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Metabolic Flux Analysis in *Streptomyces coelicolor* under Various Nutrient Limitations

Fereshteh Naeimpoor and Ferda Mavituna[1]

Department of Chemical Engineering, UMIST, P.O. Box 88, Manchester M60 1QD, United Kingdom

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Metabolic flux analysis was applied to *Streptomyces coelicolor* continuous culture data obtained under nitrogen, phosphate, sulfate, and potassium limitations. The metabolic reaction network involved more than 200 reactions describing the major pathways as well as the secondary metabolism for the production of actinorhodin and excretion of certain metabolites. Linear programming was used for the optimization of specific growth rates and energy requirements. Two types of specific growth rates, stoichiometric and theoretical, were defined, maximized, and compared in order to investigate the microbial potential. Potassium limitation led to the largest and nitrogen limitation to the smallest difference between the stoichiometric and theoretical specific growth rates. Although the value of the maximum theoretical specific growth rate was close to that of the experimental specific growth rate with potassium limitation, this difference was the largest in the case of nitrogen limitation. Energy requirements during different nutrient limitations were also investigated. The model indicated that although the highest actinorhodin production rate was with nitrogen limitation, this was accompanied with the undesired excretion of certain metabolites.

Key Words: **metabolic flux analysis; energy requirement; *Streptomyces coelicolor*; nutrient limitation.**

1. INTRODUCTION

Antibiotics are still one of the most important groups of industrially produced pharmaceuticals and the majority of these are the secondary metabolites of streptomycetes (Williams *et al*., 1983). Actinorhodin, a red/blue acid/base indicator pigment with weak antibiotic properties, is one of the four known antibiotics produced by *Streptomyces coelicolor* A3(2). This antibiotic is a member of a group of chemically related antibiotics called isochromanoquinones which are synthesized via a polyketide pathway (Rudd and Hopwood, 1979). Polyketides, in turn, are a large family of structurally diverse natural products with a broad range of biological activities including antibiotic, anticancer, antiparasitic, and immunosuppressant properties. There has been considerable research interest, therefore, in the molecular biology of antibiotic production by *S. coelicolor* as a model system for the production of natural and unnatural novel compounds using the polyketide synthesis pathway and the genetically engineered hybrid polyketide synthases. A combination of genetic, biochemical, chemical, and biochemical engineering tools can be a very effective approach to achieve this. In addition to the large amount of research in the molecular biology of *S. coelicolor*, the physiological and some of the biochemical engineering aspects of these cultures have also been investigated. For example, the regulation of actinorhodin production by carbon, nitrogen, and phosphate limitation and media composition (Hobbs *et al*., 1990; Doul and Vining, 1990; Ozergin-Ulgen and Mavituna, 1993; Melzoch *et al*., 1997; Elibol and Mavituna, 1998), effect of cell immobilization (Ozergin-Ulgen and Mavituna, 1994), oxygen limitation (Elibol and Mavituna, 1995, 1996, 1999; Ulgen and Mavituna, 1998), and mode of bioreactor operation (Ates *et al*., 1997) have been studied in order to understand the factors affecting the antibiotic production in *S. coelicolor*. Metabolic engineering tools such as metabolic flux and control analysis can indicate genetic, combinatorial, and process engineering strategies for the production of these exciting novel products such as polyketides (Stephanopoulos *et al*., 1998; Varma and Palsson, 1994).

Although most of the metabolic reactions, especially those which are related to cell growth, are common in all living organisms, there are certain metabolic pathways and regulatory functions leading to the production of secondary metabolites that are specific to each microorganism. Information on the metabolic flux distribution within the cells and especially how the carbon source is directed to biomass and secondary product formation can be used to manipulate the cells or their physicochemical environment in order to achieve higher production rates for the desired products.

In this work computational metabolic flux analysis (MFA) is applied to *S. coelicolor* experimental data

[1] To whom correspondence should be addressed. Fax: +44(0)161 200 4399. E-mail: Ferda.Mavituna@umist.ac.uk.

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obtained under different nutrient limitations focusing on biomass formation, energy requirement by cells, metabolite excretion, and actinorhodin production. Two different concepts of calculated (optimized) specific growth rates, the maximized theoretical and maximized stoichiometric, are defined depending on the constraints used in the solution of the computational model. They are compared with each other and the experimental specific growth rates at different nutrient limitations. This is the first report in the literature on the application of MFA to *S. coelicolor* culture.

