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Metabolic control analysis and its applications

Ana Meireles[1], Manuel Simões[1*]

[1]LEPABE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, s/n, 4200-465 Porto, Portugal

*Author to whom correspondence should be addressed: Manuel Simões (mvs@fe.up.pt)

ABSTRACT

Metabolic Engineering (ME) provides the know-how for the reconstruction of microorganisms, in order to provide higher production rates of biotechnological products and increase their potential application in several industries, particularly those pharmaceutical, food and environmental. However, microbial metabolic cell reconstruction has limitations. Metabolic Control Analysis (MCA) allows the evaluation of the reliability of the changes performed by ME, emphasizing the importance of the whole pathways rather than individual pathway reactions resulting from simple flux analysis. This led to an increased emphasis on the regulatory structure of the network. The use of MCA becomes indispensable to quantify metabolic parameters, particularly those related with direct genetic modifications. However, this type of analysis is not a common practice. In the present study MCA is exemplified as a tool of ME, being demonstrated its practical application in drug delivery and in the production of three relevant biotechnological products (penicillin V, l-lysine and glycerol).

Keywords: control coefficient, drug delivery, enzyme, glycerol, kinetics, l-lysine, metabolic control analysis, penicillin V.

1 LIST OF ABBREVIATIONS

6-APA	6-aminopenicillanic acid
ACVS	ACV synthetase
ADP	Adenosine diphosphate
ASA	Aspartate semialdehyde
ASD	Aspartate semialdehyde dehydrogenase
ASK	Aspartokinase
ASP	L-aspartate
AT	Acyl-CoA isopenicillin acyltransferase
ATP	Adenosine triphosphate
BAP	β-aspartylphosphate
cys	Cysteine
DAP	D, L- diaminopimelate
DAPDC	Diaminopimelate decarboxylase
DAPDH	Diaminopimelate dehydrogenase
DHAP	Dihydroxyacetone phosphate
DHP	L-dihydrodipicolinate
DHPR	Dihydrodipicolinate reductase
DHPS	Dihydrodipicolinate synthase
FCC	Flux control coefficient
glut	Glutathione
GPD	Glycerol-3-phosphate dehydrogenase
GPP	Glycerol-3-phosphatase
IPN	Isopenicillin N
IPNS	Isopenicillin N synthetase
LLD-ACV	L-α-aminoadipyl-L-cysteinyl-D-valine

Lys	L-lysine
L-α-AAA	L-α-aminoadipic acid
MCA	Metabolic Control Analysis
ME	Metabolic Engineering
MFA	Metabolic Flux Analysis
NADPH	Nicotinamide adenine dinucleotide phosphate
PERM	Permease
Pi	Inorganic phosphate
Pyr	Pyruvate
THDP	L-tetrahydrodipicolinate
val	Valine

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2 **LIST OF SYMBOLS**

c_i	Concentration of the i compound
C_{ij}^{J0}	Flux control coefficient for the *i*th enzyme on *J*th steady state flux
C_{ij}^{x0}	Concentration control coefficient for the *i*th enzyme on the *x*th metabolite
e_i^0	Enzymes activity through the *i*th pathway
J_i^0	Steady state flux through the *i*th pathway
K	Constant parameters
K_{eq}	Equilibrium constant
K_i	Inhibition constant
K_m	Michaelis-Menten constant
$L_{Lys\text{-}Thr}$	Inhibition term by lysine and threonine
r	Specific rate of the enzyme catalysed reaction
R	Response coefficient
R_{ij}^{J0}	Response coefficient for the *i*th enzyme on *J*th flux

R_{ij}^{x0}	Response coefficient for the *i*th enzyme on the *x*th metabolite
v_i	Rate of an individual reaction
v_{max}	Maximum rate of the reaction
x_i^0	Level of intracellular *i*th metabolite
ε	Elasticity coefficient
ε_{ij}^{c0}	Elasticity coefficients for the *i*th enzyme related to external effectors
ε_{ij}^{e0}	Elasticity coefficients for the *i*th enzyme related to enzyme activity
ε_{ij}^{x0}	Elasticity coefficients for the *i*th enzyme related to intracellular metabolites

1. INTRODUCTION

The most interesting feature of organisms is their ability to perform a wide variety of metabolic activities that provide the basis for synthesizing a wide range of products of commercial value. However, in their natural environment, the metabolism of a living cell is predominantly focused on survival and reproduction. In general, this means that cells use their biochemical machinery to synthesize metabolites and cellular constituents, in the minimum quantities required for their maintenance and growth. However, the requirements of a biotechnological process are contrary to these facts, as a compound of interest must be produced in large quantities in a way that the process is profitable [1, 2]. The study of cellular metabolism is a relevant strategy to understand the unique properties and behaviour of the constituent elements, particularly enzymes and their reactions. This has led to the continuous and growing interest of academic and industry researchers in Metabolic Engineering (ME) [3, 4].

