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DYNAMIC SENSITIVITY ANALYSIS OF CATHARANTHUS ROSEUS HAIRY ROOTS METABOLISM

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Abstract: A dynamic metabolic model was used to investigate the relationship between the primary metabolism and the secondary metabolites production potential for *Catharanthus roseus*. The production potential was described using an objective function including both the concentrations in precursors (tryptamine, secologanin) and the fluxes leading to these molecules. The sensitivity of this function to enzyme level and experimental conditions was tested through simulation. It was observed that the carbohydrates accumulation is an important factor for the production potential. The influx of nitrogen to the metabolism had a negative effect on production potential. These conclusions are qualitatively compared to previous experimental results.

Keywords: Dynamic Metabolic Modelling, Objective Function, Plant Cells Culture, Secondary Metabolites Production, Sensitivity Analysis

1. INTRODUCTION

The production of phytopharmaceuticals by plant cells and tissues in bioreactor cultures has been an enduring challenge in biotechnology. The poor reproducibility of the cultures complicates the scale-up and validation of a bioprocess based on plant cells. Genetic flexibility (Fienh et al., 2000) as well as inadequate culture and medium management could explain the variability observed in plant cells bioprocesses. A better understanding of the plant cells metabolism could be the answer to those issues. It has been reported in the literature that the addition of precursors for the secondary metabolism significantly increase the production potential of plant cells (El-Sayed and Verpoorte, 2001). It was also observed that cell nutrition significantly affect the production potential of plant cell cultures. *Eschscholtzia californica* cells limited in nitrogen can accumulate more intracellular sugars, providing carbon skeletons for secondary metabolites production after elicitation with chitin (Lamboursain and Jolicoeur, 2005). A metabolic model describing cell nutrition, primary, and secondary metabolism could be a powerful tool to get a better insight on this link between nutrients accumulation and secondary metabolites production potential. Using a model previously developed for *C. roseus* hairy roots (Leduc *et al.*, 2006), a framework was developed to estimate the sensitivity of the plant cell production potential to metabolic and nutritional perturbations.

2. METABOLIC MODEL FOR PLANT CELLS

The metabolic model includes the major pathways of the primary metabolism: glycolysis, pentose phosphate pathway, TCA cycle and biosynthesis of cell building blocs (amino acids, structural hexoses, phosphates, lipids, organic acids). The model also describes nutrients (phosphate, carbohydrates, and nitrogen sources) accumulation by the cell and the energetic state of the cell (ATP, ADP) and cofactors (NAD/H, NADP/H). Each flux is described by a

dynamic equation using multiple Monod kinetics and sigmoid switch functions to describe known regulatory phenomenon.

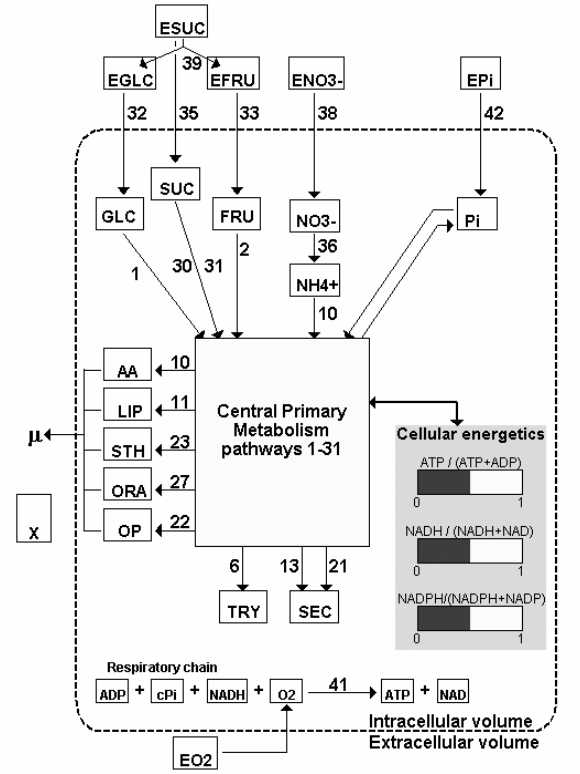

Fig. 1. Schematic view of the metabolic model

The model was presented in Leduc *et al.* (2006) with a calibration of the parameters to describe batch and medium exchange cultures of *C. roseus* hairy roots. A schematic view of the metabolic model is presented in Figure 1 and Figure 2.