METABOLIC FLUX ANALYSIS

Theoretical MFA is a methodology used for the determination of metabolic pathway fluxes, usually in terms of specific rates of reactions, through a stoichiometric model of the cellular reactions using mass balances for intracellular metabolites. Determination of fluxes allows one to assess the contribution of different pathways to the metabolism as a whole and especially to substrate consumption and product formation. Comparison of different metabolic flux distributions can show the effect of different genetic or environmental conditions on the pathways and can indicate strategies for the selection of media, strains, and genetic engineering targets and therefore for more efficient formation of desired products (Stephanopoulos *et al.*, 1998).

By performing mass balances for each intracellular metabolite with the quasi-steady-state assumption, the steady-state balance equation is written (Savinell and Palsson, 1991) as

$$\mathbf{S}.\mathbf{v} = \mathbf{b},$$

where **S** is the stoichiometric matrix, **v** is the vector of fluxes, and **b** is the vector of net specific excretion rates from the cell (in the case of nutrient uptake from the environment, the elements of **b** will be negative). Since the number of reactions is usually greater than the number of metabolites, the number of unknown fluxes will be greater than the number of balance equations. This situation gives an underdetermined system of linear algebraic equations which has infinite solutions. By using linear programming with various stoichiometric, physiological, and experimental constraints, an objective function can be defined and optimized. The objective function can be, for example, the specific growth, substrate uptake, or product formation rate. The solution not only gives the optimized value of the objective function but also the corresponding metabolic fluxes. It is noteworthy that although the optimized value of the objective function is unique, the flux distribution may not be unique. In this case adding more biological or operational constraints can give a unique flux distribution.

MODEL CONSTRUCTION

Bioreaction Network

The main metabolic pathways by which carbon-energy sources are used for the production of energy and carbon skeleton needed for the synthesis of intermediary metabolites, building blocks, and end products are considered for streptomycetes in a bioreaction network which describes the interactions among the coupled reactions. In addition to the main catabolic and anabolic pathways, the secondary metabolism involving actinorhodin production starting from one acetyl-coenzyme and seven malonyl-coenzyme molecules is included in the network. Furthermore, ammonia and sulfate assimilation reactions are linked to the metabolism. This network therefore includes the glycolysis and pentose phosphate (PP) pathways; tricarboxylic acid cycle (TCA); glyoxylate cycle; anaplerotic reactions; ammonia assimilation; sulfate assimilation; electron transport; folic acid and thioredoxin reactions; transdehydrogenase activity; the biosynthesis of aromatic, aspartate, glutamate, pyruvate, and serine family amino acids and histidine, pyrimidine, and purine nucleotides; and the biosynthesis of macromolecular components of biomass such as RNA, DNA, protein, fatty acids, phospholipids, carbohydrate, as well as actinorhodin production by *S. coelicolor* (Zubay, 1998; White, 1995; Mandelstam *et al.*, 1982; Strohl and Connors, 1992). This metabolic network involves more than 200 bioreactions.

Assumptions and Model Parameters

Biomass is assumed to consist mainly of proteins, carbohydrates, RNA, DNA, and phospholipids. The mass percentages of protein, RNA, and DNA in the biomass are taken from Shahab *et al.* (1996) for the continuous culture of *S. coelicolor* at the specific growth rate of 0.048 h^{-1} which is the nearest value to the specific growth rate used in this work. The phospholipid mass percentage of the biomass is assumed to be the same as that for *E. coli* (Ingraham *et al.*, 1983). The rest of the biomass is assumed to consist of carbohydrate. The amount of precursors required to produce 1 g of protein, RNA, DNA, and phospholipids is assumed to be the same as in *E. coli* (Ingraham *et al.*, 1983). The polymerization energies required for protein, RNA, and DNA are taken from Ingraham *et al.* (1983). The P/O ratio is assumed to be 1.9 in NADH oxidation reactions and two-thirds of this amount for $FADH_2$ oxidation reactions (electron transfer reactions).