ME is based on the manipulation of the cellular metabolism in order to obtain an organism capable of synthesizing a product of interest, at the maximum production rate, requiring the minimal amount of substrate, and therefore, providing less expensive

products [5-7]. ME is multidisciplinary and uses different knowledge from different areas, such as Genetics, Mathematics, Computer Science, Biochemistry, among others (Figure 1). To achieve the desired goals, microorganisms can be redesigned, to a certain limit, modifying the existing metabolic pathways or inserting new paths [8, 9]. If the metabolic main components are identified, the manipulation of these components and the study of the metabolic fluxes can be analysed, but not fully constructed. As genetic modifications are not predictable, the random modification in a gene is not a sufficient strategy to achieve a particular purpose. Therefore, it is necessary to understand the subsequent interactions of this genetic manipulation on the microbial physiology and behaviour [6]. Moreover, to make the desired changes, the knowledge of Transcriptomics, Proteomics, Metabolomics and Fluxomics is essential information [10].

After disrupting the natural system of organisms, research and experiments are still needed, to determine how to adjust the metabolic processes according to the manipulations. This process is a continuous and constructive cycle that permits to acquire more information every time a cycle is completed [1, 11].

ME involves mechanisms of analysis and synthesis. Therefore, ME focuses on kinetics, enzymatic catalysis and in stoichiometry reactions [6, 12]. The enzymatic profile is crucial in identifying the steps that origin the desired product. ME defines the steps that contribute most to the development of the final product; which are the limiting steps (analysis); and executes the changes (synthesis) to achieve the desired objective [6].

The determination of metabolic fluxes is carried out by Metabolic Flux Analysis (MFA). The fluxes are considered the key element of metabolism and this analysis is strategic, since the fluxes contain the minimum information necessary to describe the metabolism [2, 6, 10].

Metabolic Control Analysis (MCA) can quantify the metabolic fluxes, that are nonetheless than the rate of components production in the metabolic pathway [12]. MCA can help the manipulation of certain pathways in order to access the control that an enzyme exerts in the fluxes [13]. The software available for MCA are typical tools that can be applied in ME, such as CellDesigner, Wolfram Mathematica, Matlab, The R Project for Statistical Computing [4, 14-16]. However, there are specific metabolic simulators, such as SCAMP [17], MetaModel [18], Gepasi [19, 20], as well as MCA coefficients calculators, like CONTROL [21] and MetaCon [22].

2. MCA GUIDE

The biochemical pathways of microorganisms are not yet fully understood, mainly due to the fact that the methods applied to uncover the biochemical systems are minimalists, regarding the representation of the complexity of metabolic pathways and their intervenients [23, 24]. Therefore, there are mathematical methods, such as MCA, that are used to characterise and quantify the flux changes, by acting as a control. A MCA approach allows the quantification of cellular changes that may occur: metabolite concentration variation; changes in metabolic fluxes in response to changes in growth conditions; and enzyme activity (Figure 2) [25].

The concepts used in MCA have some gaps: they focus on the regulation of metabolic flux in an enzyme, and this cannot be considered absolutely correct, since the enzyme may not exercise control over the complete metabolic pathway. Despite the fact that a cell directs its machinery to regulate a particular enzyme, it does not mean that an increase or decrease in its activity will affect the flux significantly, the rate of the reaction can be affected by other factors, such as environmental, physiological or genetic [12]. Another disadvantage of this theory lies in the fact that there is not

sufficient knowledge about enzyme kinetics, genetics, epigenetics and response to environmental changes. This gap led to the development of various computational tools that allow the study of metabolism, due to the variation of certain on and the determination of the variables involved [4, 26].

Despite the limitations, this type of analysis is an advantageous tool for controlling the metabolic flux of individual reactions and allows the verification of different concentrations of intracellular metabolites and the enzyme activity [12].

MCA is only applied to reactions in the steady state or pseudo-stationary state [27]. It is considered that the reactions studied are defined only by the activity of enzymes that act at different stages of the pathway [12]. There are databases, such as MetaCyc (www.metacyc.com) and BioCyc (www.biocyc.com) where all the information about the metabolic pathways and enzymes can be accessed [28].

The enzyme kinetics is crucial to apply MCA [29]. It is necessary to determine the metabolic control coefficients: control and elasticity coefficients (which are interrelated) [27]. Therefore, MCA defines the quantitative relationship between the flux of a metabolic pathway, and the activity of an enzyme, in terms of flux control coefficient (FCC) [30]. Consequently, FCC evaluates the influence of an enzyme in the pathway flux [25].

The control coefficient allows the characterization of the systemic response of the system variables, such as metabolic flux and/or the concentration of metabolites. It correlates the changes observed with the disturbances imposed. The variation of the rate of a single step reaction, regarding a metabolite, is expressed by the elasticity coefficient [25, 31, 32].

These coefficients are dimensionless [25] and are associated with the enzymes activities (e_j^0), fluxes (J_i^0), levels of intracellular metabolites (x_i^0) and external factors (c_i^0). The FCC's (C_{ij}^{J0}) can be calculated from equation 1 [33].

$$C_{ij}^{J0} = \frac{e_j^0}{J_i^0}\frac{dJ_i}{de_j} \quad (1)$$

Where C_{ij}^{J0} is the FCC for the *i*th enzyme on J flux [33].

This coefficient is considered the most important of the control coefficients and for a linear pathway, has values between 0 and 1. The enzyme that has the higher value of FCC is the enzyme that has a higher flux control and an increase in this enzyme activity will result in the flux increase [12, 34].