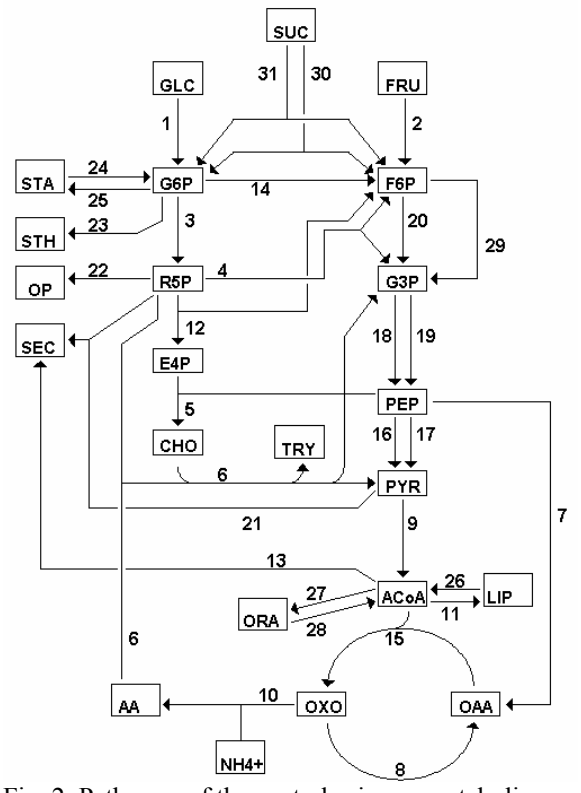

Fig. 2. Pathways of the central primary metabolism

In the aforementioned manuscript, a steady-state hypothesis on the central primary metabolism was considered in order to perform a pathway reduction. To widen the range of application of this model, it was decided to remove this hypothesis. Thus, in this work, the same model is used, but with a description of the kinetics of each enzymatic reaction of the central primary metabolism (pathways 1 to 31). The reactions of the model are presented in Table 1. A full description of the model and simulations of batch and medium exchange cultures can be found in Leduc *et al.* (2006).