Experimental Data

The experimental data of Melzoch *et al.* (1997) analyzed here were obtained from the chemostat cultures of

S. coelicolor A3(2), M145, at 28°C, pH 7, and a dilution rate of 0.06 h^{-1} under various nutrient limitations in minimal medium described by Evans *et al.* (1970). The main nutrients in the original medium were 20 g/liter glucose, 100 mM ammonium chloride, 10 mM sodium phosphate, 2 mM sodium sulfate, 10 mM potassium chloride, and 1.25 mM magnesium chloride. To obtain different nutrient limitations, Melzoch *et al.* (1997) altered the concentrations of these nutrients by reducing N, P, S, K, and Mg concentrations by one-tenth and C concentration by one-fourth each at a time while keeping the remaining nutrient concentrations at the original level. Each nutrient limitation was studied at least twice. Each steady state was maintained for at least 5 consecutive days and samples were taken every day. From the average of all measurements at a particular steady state, Melzoch *et al.* (1997) calculated the specific rates of glucose and oxygen consumption, carbon dioxide, and actinorhodin production and presented these in a table in their paper. They reported that the standard deviation of all data presented was maximally 5%.

Energy Requirements

Energy requirements of the cell can be categorized into three groups. First, it is required for the biosynthesis of precursors of macromolecules within the cell in terms of ATP (or other cell energy currencies) consumed in bioreactions and the polymerization energy needed for the biosynthesis of macromolecules. This amount is a linear function of the specific growth rate, assuming that cell composition is constant and it is called the growth-dependent energy requirement. Second, energy is also required for the formation and secretion of products that are not growth dependent. In this case, the energy requirement depends on the specific product formation and excretion rates. The third category of energy requirement includes the maintenance energy such as energy used for repairing the damages and controlling the osmotic pressure and the motility of cells. The maintenance energy requirement can depend on the medium concentration and hence it is an important issue when it comes to using the nutrient-limited media. In this work we assumed that the maintenance energy was a variable in order to check how it varied with the media composition.

Metabolite and Product Excretion

Wild-type cultures tend to direct most of their resources toward biomass production when they are cultivated under proper physicochemical conditions using readily available nutrients (Shapiro, 1989). However, this does not necessarily mean that no product, either desired or undesired, is excreted during their growth. One can divide products into three groups: primary metabolites, secondary metabolites, and overflow metabolites. A common example of the first group is the undesired production of carbon dioxide by microorganisms. Another example is the anaerobic growth producing desired products such as ethanol or lactic acid as end products of primary metabolism. The second group consists of the secondary metabolites which are usually produced during the slow growth rates or stationary phase when one of the essential nutrients is exhausted (Shapiro, 1989). The third group includes the excretion of some intermediate metabolites as a result of metabolic imbalance when the utilized substrate cannot be directed into biomass (Neijssel *et al.*, 1993). *Streptomycetes* spp. are well known for the production of a variety of secondary products, many of which are antibiotics (Shapiro, 1989). These species are also known to show overflow metabolism under carbon-excess conditions leading to the excretion of some organic compounds the type of which is profoundly dependent on the nature of the growth limitation and the physiology of species (Madden *et al.*, 1996; Hobbs *et al.*, 1992; Delkleva and Strohl, 1987; Surowitz and Pfister, 1985; Ahmed *et al.*, 1984).

Using the data reported by Melzoch *et al.* (1997) under various nutrient limitations (mostly carbon-excess) and assuming lactate, formate, and acetate excretion, we calculated the theoretical excretion rates of these metabolites.

SOLUTION

Our mathematical model was programmed in a GAMS (general algebraic modeling system) environment (Brooke *et al.*, 1992), which is used for optimization purposes. The constructed GAMS model was then solved via linear programming (simplex method) to maximize the defined objective function such as the specific growth rate and the variable energy requirement under different conditions. The corresponding solutions also contain the values of the specific metabolic rates for over 200 reactions. In this article, however, in the flux distribution figures, we included only a summary of fluxes of the important metabolic pathways.

Regarding the specific growth rate, two cases are considered here. The maximized stoichiometric specific growth rate is the optimized value of the specific growth rate when only the stoichiometric constraints and the experimental value of the specific glucose uptake rate are used and the other specific rates such as oxygen uptake, carbon dioxide, and actinorhodin production are left as variables to be calculated by the program. In this case, the highest portion of specific carbon uptake rate is directed to biomass formation. The maximized theoretical specific growth rate on the other hand is the optimized value of the specific growth rate using the stoichiometric constraints as well as the

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experimental values of the specific glucose and oxygen uptake, carbon dioxide, and actinorhodin production rates. In this case, the portion of carbon which is directed to biomass formation is constrained by the experimental specific oxygen uptake, carbon dioxide, and actinorhodin production rates.