The concentration control coefficients (C_{ij}^{x0}) are determined by equation 2 [12, 33, 35].

$$C_{ij}^{x0} = \frac{e_j^0}{x_i^0}\frac{dx_i}{de_j} \quad (2)$$

Where C_{ij}^{x0} is the concentration control coefficient for the *i*th enzyme on the x metabolite; x_i^0 are the levels of intracellular metabolites [12].

The elasticity coefficients (ε) can be calculated from equations 3, 4 and 5, related to intracellular metabolites (ε_{ij}^{x0}), enzyme activity (ε_{ij}^{e0}) and external effectors (ε_{ij}^{c0}), respectively, as in any compound that modifies the reaction rate [12, 33].

$$\varepsilon_{ij}^{x0} = \frac{x_j^0}{J_i^0}\frac{\partial v_i}{\partial x_j} \quad (3)$$

$$\varepsilon_{ij}^{e0} = \frac{e_j^0}{J_i^0}\frac{\partial v_i}{\partial e_j} \quad (4)$$

$$\varepsilon_{ij}^{c0} = \frac{c_j^0}{J_i^0}\frac{\partial v_i}{\partial c_j} \quad (5)$$

Where v_i represents the rate of an individual reaction, ε_{ij}^{x0} the elasticity coefficient for the *i*th enzyme related to intracellular metabolites, ε_{ij}^{e0} the elasticity coefficients for the *i*th enzyme related to enzyme activity and ε_{ij}^{c0} the elasticity coefficients for the *i*th enzyme related to external effectors [12, 33].

The response coefficients (R) can also be defined, allowing to verify the effect of a change in an external parameter. For the fluxes is set equation 6, and for the concentration is established equation 7 [33, 35].

$$R_{ij}^{J0} = \frac{c_j^0}{J_i^0}\frac{dJ_i}{dc_j} \quad (6)$$

$$R_{ij}^{x0} = \frac{c_j^0}{x_i^0}\frac{dx_i}{dc_j} \quad (7)$$

Where R_{ij}^{J0} is the response coefficient for the *i*th enzyme on J flux and R_{ij}^{x0} is the response coefficient for the *i*th enzyme on the x metabolite [33, 35].

The elasticity coefficients are local properties, and take into account the variation of one effect (keeping the others constant), while the response coefficients are global, and evaluate all the effects imposed [33]. On the other hand the control coefficients have systemic properties and can only be compared with the control coefficients of the same metabolic pathway [12].

Two theorems of flux control were introduced in detail by Stephanopoulos *et al.* [12]: the Summation Theorem and the Connectivity Theorem [12, 33, 36]. For the

Summation Theorem, the control of a metabolic pathway is distributed by the enzymes that constitute it. This means that the sum of fluxes must be equal to one (equation 8) [12, 33, 34].

$$\sum C_{ij}^{J0} = 1 \quad (8)$$

If the pathway is very long, the FCC's will have a small value, but there has to be a particular step in the pathway that exerts the control over the fluxes, if the FCC value is considerably higher than the others. The FCC's with a small value, in a long pathway, are the explanation for the numerous and consecutive mutations required to improve strains to increase the production of selected metabolites [12].

Additionally, it is perceptible that one of the enzymes in the reaction has to exert negative control over the metabolites. Picturing a simple reaction, if the level of the enzyme increases and the metabolite concentration decreases the sum of the concentration control coefficients is zero (equation 9) [12, 33, 36].

$$\sum C_{ij}^{x0} = 0 \quad (9)$$

The response coefficient can be calculated by the multiplication of FCC's and elasticity coefficients of the same enzyme (equation 10) [12, 33].

$$R_{ij}^{J0} = C_{ij}^{J0} \cdot \varepsilon_{ij}^{x0} \quad (10)$$

When the action of more than one enzyme is present, it is used the sum of responses for each enzyme (equation 11) [12, 33].

$$R_{ij}^{J0} = \sum C_{ij}^{J0} \cdot \varepsilon_{ij}^{x0} \quad (11)$$

The elasticity is related to flux control through the Connectivity Theorem presented in its mathematical form in equation 12 [12, 33].

$$\sum C_{ij}^{J0} \cdot \varepsilon_{ij}^{x0} = 0 \quad (12)$$

This theorem shows how the enzyme kinetics affects the flux control [12, 33]. Higher values of elasticity are reflected in lower FCC's. This gives an indication if the flux control of certain reactions will be low or high, according to the elasticity [12, 33, 37]. For example, to block a pathway of a pathogen is important to identify which enzymes have the highest FCC's values. It is supposed that the inhibition of these enzymes reduces the flux of the pathway, controlling the pathogen proliferation [31].

The control coefficients can be determined by direct methods (titration with specific inhibitors, or genetic alteration of enzyme activity) or by indirect methods (SCAMP [17], MetaModel [18], Gepasi [19, 20]) and the elasticity coefficients can be calculated from kinetic models [12, 38], such as MetaCon [22] and CONTROL [21]. This software is based on the matrix method, therefore, it allows the determination of the matrix in a more simple approach than what was previously demonstrated [39].

