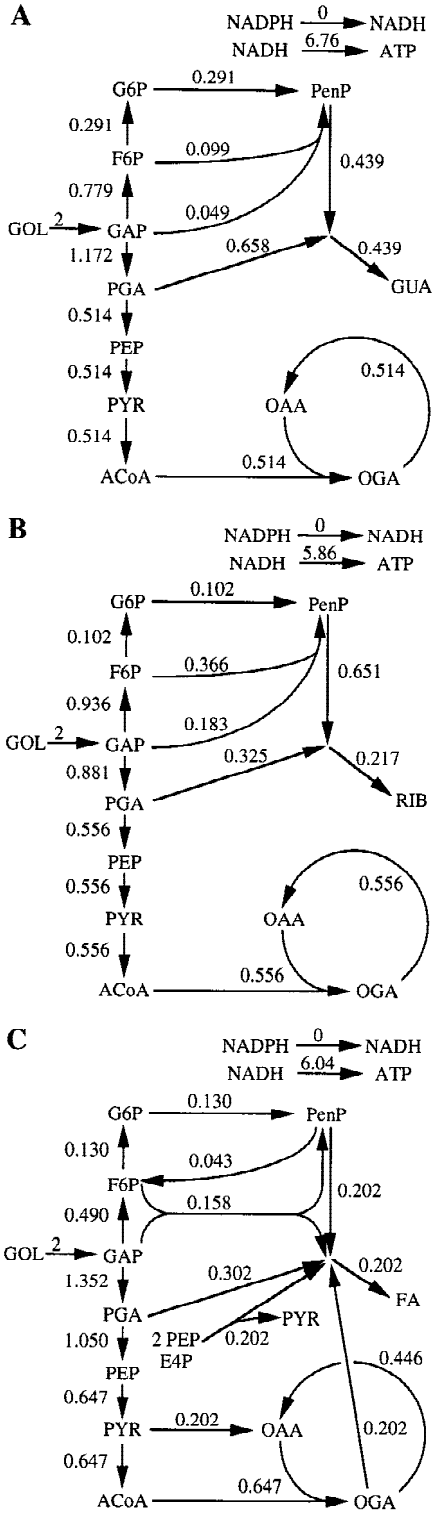

Figure 5. Flux distribution for maximum guanosine **(A),** riboflavin **(B),** and folic acid **(C)** production in *Bacillus subtilis* using glycerol as the carbon source and ξ_{GOL} equal to 4.4. The solutions are presented for the uptake of two molecules of glycerol (6 moles carbon).

DISCUSSION

The presented stoichiometric model was shown to be an effective analytical tool in fermentation process optimization. The computed maximum yields and the corresponding carbon flux distributions are valuable for assessing whether there is room for genetic or process engineering in product yield optimization. Provided that product biosynthesis has been thoroughly optimized in an organism with the specified energetic efficiency, these yields could potentially be reached under process conditions that promote high carbon conversion efficiency, such as carbon-limited fed-batch cultivations. To estimate the upper bounds on product yields, nongrowing cells were considered here. In practice, however, the product yield will also be affected by simultaneous biomass generation, which cannot be completely uncoupled from product formation and will depend on the process conditions. To evaluate the metabolic limitations for an actual fermentation process, maximum product yields could easily be computed using the observed substrate uptake and specific growth rates.

The simulations indicate that the maximum yield of purine pathway-related biochemicals on glucose is limited by pathway stoichiometry, provided the host organism generates sufficient energy. Stoichiometric limitations were found to be more relevant for purine nucleosides and riboflavin, as indicated by the higher ξ^*_{GLC} values, when pyruvate carboxylase is used as the anaplerotic reaction. When glucose is processed by an *E. coli* network with PEP carboxylase as the anaplerotic reaction, the flux distribution changes only locally in the reactions connecting PEP, pyruvate, ocaloacetate, and malate. Using this substrate, however, the *E. coli* network cannot provide as much PEP for biosynthesis as the *B. subtilis* network, which results in reduced maximum folic acid yields on glucose in the *E. coli* case. The *B. subtilis* results show that stoichiometric constraints limit maximum product yields already at less than optimal energetic efficiency. These stoichiometric limitations could potentially be improved by either of two strategies. The first, and most simple, would employ alternative

carbon substrates, such as glycerol or sucrose, with reduced or non-existing stoichiometric limitations. The second would involve metabolic engineering, such as replacing the glucose uptake system by a non-PTS system, which would potentially relieve the stoichiometric limitations of using glucose. However, the calculations indicate that either strategy can be effective only in organisms with adequate energetic efficiency.

