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Applications of Metabolic Engineering to Fermentation Technology

Ferda Mavituna

Professor of Biochemical Engineering
Department of Chemical Engineering, UMIST, Sackville Street,
PO Box 88, Manchester, M60 1QD, UK
*Telephone: 44 161 200 4372
*Fax: 44 161 200 4399
*E-mail: F.Mavituna@umist.ac.uk

Metabolic engineering can be broadly defined as the study of microbial, plant and animal metabolism using scientific and engineering tools in order to;

a) understand the metabolism better, under normal and abnormal (mutant, genetically altered, diseased) conditions,
b) identify and test metabolic bottlenecks for targeted genetic engineering,
c) design and genetically modify metabolic pathways for the increased the formation of desired metabolites and decreased formation of unwanted metabolites in bioprocesses or in cells, tissues and organs.

Metabolic engineering can also be used very effectively to understand the actions of hormones, drugs and chemical agents. It can therefore, aid the search for drugs that affect metabolism (host or pathogen) by identifying targets.

Metabolic flux analysis (MFA) and metabolic control analysis (MCA) form two very powerful tools of metabolic engineering. Metabolic flux analysis (MFA) is a method for the determination of metabolic pathway fluxes (specific rates of reactions) through a stoichiometric model of the cellular pathways, using mass balances for intracellular metabolites. It is a powerful tool for the identification of the important bioreactions in the metabolism for further genetic manipulations and for selecting the physicochemical conditions for improved bioprocesses.

The cellular reaction rates can be expressed as the specific rate of reaction (fluxes). By performing mass balances for each intracellular metabolite under quasi-steady state assumption the steady state balance equation is written as:

$$\mathbf{S} \cdot \mathbf{v} = \mathbf{b}$$

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).

We have developed theoretical and computational models for metabolic flux analysis in *Brevibacterium flavum*, *Bacillus licheniformis*, *Saccharomyces cerevisiae*, *Streptomyces coelicolor* and *Catharanthus roseus*, each involving between 100 to 200 reactions of the major metabolic pathways. These include glycolysis, TCA cycle, pentose phosphate shunt, electron transport system, respiration, and the biosynthesis of amino acids, cofactors, nucleotides, fatty acids, phospholipids, and additionally for *C. roseus*, the secondary metabolism and photosynthesis, cellulose and lignin

synthesis. Some examples will be presented from these studies which can indicate how metabolic engineering can be applied to fermentation technology.

Streptomyces coelicolor A3(2) produces four known antibiotics; actinorhodin, undecylprodigiosin, methylenomycin and the calcium-dependent antibiotic (CDA). Actinorhodin is red in acid and blue in alkali while undecylprodigiosin is normally red but tends to turn orange at low pH. Actinorhodin is a polyketide, a dimeric molecule derived from one acetyl-CoA precursor and seven malonyl-CoA extender units. Since these are weak antibiotics without commercial value and the pigmentation allows visual observation of the onset of antibiotic production, *S. coelicolor* A3(2) has been used as a model microorganism for detailed and extensive genetic and physiological studies. *Streptomyces coelicolor* A3(2) is therefore, genetically the most-studied strain in streptomycetes along with *Streptomyces griseus*.

Metabolic flux analysis was applied to *Streptomyces coelicolor* continuous culture data obtained under nitrogen, phosphate, sulphate 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 optimisation of specific growth rates and energy requirements. Two types of specific growth rates, stoichiometric and theoretical, were defined, maximised 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. Metabolic flux distributions in *Streptomyces coelicolor* during growth under nitrogen limitation with nitrate or ammonia as the nitrogen source were also investigated. Use of nitrate resulted in lower specific growth rates compared to ammonia. Excretion of some organic metabolites was observed in both limitation cases.

Furthermore, we constructed a comprehensive metabolic network consisting of 110 reactions to represent the metabolism of glutamic acid bacteria and used this in a stoichiometrically based flux balance model for L-glutamate production. The theoretical results were compared with the experimental results obtained from the batch fermentations of *Brevibacterium flavum*. The stoichiometrically balanced reactions involved major pathways of catabolism, anabolism including energy and redox balances for glutamic acid bacteria. An optimisation programme was used to solve the pseudo- state steady metabolic flux balance equation for different objective functions.

In the batch fermentation of *B. flavum*, the concentrations of carbohydrate and organic acids were analysed by HPLC. Theoretical metabolic flux distributions were optimised for several conditions and objective functions. These results were compared with experimentally measured parameters, such as specific substrate uptake and product formation rates. The flux distribution maps showed that the cells utilised the TCA cycle in part, whereas the glyoxylate bypass was active throughout the

fermentation. The results also indicated that the phosphate pentose shunt played an important role in glutamate fermentation. Comparison of the theoretical metabolic flux distributions with experimental results indicated some targets for genetic engineering as well as physiological engineering by changing the physico-chemical environment of the cultures for improved glutamate production.

Catharanthus roseus (Madagascar periwinkle) is the source of at least 200 pharmaceutical agents such as vincristine, vinblastine, ajmalicine, serpentine, catharantine and vindolene. Yields of these compounds when extracted from the whole plant are very low: 0.3- 0.5% for ajmalicine and serpentine, 0.001% for catharanthine and vindolene, 0.0005% for vinblastine and vincristine, based on the plant dry weight. Metabolic engineering can therefore be a very effective approach to improve yields either in the plants or/in plant cell/tissue cultures via targeted genetic engineering or bioprocess engineering optimisation. For this purpose, we developed a computational model using a commercial optimisation software in order to obtain metabolic reaction fluxes for different physiological and process conditions and compared the results with our experimental data obtained from *C. roseus* cell cultures. In the model, we described the metabolism of *Catharanthus roseus* using more than 170 stoichiometrically balanced equations. These involved glycolysis, TCA cycle, pentose phosphate shunt, electron transport system, respiration, photosynthesis, the biosynthesis of amino acids, cofactors, nucleotides, fatty acids, phospholipids, cellulose, as well as the secondary metabolism leading to the production of ajmalicine, serpentine, catharanthine, vindolene, vincristine and vinblastine. The optimisation programme was then used to solve the pseudo-steady state metabolic flux balance equation. The solution gave the values of the metabolic fluxes (specific reaction rates) for 170 reactions including the substrate uptake and product excretion rates. The comparison of these with the experimental values indicated the biological potential as well as some interesting pinch points. These can be used for targeting genetic engineering manipulations as well as designing better media, strategic planning of precursor feeding and in vitro operating conditions for increased product formation.

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