3. MCA Successful Applications

The practical applications of MCA are mainly related with the medical field, allowing diagnose of diseases that are related to enzyme deficiencies by the identification of the cause of the metabolic pathway malfunction [40, 41]. MCA also allows the deletion of specific metabolic pathways in pathogens [31] and is applied to the study of cancer treatment [42] and drug delivery [43], which are described below.

Since the industry is interested in obtaining large quantities of particular biotechnological products, penicillin-V, l-lysine and glycerol were selected for this study as cases of MCA successful applications [12, 23].

3.1. DRUG DELIVERY

The drug delivery is an important pharmacology issue both quantitatively and qualitatively. MCA can be valuable to quantify and determine which components are more important for the functioning of the system [40, 44].

In order to a drug be delivered properly, the targeting is essential. To determine if the target is the most appropriate, one can use the control coefficients information. A good target is the one with the highest value of control coefficients [44].

In the case of cancer, the targeting is very important due to the fact that tumour cells are very similar to the non-tumour cells [44]. The conventional treatments are based in the tumour cells susceptibility towards irradiation and chemical compounds. The main problem with these treatments is the fact that it is not specific and it also affects the non-tumour cells. These solutions can also lead to resistant tumour cells due to mutation. With these problems new solutions are being developed. In this way, MCA is a very important tool, since it permits to follow which reactions are controlling the processes. This control is not uniform and it belongs to multiple enzymes, which means that inhibiting or altering more than one enzyme is more efficient [42, 45]. In other words, the rate limiting step is not a single step, but multiple steps, since the enzymes share the control of energy metabolism [42].

Özbayraktar and Ülgen [43] used two computational methods (MCA and metabolic pathway analysis) with the purpose of identifying enzymes of the sphingolipid pathway, which can be used as targets in the cancer therapy. Sphingolipids are a very important

piece in cancer development and treatment. The metabolic significant reactions were identified by MCA and the target enzymes, responsible for these reactions, were selected and manipulated to accumulate ceramide – simple sphingolipid that induces apoptotic responses. These authors propose the increasing activity of certain enzymes (glycerol-3-phosphate, 4-hydroxylase, ceramide synthase, acetyl-coenzyme A synthetase, etc.) and the decreasing activity of others (phosphoserine-phosphatase, serine palmitoyltransferase, sphingolipid long chain base kinase, etc.) resulting in a multiple response [43].

Type 2 diabetes mellitus is another disease where MCA was already used to identify therapeutics strategies. Trombetta *et al.* [46] applied MCA to quantify the control coefficients in the intravenous glucose tolerance test. This test evaluates the plasma glucose concentration and is used to diagnose the disease. The strategy was to target the points with highest control coefficients and restore the primary control [46].

3.2. PENICILLIN V PRODUCTION

An example of MCA application is the penicillin V (phenoxymethylpenicillin) production by *Penicillium chrysogenum*. The β-lactam antibiotics are used to treat various infectious diseases and, therefore, it becomes imperative to optimize the industrial production, being inevitable the application of ME [47-50].

The metabolic pathway of biosynthesis of penicillin V (Fig. 3) has three enzymatic steps. The first step is the condensation of three amino acids: L-α-aminoadipic acid (L-α-AAA), L-cysteine and L-valine, forming L-α-aminoadipyl-L-cysteinyl-D-valine (LLD-ACV). This first reaction is catalysed by the enzyme ACV synthetase (ACVS). This enzyme also modifies the L-valine into D-valine running an epimerization [47, 49].

In the second step, the ring of LLD-ACV is closed. This reaction is catalysed by isopenicillin N synthetase (IPNS) in the presence of oxygen, forming the isopenicillin N (IPN) [47, 49].

The last step can occur in two different ways: two-step reaction and one-step reaction. In the two-step reaction, the molecule of L-α-AAA of IPN is cleaved and 6-aminopenicillanic acid (6-APA) is released. If available a precursor phenoxyacetyl-CoA is available, it can bind to the enzyme acyl-CoA isopenicillin acyltransferase (AT) and turn into penicillin V [11]. In the reaction with only one step, the hydrophilic chain of L-α-AAA of IPN is exchanged for an added precursor (phenoxyacetic acid), resulting in penicillin V, without release of 6-APA [47, 49].

The molecule of L-α-AAA is released and can be reused for the synthesis of LLD-ACV. However, part of this molecule undergoes cyclization to form 6-oxopiperide-2-carboxylic acid which is excreted into the medium, with consumption of L-α-AAA, implying that this molecule (L-α-AAA) should be replaced during the biosynthesis of penicillin V [50].

This pathway has negative feedback inhibition by the first enzyme (ACVS) through the LLD-ACV. Therefore, to obtain a high production of penicillin V, it becomes obvious that it is important to keep the concentration of LLD-ACV low, in order that the flux of production becomes higher, and that through the enzyme IPNS (which consumes LLD-ACV) there is a metabolic flux control [50].

According to different authors, the kinetic expressions are only projected to ACVS and IPNS, due to the greatest control of metabolic flux of these enzymes [12, 49, 51-54].