The maximization of the variable maintenance energy requirement, subject to stoichiometric constraints, experimental specific glucose and oxygen uptake, carbon dioxide, and actinorhodin production rates, as well as the experimental specific growth rate leads to the upper limits of energy requirement in different nutrient limitations since the optimization program selects the most energy-efficient pathways for biomass formation and metabolite excretion.

RESULTS AND DISCUSSION

Experimental Data from Continuous Culture

In Fig. 1 and Fig. 2, we plotted the tabulated data of Melzoch *et al.* (1997) on the specific glucose and oxygen consumption, carbon dioxide, and actinorhodin production rates in carbon-, nitrogen-, phosphate-, sulfate-, and potassium-limited media under steady-state conditions for the dilution rate $D = 0.06\ \mathrm{h}^{-1}$.

Biomass Formation and the Maximum Stoichiometric Biomass Yield

To calculate the maximum stoichiometric biomass yield, defined as g DW cells produced per millimole of glucose consumed, the specific growth rate was maximized at various specific glucose uptake rates. Not surprisingly there was a linear relationship between the maximized stoichiometric specific growth rates and specific glucose uptake rates leading to a constant maximum stoichiometric biomass yield (Table 1). The values of $Y_{O_2/Glc}$ and $Y_{CO_2/Glc}$ defined as moles of oxygen taken up and moles of carbon dioxide produced per mole of glucose consumed, respectively, at the maximized stoichiometric growth rate are given in Table 1.

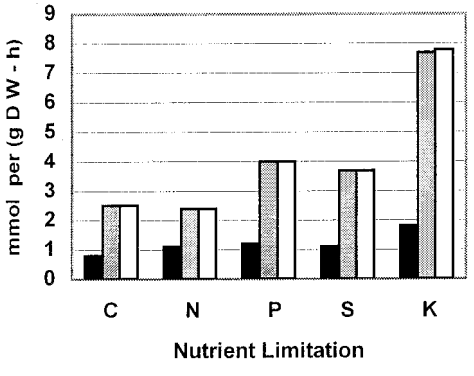

FIG. 1. Experimental specific glucose and oxygen uptake and carbon dioxide production rates at a dilution rate of 0.06 h^{-1} (data taken from Melzoch *et al.*, 1997). ■, Glc; ■, CO_2; □, O_2.

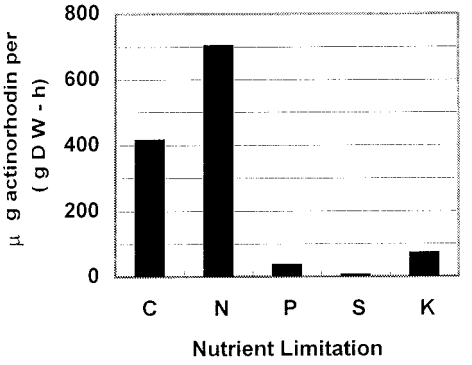

FIG. 2. Experimental specific actinorhodin production rate at a dilution rate of 0.06 h^{-1} (data taken from Melzoch *et al.*, 1997).

To review the metabolic fluxes within the cell, some parts of the normalized flux distribution at the maximized stoichiometric specific growth rate are given in Fig. 3. In this figure, inputs and outputs of the pentose phosphate pathway show that no glucose 6-phosphate enters this pathway; instead, fructose 6-phosphate and glyceraldehyde 3-phosphate are used as inputs. Thus, the pentose phosphate pathway is driven in the reverse direction. The TCA cycle is completely active. In addition to producing redox power and carbon dioxide, the TCA cycle produces some metabolites required for cell growth.

Maximum Theoretical Specific Growth Rate under C, N, P, K, and S Limitations

If in addition to the stoichiometric constraints the experimental specific consumption and production rates are used and the objective function is the specific growth rate, the maximized values of the specific growth rate (referred to as the maximized theoretical specific growth rate, μ_T, in this work) will be different from the maximized stoichiometric specific growth rates, μ_S, for each nutrient limitation. Figure 4 shows the comparison between these two cases. The theoretical specific growth rate which depends on the experimental measurements is lower than the stoichiometric specific growth rate. This means that the experimental

TABLE 1

Yields at Maximized Stoichiometric Speficic Growth Rate

$Y_{Biomass/Glc}$ (g D W cells/mmol glucose)	$Y_{O_2/Glc}$ (mol/mol)	$Y_{CO_2/Glc}$ (mol/mol)
0.1215	0.8027	1.0534