Table 1 Reactions of the metabolic model

No.	Reaction
1	GLU + ATP ➔ G6P + ADP
2	FRU + ATP ➔ F6P + ADP
3	G6P + 2 NADP ➔ R5P + 2 NADPH
4	3 R5P ➔ 2 F6P + G3P
5	E4P + 2 PEP + ATP + NADH ➔ CHO + 4 Pi + ADP + NAD
6	CHO + 2 AA + 2 ATP + R5P ➔ PYR + 2 ADP + PPi + G3P + TRY
7	PEP + CO2 ➔ OAA + Pi
8	OXO+2 NAD + ADP + Pi ➔ OAA + 2 NADH + ATP
9	PYR + NAD ➔ NADH + ACOA
10	OXO + NH_4 + 3 NADPH + 3 ATP ➔ AA + 3 NADP + 3 ADP + 3 Pi
11	ACOA + ATP + 2 NADPH ➔ LIP + ADP + Pi + 2 NADP
12	2 R5P ➔ F6P + E4P
13	3 ACOA + 2 NADPH + 3 ATP ➔ SEC + 2 NADP + 3 ADP + 3 Pi
14	G6P ➔ F6P
15	ACOA + OAA + NAD ➔ OXO + NADH
16	PEP + ADP ➔ PYR + ATP
17	PEP ➔ PYR + Pi
18	G3P + Pi + ADP + NAD ➔ PEP + ATP + NADH
19	G3P + NADP ➔ PEP + NADPH
20	F6P + ATP ➔ 2 G3P + ADP
21	R5P + PYR + ATP ➔ SEC + ADP + 2 Pi
22	R5P + 3.75 AA + 7 ATP + 0.25 NAD ➔ 7 ADP + 3.5 Pi + 1.75 Ppi + 0.25 NADH + OP
23	G6P + 2 ATP + NADPH ➔ STH + 2 ADP + NADP + Pi + Ppi
24	STA + Pi ➔ G6P
25	G6P + ATP ➔ STA + ADP + Ppi
26	LIP + 2 ATP + NAD ➔ 2 ADP + Ppi + NADH
27	ACOA ➔ ORA
28	ORA ➔ ACOA
29	F6P + Ppi ➔ 2 G3P + Pi
30	SUC + 2 ATP ➔ G6P + F6P + 2 ADP
31	SUC + PPi ➔ G6P + F6P
32	EGLC + ATP ➔ GLC + ADP + Pi
33	EFRU + ATP ➔ FRU + ADP + Pi
34	OP ➔ Pi
35	ESUC + ATP ➔ SUC + ADP + Pi
36	NO_3 + NADH + 3 NADPH ➔ NH_4 + NAD + 3 NADP
37	PPi ➔ 2 Pi
38	ENO_3 + ATP ➔ NO_3 + ADP + Pi
39	ESUC ➔ EGLC + EFRU
40	ATP ➔ ADP + Pi
41	2.5 ADP + 2.5 Pi + NADH + O2 ➔ 2.5 ATP + NAD
42	EPi + 2 ATP ➔ 3 Pi + 2 ADP
43	AA + LIP + ORA + STH + OP ➔ X

3. A FRAMEWORK TO STUDY THE CELL PRODUCTION POTENTIAL

The model describes the production and accumulation of secondary metabolites precursors tryptamine and secologanin. These two molecules feed the indole alkaloid pathways in *C. roseus*. However, the model does not include the description of the secondary metabolism pathways since these are mostly unknown or poorly described. The secondary metabolites precursors will be used to describe root cells production potential in secondary metabolites. The precursors for the secondary metabolism are the model state variables TRY (tryptamine) and SEC (secologanin) and they are produced by fluxes v_6 (tryptamine) and v_{13} and v_{21} (secologanin).

3.1 Construction of a metabolic objective function to describe the cell potential for secondary metabolites production

The construction of an objective function to describe cell production potential is a subjective task. Most of the literature on metabolic objective functions is related to the flux balance analysis method where a set of fluxes is optimized to describe cellular behaviour (growth, production). Many applications of metabolic objective functions to bacterial fermentation problems can be found in the literature. Edwards and Palsson (2000) developed an objective function to describe *E. coli* growth. Radakrishnan et al. (2002) developed objective functions to describe diauxic growth of *E. coli*. Objective functions can also be used to develop error minimization routines. Muzic and Christian (2006) presented a framework to evaluate the capacity of objectives functions to estimate pharmacokinetic parameters. Polisetty et al. (2006) presented a method to identify metabolic parameters using objective functions.

Metabolic objective functions can also be used to describe and optimize cell productivity. However, if the pathways and enzyme kinetics are known the use of a metabolic objective function to optimize the production rate is not necessary as seen by Hatzimanikatis *et al.*, (1998). The problem of the description of plant cell production potential is a different matter for which a descriptive solution does not exist at the moment.

A metabolic objective function usually contains a set of fluxes. To describe the plant cell production potential, the use of the concentrations in precursors and production fluxes is suggested. The concentrations in precursors are the pools that the cell can use to produce secondary metabolites. It has been reported that an increase in precursor pools can lead to an increase in cell productivity for secondary metabolites (El-Sayed and Verpoorte, 2001). The fluxes v_6, v_{13} and v_{21} were considered in the objective function since they represent the rates at which the cell can replenish the precursor pools. Each term of the objective function is weighted with the inverse of its mean value over a non-perturbed simulation. The summation of the terms is considered as the production potential of the cell, *P(t)*, defined as :

$$P(t) = w_1 * SEC(t) + w_2 * TRY(t) + w_3 * v_6(t) + w_4 * [v_{13}(t) + v_{21}(t)] \quad (1)$$

Although this equation is an abstract description of the production potential, it has a phenomenological basis. Since the numerical resolution of the model describes both the concentrations and fluxes over time, it is possible to evaluate *P(t)* at any point during a simulated experiment.