For ACVS, for the production of LLD-ACV, it was found that it follows the Michaelis-Menten kinetics, resulting in the equation 13.

$$r_{LLD-ACV}^{ACVS} = \frac{\nu_{max}}{1 + K_{L-\alpha-AAA} \cdot c_{L-\alpha-AAA}^{-1} + K_{cys} \cdot c_{cys}^{-1} + K_{val} \cdot c_{val}^{-1}} \times \frac{1}{1 + K_{LLD-ACV}^{-1} \cdot c_{LLD-ACV}} \quad (13)$$

Where r is the specific rate of the enzyme catalysed reaction, ν_{max} represents the maximum rate of the reaction, K are constant parameters related to the compounds (L-α-AAA, cysteine, valine and LLD-ACV) and c_i refers to the concentration of the *i*th compound (L-α-AAA, cysteine, valine and LLD-ACV).

Regarding IPNS, for the production of IPN, is applied by equation 14 [12].

$$r_{LLD-ACV}^{IPNS} = \frac{\nu_{max} \cdot c_{LLD-ACV}}{c_{LLD-ACV} + K_m\left(1 + c_{glut}K_i^{-1}\right)} \quad (14)$$

Where K_m is the Michaelis-Menten constant [12].

For the elasticity coefficients, the expressions that allow their determination are given by equations 15 and 16 [53].

$$\varepsilon_{LLD-ACV}^{ACVS} = -\frac{K_{LLD-ACV}^{-1} \cdot c_{LLD-ACV}}{1 + K_{LLD-ACV}^{-1} \cdot c_{LLD-ACV}} \quad (15)$$

$$\varepsilon_{LLD-ACV}^{IPNS} = \frac{K_m\left(1 + c_{glut}K_i^{-1}\right)}{c_{LLD-ACV} + K_m\left(1 + c_{glut}K_i^{-1}\right)} \quad (16)$$

There are several published studies in which genetic engineering is applied to increase the productivity of penicillin V [47, 49, 55, 56]. However, there are no published works, other than those mentioned previously [12, 49, 51-54], using MCA to analyse the metabolic pathway and assess possible changes to be implemented.

3.3. L-LYSINE PRODUCTION

Lysine is an essential amino acid that has applications in the pharmaceutical area, as well as in feed and food products [57, 58]. Many studies have been made with the

purpose of optimizing lysine production. In spite of this advances, it has not been found a modified microorganism providing better results than the traditional strains [59-62].

MCA is applied to improve lysine production and to quantify the modifications imposed. The metabolic pathway for lysine production for *Corynebacterium* species is represented in Figure 4. As can be seen, the synthesis has successive catalysed reactions. First, L-aspartate is activated by aspartokinase (ASK) and reduced by aspartate semialdehyde dehydrogenase (ASD). Dihydrodipicolinate synthase (DHPS) and dihydrodipicolinate reductase (DHPR) catalyse the subsequent steps in the pathway [59, 60]. At this point, L-tetrahydrodipicolinate (THDP) can be transformed into l-lysine by two different ways: the dehydrogenase pathway (catalysed by diaminopimelate dehydrogenase (DAPDH)) or the succinylase pathway, that operate at the same time [62, 63]. L-lysine is then obtained from D, L-diaminopimelate decarboxylation by diaminopimelate decarboxylase (DAPDC) [59, 60].

The kinetic expressions are not projected to ASD and DHPR, due to the fact that they operate near equilibrium [64]. For ASK, the production of aspartate semialdehyde (ASA) is represented by the specific rate of the enzyme catalysed reaction, according to equation 17 [64, 65].

$$r_{ASA}^{ASK} = \frac{v_{max}\left(c_{ASP}c_{ATP} - \frac{c_{ADP}c_{BAP}}{K_{eq,ASK}}\right)}{K_{ASP}K_{ATP} + K_{ATP}c_{ASP} + K_{ASP}c_{ATP} + c_{ASP}c_{ATP} + \frac{K_{ASP}K_{ATP}c_{ADP}}{K_{ADP}} + \frac{K_{ASP}K_{ATP}c_{BAP}}{K_{BAP}} + \frac{K_{ASP}K_{ATP}c_{ADP}c_{BAP}}{K_{ADP}K_{BAP}}} \times \frac{1}{1 + L_{Lys-Thr}\left(1 + \frac{c_{Lys}}{K_{Lys}}\right)^{8}} \quad (17)$$

Where $L_{Lys\text{-}Thr}$ is the inhibition term by lysine and threonine and c_{BAP} is defined in equation 18 [64, 65].

$$c_{BAP} = \frac{c_{ASA} c_{NADP^+} c_{Pi}}{K_{eq,ASD} c_{NADPH} c_{H^+}} \quad (18)$$

For DHPS, for the production of L-dihydrodipicolinate (DHP), is represented in equation 19 [64, 65].

$$r_{DHP}^{DHPS} = \frac{v_{max}(c_{Pyr} c_{ASA})}{K_{m,ASA} c_{Pyr} + K_{m,Pyr} c_{ASA} + c_{Pyr} c_{ASA} + c_{DHP}\left(\frac{K_{m,Pyr} K_{i,ASA}}{K_{i,DHP}} + c_{ASA}\frac{K_{m,Pyr}}{K_{i,DHP}}\right)} \quad (19)$$

Where c_{DHP} is defined in equation 20 [64, 65].