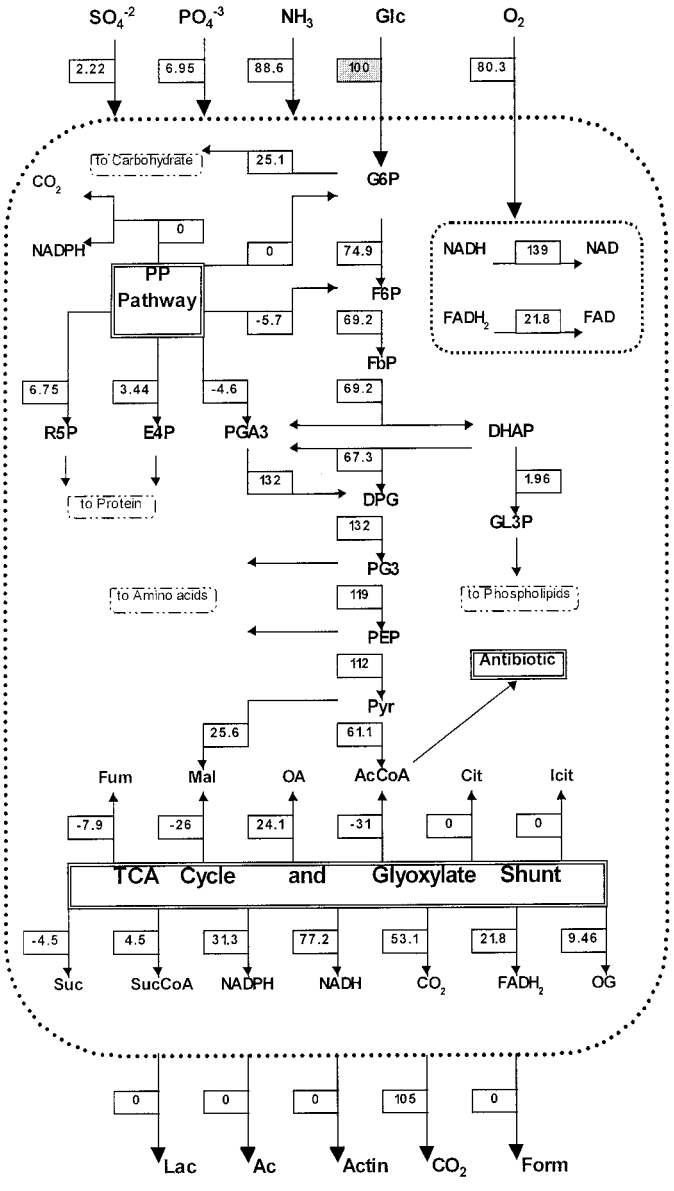

FIG. 3. Normalized metabolic flux distribution at maximized stoichiometric specific growth rate based on the specific glucose uptake rate taken as 100. The value on gray background is given as a constraint to the program, while others are those calculated. A negative sign indicates that the reaction proceeds in the opposite direction of the arrow.

values of specific carbon dioxide and actinorhodin production impose a limit on the amount of glucose that could be directed into biomass formation. Since the specific glucose uptake rates are the same in both cases for each nutrient limitation, the maximum theoretical biomass yields on glucose will be lower than the maximum stoichiometric biomass yields.

The difference between the maximized theoretical specific growth rate and the experimental specific growth rate, μ_E,

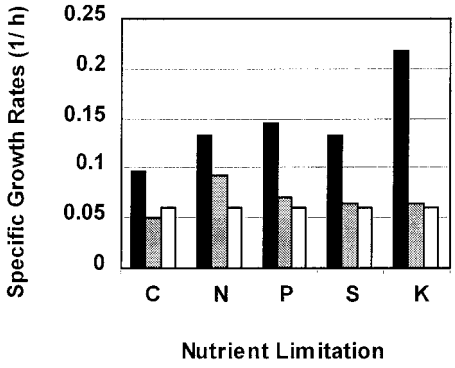

FIG. 4. Comparison of the maximized stoichiometric, $\mu_{Max,S}$, the maximized theoretical, $\mu_{Max,T}$, and the experimental μ_E, specific growth rates. ■ $\mu_{Max,S}$; ■, $\mu_{Max,T}$; □ μ_E.

is the largest for nitrogen limitation, the smallest for both potassium and sulfate, and in between for phosphate limitation. This reinforces the fact that nitrogen and phosphate are the two most important nutrients for biomass formation. In carbon limitation, the maximized theoretical specific growth rate is less than the experimental dilution rate, which is probably the result of some inaccuracies in the experimental data (Melzoch *et al.*, 1997). Therefore, carbon limitation is not considered further here.