Optimization calculations for metabolic variants of *B. subtilis* which include physiological information, such as preferential pathway usage and a realistic ξ_{GLC}, indicate that maximum yields on glucose are mainly energetically limited. Thus, energetic limitations will be even more important in a production process with realistic product yields below Y^{max}. Hence, improvements in biochemical yields on glucose in *B. subtilis* are more likely to come from process strategies that aim for highest efficiency of carbon conversion. For instance, in our calculations the yields were found to be improved by 25 to 40% in a *B. subtilis*-based production system using glycerol as the substrate. For a commercial process, the economic benefits from potential increases in final product yields need to be balanced with higher substrate costs. As a lower-cost alternative, sucrose could also relieve the stoichiometric limitations of glucose. However, because of the insufficient energetic efficiency of *B. subtilis*, the expected yield improvement is relatively small. To take full advantage of the sucrose potential for commercial applications, a host organism with high bioenergetic efficiency would need to be employed.

In principle, the described analyses can also be applied to organisms that are less well characterized than *B. subtilis.* Alternative metabolic networks may have to be analyzed if there is uncertainty about the presence or absence of reactions in central carbon metabolism, but the required information to estimate the ξ parameter could quickly be obtained from growth experiments. Due to their commercial history, other bacilli are potentially interesting hosts for the purpose of making purine pathway-related biochemicals (Kuninaka, 1996; Miyagawa and Kanzaki, 1991). In particular, *B. licheniformis* is an interesting candidate with well characterized bioenergetics (Bulthuis et al., 1989; Frankena et al., 1986; Frankena et al., 1985). Unfortunately, no detailed studies of its metabolism are available. Assuming an identical biochemical reaction network as in *B. subtilis*, a fit of the model to the experimentally determined Y^{max}_{GLC} value of the closely related organism *B. licheniformis* (Frankena et al., 1985) would yield an ξ_{GLC} of 5.2. Similarly, ξ_{GLC} values of 9.9, 9.3, and 8.2 were obtained for the thermophilic *B. stearothermophilus* growing at 45, 55, and 63°C, respectively (Pennock and Tempest, 1988). In *B. stearothermophilus*, product yields in all cases were found to be very similar to the *B. subtilis* values, because of only minor differences in the estimated energetic efficiency parameter (ξ). On the other hand, *B. licheniformis* would be a more promising host for biochemical production. With glucose as the substrate, maximum product yields are not improved in *B. licheniformis* compared to the *B. subtilis* values, because the maximum yield on glucose is limited by stoichiometry. A small improvement of about 2–10% was found only when the information on preferential pathway usage of *B. subtilis* growing on glucose was also used for *B. licheniformis.* However, non-maximum product yields in an actual biochemical production process using glucose are likely to be energetically limited because concomitant biomass formation will increase the energetic burden. In this case, the use of a *B. licheniformis* host has the potential to improve the yield of a *B. subtilis*-based process. Using sucrose, which exhibits reduced stoichiometric limitations, a yield improvement of 28%, 37%, and 25% for purine nucleosides, riboflavin, and folic acid, respectively, was found, compared to the *B. subtilis* glucose solution (Table V).

A transhydrogenase-like activity is not required for optimal biomass formation, presumably the normal objective in microbial growth. Thus, NADPH formation in primary metabolism appears to be well balanced with the requirements for biomass generation. This result is consistent with the apparent lack of such NADPH-oxidizing activity in several microorganisms (Vallino and Stephanopoulos, 1990; van Dijken and Scheffers, 1986). However, attempts to overproduce biochemicals will cause radical alterations in the carbon-flux distribution, which, in turn, affect the NADPH balance. In this study, the formation of products requiring intermediates from the PPP, in particular, were shown to generate excess NADPH on glucose substrates. Consequently, significant transhydrogenase fluxes were computed for the optimal solutions, indicating that the metabolic capacity for the reoxidation of NADPH is an important determinant of suitable hosts for the production of purine pathway-related biochemicals. The reported inherent flexibility of *B. subtilis*, and possibly other bacilli, for the reoxidation of NADPH (Sauer et al., 1997) may be the physiological basis for the successful role of these organisms in the manufacture of biochemicals from the purine pathway (Arbige et al., 1993; Kuninaka, 1996; van Loon et al., 1996).