$$c_{DHP} = \frac{c_{THDP} c_{NADP^+}}{K_{eq,DHPR} c_{NADPH} c_{H^+}} \quad (20)$$

Concerning DADPH, for the production of D, L-diaminopimelate (DAP) the specific rate of the enzyme catalysed reaction is calculated through equation 21 [64, 65].

$$r_{DAP}^{DADPH} = \frac{v_{max}}{\left(1 + \frac{K_{NADPH}}{c_{NADPH}}\right)\left(1 + \frac{K_{NH_4^+}}{c_{NH_4^+}}\right)\left(1 + \frac{K_{THDP}}{c_{THDP}}\right)} \quad (21)$$

For DAPDC, the production of l-lysine (Lys), was found to follow a Michaelis-Menten kinetics, resulting in equation 22 [64, 65].

$$r_{Lys}^{DAPDC} = \frac{v_{max} c_{DAP}}{K_{m,DAP} + c_{DAP}} \quad (22)$$

The first step of the reaction is the rate limiting step, consequently the control is mainly exerted by ASK [64]. The kinetic parameters can be estimated by different software [64]: CONTROL [21] and MetaCon [22]. The first uses the matrix method to calculate control coefficients [21] and MetaCon allows the calculation of algebraic expressions for the control coefficients [22]. The application of MCA for the production of desirable

industrial products is a future proposition. To achieve higher concentrations of products, it is necessary to use simulation to evaluate the magnitude of the changes applied [16]. Moreover, the productivity of l-lysine can be enhanced through genetic engineering techniques, such as coordinated gene overexpression [61].

3.4. GLYCEROL PRODUCTION

Glycerol is a by-product commonly formed by *Saccharomyces cerevisiae* during alcoholic fermentation [66]. It is used to synthetize products like lubricants and cosmetics [67] and it is also used as an antifreeze in chemical industry [68].

This promising product is synthetized by the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate, by glycerol-3-phosphate dehydrogenase (GPD). Glycerol-3-phosphate is then dephosphorylated to glycerol by glycerol-3-phosphatase (GPP) (Figure 5) [66, 67]. *S. cerevisiae* produces this compound in response to osmotic stress or anaerobiosis. The quantity of glycerol naturally produced is small, therefore, research has been made to direct the sugar metabolism to glycerol production [67].

The increase in glycerol production was accomplished by the sulfite process and by the application of recombinant DNA technology. In the sulfite process, sulfite is added to the fermentation process and it stays connected with acetaldehyde. With this bond the acetaldehyde is not able to be an electron acceptor in the reoxidation of NADH. The NADH is then reoxidized but in glycerol production [67]. When using engineered strains it was found that glycerol is accumulated intracellularly. Using MCA, it was found that the glycerol efflux is the rate limiting step of this reaction [66]. Other recombinant techniques applied were the reduction of pyruvate decarboxylase expression and deletion of alcohol dehydrogenase genes [67].

Cronwright *et al.* [69] determined a model with MCA and concluded that several parameters affect glycerol production, and that some parameters may have a certain effect when altered in the beginning of the fermentation process and the same parameter may not affect the fermentation in the end of the process. This shows that despite the fact that MCA is a powerful tool, not everything can be predicted [69].

CONCLUDING REMARKS AND FUTURE OUTLOOK

ME applies multidisciplinary techniques, including computer and experimentation, allowing the study of cell dynamics and function. This knowledge enables the modification of microorganisms in order to improve the productivity of a particular metabolic product. In this work it was presented the application of MCA to study the metabolic pathways of microorganisms. This strategy allows to identify which are the enzymes that have higher control coefficients, in order to control the metabolic performance of the organisms. The enzymes and the pathways involved are the key to improve the common methods and applications.

Complete success in ME will only be achieved if the changes in metabolism can accomplish industrial application. However, although the study of kinetics is a slow process, this technique is the one that helps the reliable design of the desired strain, with the highest metabolic performance and, thus the highest productivity. To improve the knowledge on enzyme kinetics, at a large scale, mathematical simulation is a required tool.