Energy Requirements

Maximizing the stoichiometric specific growth rate as the objective function at a given specific glucose uptake rate results in a zero maintenance energy requirement according to the solution of the model. However, for the maximum theoretical specific growth rates using the experimental data for different nutrient limitations, the calculated maintenance energy requirements are different (maintenance energy I in Table 2). This difference is due to the different maximum theoretical specific growth rates and media composition. To cancel the effect of different specific growth rates, they were fixed at the experimental dilution rate of 0.06 h^{-1} and the objective function was altered to be the variable energy requirement. To have the most energy-efficient metabolic network in terms of primary and secondary metabolism, maximization of the maintenance energy requirement was chosen. The results are shown as "maintenance energy II" in Table 2 for comparison with maintenance energy I of the previous case.

The highest values of maintenance energy in Table 2 are related to K limitations. This emphasizes the fact that potassium ions in the medium play an important role in cell survival and also explains why the experimental specific glucose uptake and carbon dioxide production rates are highest in K limitation compared with other nutrient limitation cases (Fig. 1). Most of the excess glucose consumed is

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TABLE 2
Calculated Maintenance Energy Requirements (mmol ATP/g DW Cells) and Specific Growth Rates at Various Nutrient Limitations

Limitation	Maximized objective function: Theoretical specific growth rate		Maximized objective function: Maintenance energy requirement	
	$\mu_{Max,\,T}$	Maintenance energy I	μ_E	Maintenance Energy II
N	0.093	6.39	0.060	10.39
P	0.071	13.56	0.060	15.89
S	0.065	12.61	0.060	14.24
K	0.065	28.53	0.060	31.55

oxidized to produce ATP. One can realize that even by considering the experimental measurements as constraints, the calculated maximum theoretical specific growth rate (Fig. 4) is not far from the experimental dilution rate of 0.06 h^{-1}. Setting the value of specific growth rate to the experimental dilution rate in K limitation increases the energy requirement by only 10.6%. N limitation on the other hand gives the lowest value of the maintenance energy requirement which can be explained by the highest maximum theoretical specific growth rate obtained previously for N limitation when experimental measurements were considered as constraints (Fig. 4). With N limitation, setting the specific growth rate to 0.06 h^{-1} increases the calculated maximum maintenance energy requirement by 62%to compensate for the unrealistically high theoretical specific growth rate. This confirms that with N limitation, excess carbon cannot be directed into the biomass because nitrogen is lacking for the biosynthesis of proteins, which constitute nearly half of the biomass. P and S limitations fit between the two limitations discussed above (Table 2).

Metabolite Excretion

In Fig. 5, the calculated fluxes of formate and acetate excretion at maximized maintenance energy requirements are shown. The calculated lactate excretion was zero; therefore, it is not shown. The highest specific acetate excretion rate and the highest total specific metabolite excretion rates are seen in nitrogen limitation. This can be explained by the observation of many researchers that high C : N ratios lead to the excretion of organic metabolites. Since nitrogen is essential for protein biosynthesis, when nitrogen is limited, the excess carbon cannot be consumed for protein formation and hence it does not lead to the production of biomass. Therefore, excess carbon could be excreted as organic acids if not oxidized to CO_2 for energy production. Calculated carbon losses as formate and acetate are 26.1, 10.5, 6.9, and 6.0% of the utilized carbon for N, P, S, and K limitations, respectively, confirming that N and P are the two most important nutrients in biomass formation among the nutrient limitations considered.

As nitrogen limitation shows the highest metabolite excretion rates, its corresponding flux distribution is given in Fig. 6. These excretions are an inevitable result of the carbon, energy, and redox balances when the specific growth, glucose and oxygen uptake, carbon dioxide, and actinorhodin production rates are fixed as model constraints at their experimental values. When the specific actinorhodin production rate is set to zero without changing the other constraints, the resulting flux distributions appear as presented in Fig. 7. Since the experimental value of the specific actinorhodin production was so low, setting it to zero did not result in any significant change in the metabolic fluxes as can be deduced from the comparison of Fig. 7 with Fig. 6.