A recently published study, that was also based on a stoichiometric model, reported a value of 0.80 g biomass C/g glucose C as the maximum biomass yield of *B. subtilis* (Lee et al., 1997). Under the assumption used in this study that biomass consists of 50% C, this value translates into 115.2 g biomass/mol glucose, which is less than the maxi-

Table V. Maximum theoretical biochemical yields with *Bacillus subtilis* or *Bacillus licheniformis* using sucrose in the absence of growth.[a]

Product	Maximum yield in *B. subtilis* (mol/0.5 mol sucrose)	Maximum yield in *B. licheniformis* (mol/0.5 mol sucrose)
Inosine	0.367	0.448
Guanosine	0.360	0.440
Riboflavin	0.178	0.219
Folic acid	0.164	0.201

[a]Same stoichiometric constraints as in Table IV. Energetic efficiency on sucrose was assumed to be identical to the estimated ξ_{GLC} values for both organisms.

mum biomass yield of 121.9 g biomass/mol glucose calculated here. However, in the model of Lee et al. (1997) the detailed biomass composition of *E. coli* (Neidhardt et al., 1990) was used, which we calculated to contain about 45% C in biomass (data not shown). Thus, the reported value corresponds to a maximum biomass yield of 127.9 g biomass/mol glucose. The differences in the predicted maximum yield in both models is related to three differences between the networks considered. First, in our case we employed the biomass composition of *B. subtilis* which has a significantly higher cell wall content and different precursor requirements for lipid biosynthesis compared to *E. coli* (Sauer et al., 1996). Second, we assumed that at most two ATP can be generated per FADH compared to one assumed by Lee et al. (1997). Third, in the study of Lee et al. (1997) the anaplerotic reaction of *B. subtilis,* catalyzed by the pyruvate carboxylase, was considered to be ATP-independent, contrary to the reported mechanism of this enzyme (Gottschalk, 1986). Omitting this ATP requirement in our *B. subtilis* network increases the maximum biomass yield to 126.6 g biomass/mol glucose, thus indicating that the ATP demand for this anaplerotic reaction has a profound effect on the maximum biomass yield. Similar to the study by Lee et al. (1997), we obtain reduced maximum biomass yields for a network with the *E. coli* alternative of the anaplerotic reaction, due to stoichiometric limitations. However, their solution is most likely an overestimation of the effect on the *E. coli*-like network because an energy-requiring anaplerotic reaction was assumed for *E. coli* (one ATP equivalent in PEP) and an unrealistic energy-independent reaction was used for *B. subtilis.*

Purine nucleoside fermentations have previously been studied from the perspective of theoretical product yields, but only the stoichiometric mass balances for the purine biosynthesis were used without considering central metabolism (Acevedo, 1987; Muzzio and Acevedo, 1985). By comprehensively including primary metabolism and energetics, we have shown that the product yield on glucose is much more limited in *B. subtilis* and *B. licheniformis* and has a maximum value of 0.30 and 0.40 mole per mole glucose, respectively. Therefore, the previously reported maximum yield for IMP and GMP of 0.58 mole per mole glucose is a clear overestimation of the metabolic capabilities of the bacterial species generally used for the manufacture of these chemicals. Theoretical yields higher than 0.5 mole per mole glucose would only be possible in a metabolic variant with a non-PTS glucose uptake system and nearly optimal energetic efficiency. This example clearly illustrates the importance of considering stoichiometric coupling of biosynthesis with central metabolic pathways and energetics of bacterial metabolism for estimating bounds on practically feasible product yields. Yields actually achieved of course depend not only on stoichiometric and energetic limitations but also on the kinetics of metabolic processes.

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