3.2 Application of the objective function to simulated cultures of C. roseus

The response of *P(t)* to a change in a model parameter can be used to calculate the sensitivity of the production potential to this parameter. The parameters of interest for this study are the maximum reaction rates of the fluxes and the initial concentrations in metabolites and nutrients (intracellular and extracellular). These parameters can be manipulated by experimental techniques. The maximum reaction rates are proportional to enzyme level, which can be modulated through gene knock-out or over-expression. The initial conditions can be manipulated by changing the culture medium composition. The relative sensitivity of the production potential to a change in parameter or initial condition can be defined by equation 2.

$$\Delta P(t) = \left(\frac{P(t)}{P_0(t)} - 1 \right) * 100 \quad (2)$$

Where $P_0(t)$ is the production potential of an unperturbed simulation over a 50 days batch culture. *P(t)* is the simulated production potential when a v_{max} or initial concentration is changed. A simulation time of 50 days was considered since it represents a typical batch culture of *C. roseus* on the medium used by Leduc *et al.* (2006). In this work, the parameters were changed sequentially and one at a time. Because of the absence of steady-state in a batch culture of roots cells, it is necessary to represent the results of the sensitivity analysis over the whole range of the culture time. Radakrishnan *et al.* (2002) observed that for the diauxic growth of *E. coli*, the use of a dynamic (instantaneous) objective function lead to a better prediction than an end-point objective function. Moreover, it was observed experimentally that the optimal production potential of plant cells can shift in time from a nutritional perturbation (Lamboursain and Jolicoeur, 2005).

4. RESULTS AND DISCUSSION

The maximum reaction rates and initial conditions were submitted to +50% and -50% variations and the

resulting effect on production potential (equation 2) is plotted with time. A variation of 50% for the maximum reaction rates was considered since the experimental modulation of enzyme level in plant cell is rarely precise below that level. Since the model contain 42 reactions and 38 metabolic species, only the most sensitive parameters (>10% variation in production potential) are presented.

4.1 Perturbation of carbon-related fluxes

Figure 3 presents the effect of a perturbation of carbon-related fluxes on the production potential sensitivity.

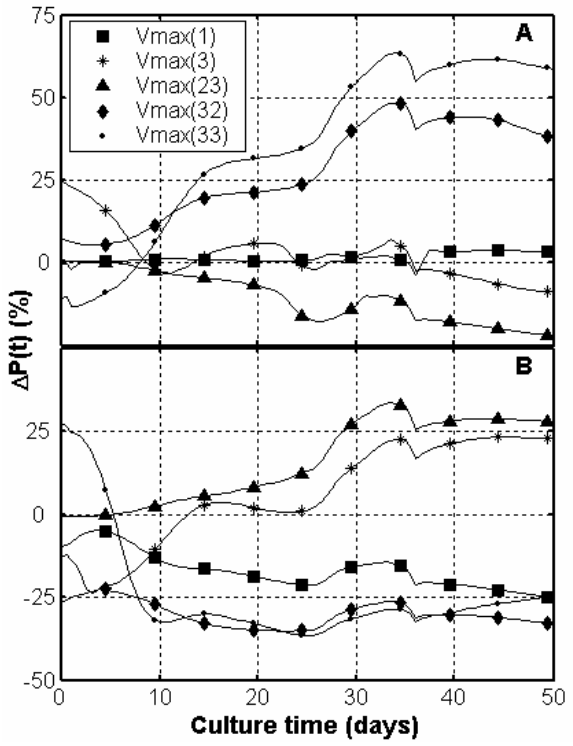

Fig. 3. Sensitivity of *P(t)* to carbon-related fluxes. A: +50% variation of v_{max}. B: -50% variation of v_{max}. Lines represent simulation results and dots are added for clarity.