Model Sensitivity

To investigate the effect of experimental data errors on the value of the optimized objective function, the shadow prices of linear programming (Savinell and Palsson, 1992) were used. The shadow prices are calculated by the solver each time the program is run. For comparison purposes,

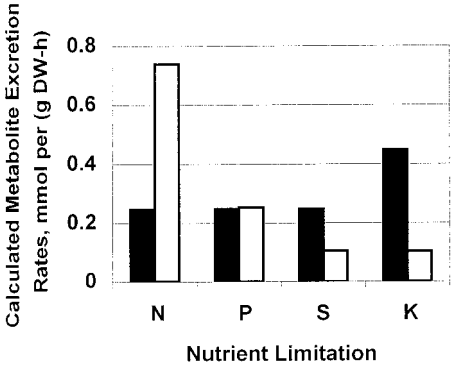

FIG. 5. Specific formate and acetate excretion rates at maximized maintenance energy requirement. □ formate; ■ acetate.

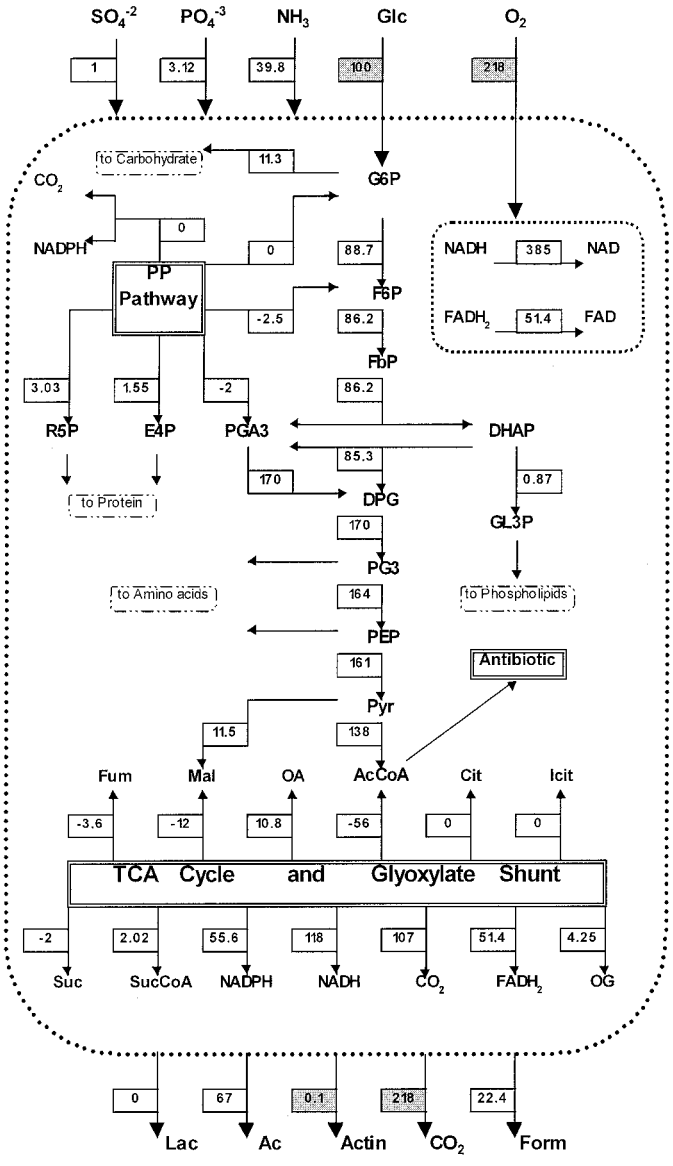

FIG. 6. Normalized metabolic flux distribution at maximized maintenance energy requirement for nitrogen limitation based on the specific glucose uptake rate taken as 100. The values on gray background are given as constraints to the program, while others are those calculated. Negative values indicate that the reaction proceeds in the opposite direction of the arrow.