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Figures and Tables

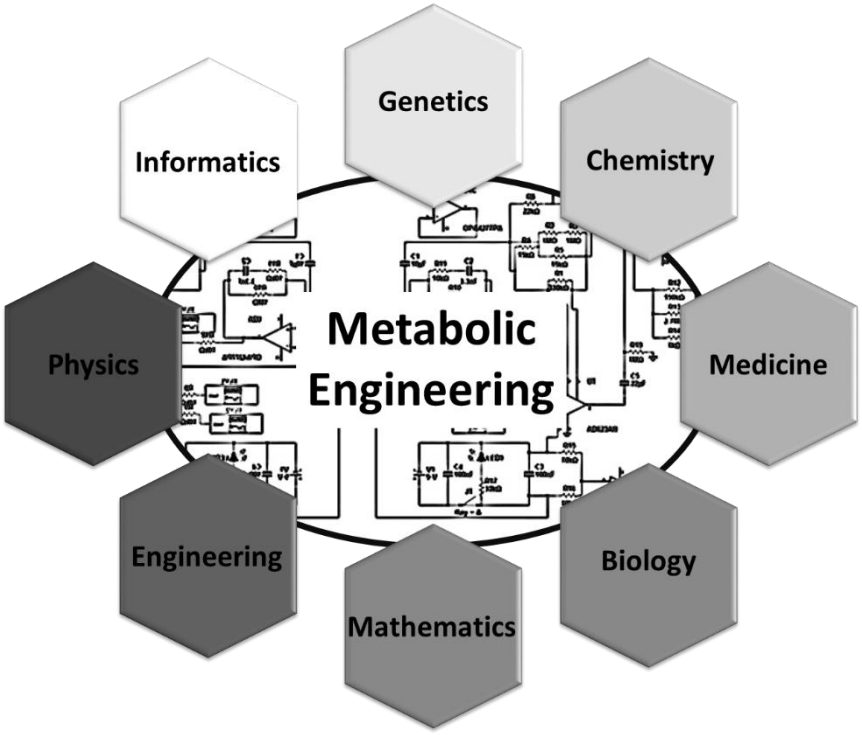

Figure 1 – Schematic representation of Metabolic Engineering multidisciplinarity (adapted from [70]).

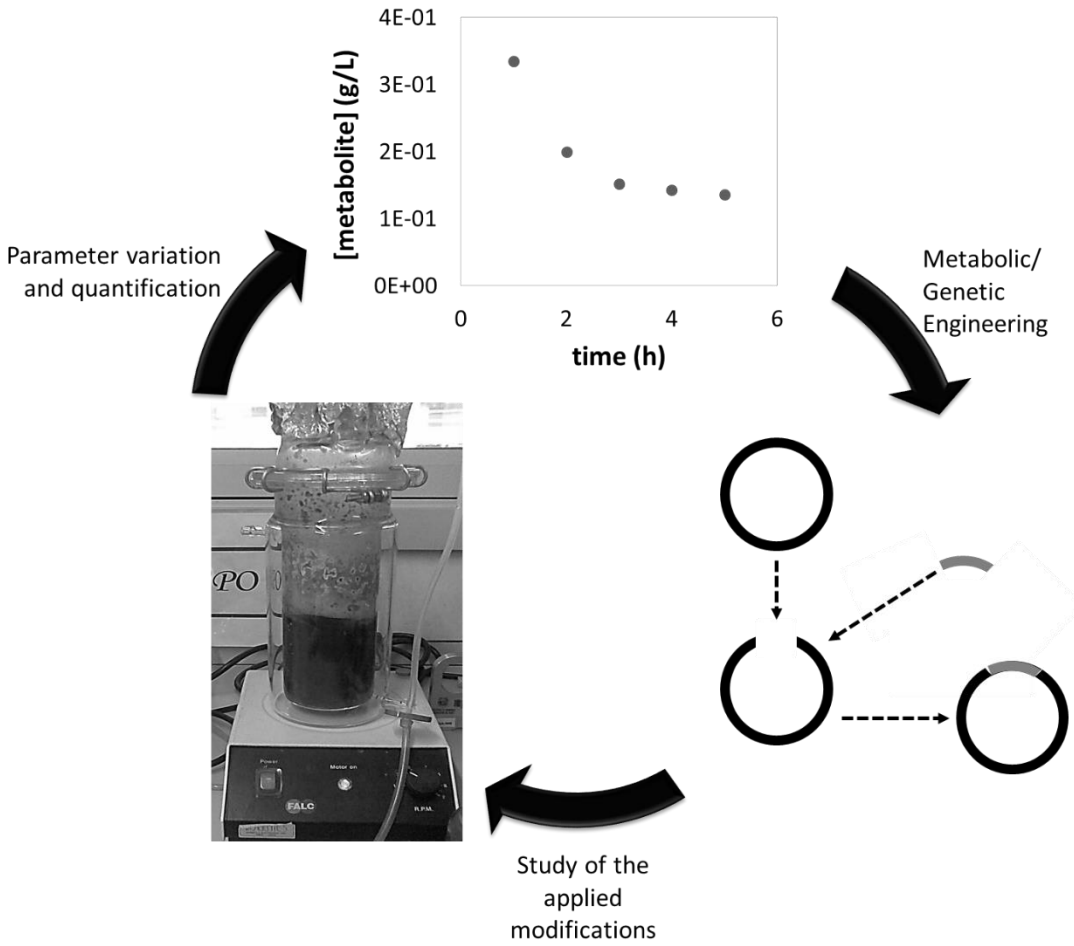

Figure 2 – Schematic representation of a strategy MCA.

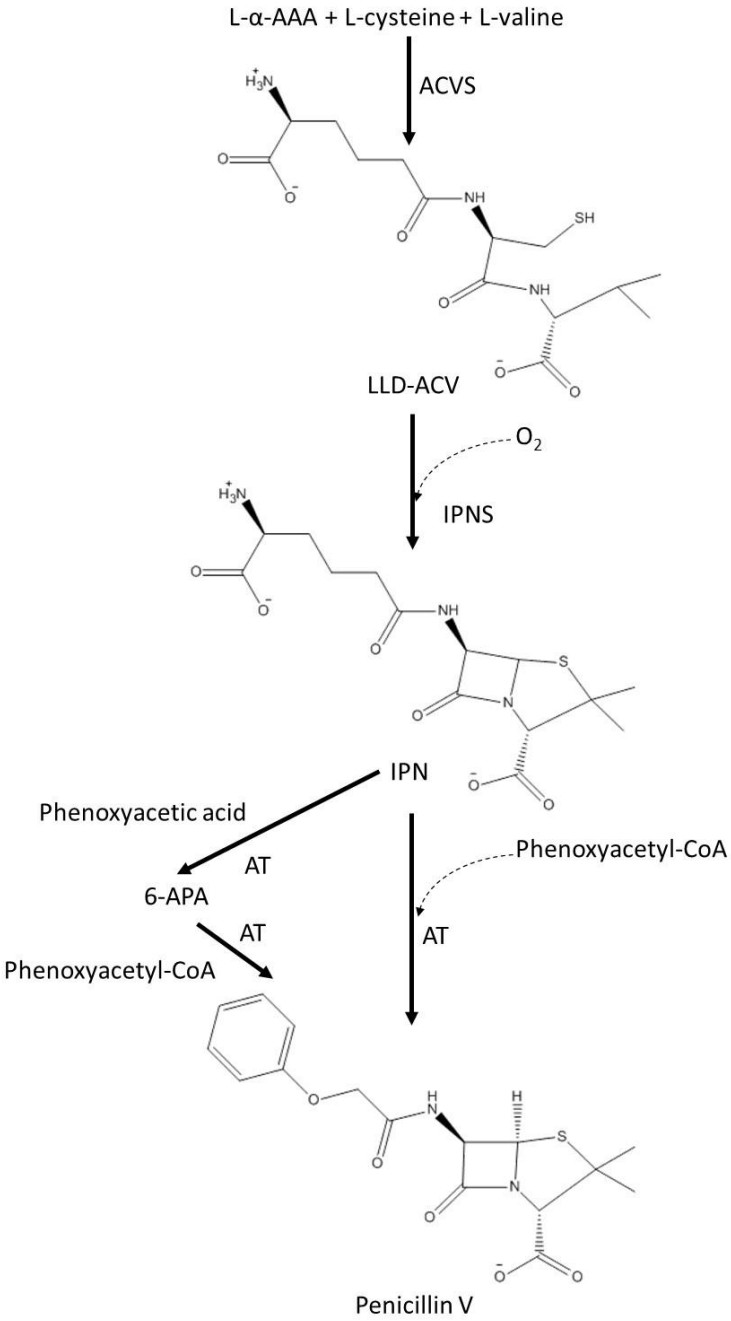

Figure 3 – Metabolic pathway for penicillin V synthesis (adapted from [51]).

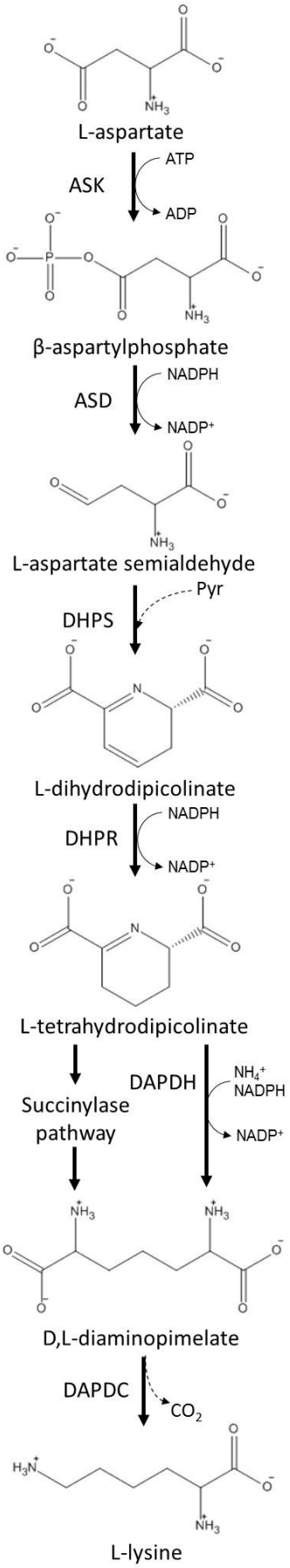

Figure 4 – Metabolic pathway for l-lysine production (adapted from [64, 65]).

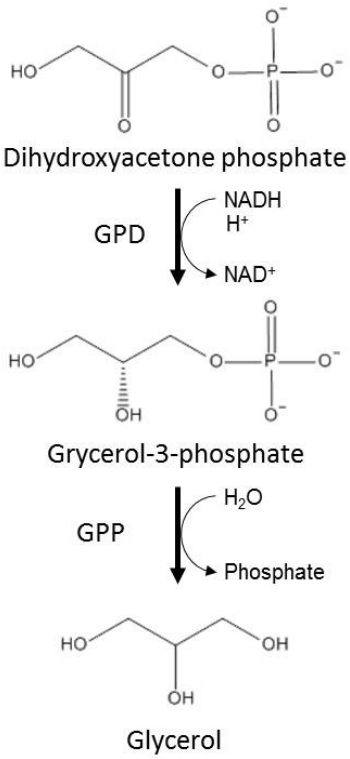

Figure 5 – Metabolic pathway for glycerol production (adapted from [67]).