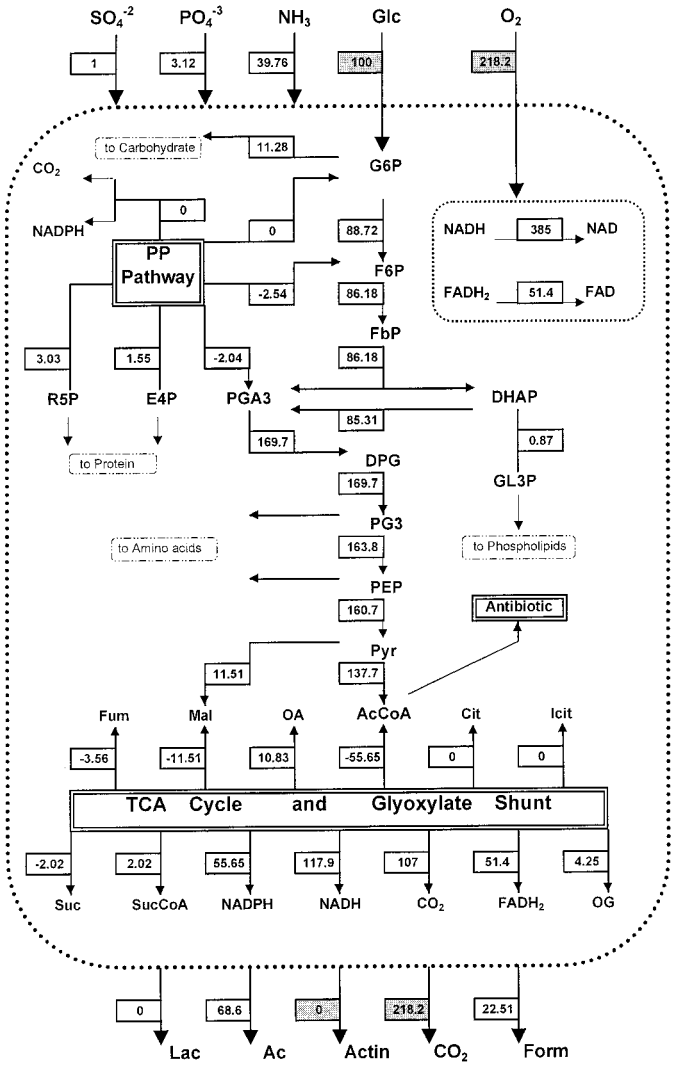

FIG. 7. Normalized metabolic flux distribution at maximized maintenance energy requirement for nitrogen limitation based on the specific glucose uptake rate taken as 100 and the specific actinorhodin production rate set to zero. The values on gray background are given as constraints to the program, while others are those calculated. Negative values indicate that the reaction proceeds in the opposite direction of the arrow.

these values were processed further to give the logarithmic sensitivities (Varma and Palsson, 1993) as the ratio of the percentage change in the value of the objective function to the percentage change (or error) in the experimental data used in the program. The logarithmic sensitivities for nitrogen limitation were calculated as 0.82, 0.47, −0.20, 0.0048, and −0.49 for the specific rates of glucose and oxygen consumption, carbon dioxide, and actinorhodin production and growth, respectively. These indicate that the objective function is most sensitive to the errors in the specific glucose uptake rate. Errors in the specific actinorhodin production rate on the other hand do not have any significant effect on the value of the objective function.

CONCLUSIONS

The metabolic reaction network constructed in this work consists of more than 200 reactions and can account for the primary and secondary metabolism of *S. coelicolor*. Since this model is underdetermined, by using linear programming with various stoichiometric and experimental constraints,

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various objective functions can be defined and optimized. In this article we reported the results of optimization of the specific growth rate and energy requirements of the cell. We also included the flux distribution maps for some cases.

Depending on the constraints used, two different concepts of optimized specific growth rates, stoichiometric and theoretical, were introduced. The maximum stoichiometric yields were 0.12 g DW cells produced/mmol glucose used, 0.8 mol oxygen consumed/mol glucose used, and 1.05 mol carbon dioxide produced/mol glucose used. Compared to the experimental growth rate of 0.06 h^{-1}, the maximum theoretical growth rate was 0.093 h^{-1} for nitrogen limitation, 0.071 h^{-1} for phosphate limitation, and 0.065 h^{-1} for both sulfur and potassium limitations.

According to our model, the maintenance energy requirement of the *S. coelicolor* culture was dependent on the media concentrations of which potassium concentration had the most profound effect among the nutrient limitations considered. Although nitrogen limitation gave the lowest maintenance energy requirement and the highest specific actinorhodin excretion rate, the total specific excretion rate of other primary metabolites in this case was the highest. This meant that a higher actinorhodin production rate with nitrogen limitation was at the expense of undesired excretion of certain metabolites in carbon-excess medium.

In conclusion, such comparisons of the experimental and calculated optimum results are very useful since they indicate the biological potential of the particular culture. One of the more important purposes of this type of study is to indicate the targets and strategies for genetic and process-engineering manipulations in order to decrease the difference between the experimental and calculated optimal results. Our current work involves metabolic flux analysis for the production of secondary metabolites by *S. coelicolor* and a link between primary and secondary metabolism including the effect of precursors.

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