An increase in the maximum uptake rate of glucose (Vmax(32)) and fructose (Vmax(33)) lead to a sustained increase in production potential. For these two perturbations, the production potential is 25-30% higher in the middle phase of the culture (15-25 days) and up to 45-55% higher at the end of the culture. A 50% decrease of Vmax(32) and Vmax(33) lead to a 25-30% decrease in production potential. The capacity of the cell to maintain the intracellular pools in free sugars seems to be an important factor on the cell production potential. An increase of Vmax(23), the flux that fixes carbohydrates in the form of structural hexoses (eg. cellulose) lead to a decrease in production potential and inversely, a decrease in Vmax(23) lead to an increase in production potential. These observations on Vmax(23), Vmax(32) and Vmax(33) are in accordance with the conclusions of Lamboursain and Jolicoeur (2005) who experimentally observed that the intracellular pool in free glucose has a significant positive effect on the production potential of *E. californica* cells. The capacity of the cell to assimilate intracellular glucose through glucokinase (Vmax(1)) is also affecting the production potential. A 50% decrease of Vmax(1) lead to a 25% decrease in production potential. A decrease of Vmax(3) (pentose phosphate pathways) has a positive effect on the production potential of the cell. However, this positive effect is seen only in the second half of the simulated experiment (after 30 days).

4.2 Perturbation of nitrogen-related fluxes

Figure 4 presents the production potential sensitivity to nitrogen related fluxes.

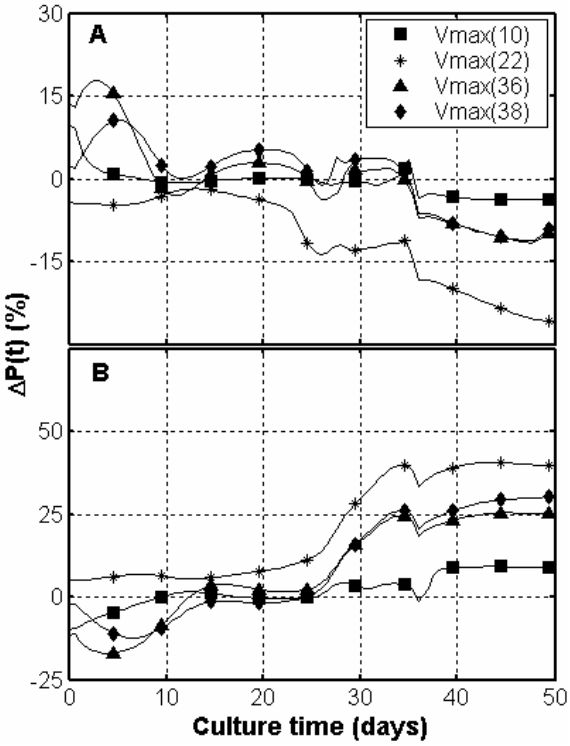

Fig. 4. Sensitivity of *P(t)* to nitrogen-related fluxes. A: +50% variation of Vmax. B: -50% variation of Vmax. Lines represent simulation results and dots are added for clarity.

The four parameters considered (Vmax(10), Vmax(22), Vmax(36) and Vmax(38)) all have an inverse effect on the production potential after 50 days. A decrease in Vmax(36) and Vmax(38) lead to a 25-30% increase in production potential. The consumption of amino acids by the cells (Vmax(22)) also showed to affect cell productivity, with a 40% increase in production potential for a reduction of Vmax(22). The better assimilation of nitrogen might help the cell to channel carbon sources to growth, which would explain the observed decrease in production potential. Moreover, it was observed experimentally that *E. californica* cells limited in nitrogen have a higher production potential (Lamboursain and Jolicoeur, 2005). The observations coming from the simulated production potential are thus in accordance with experimental results.

4.3 Perturbation of phosphate-related and energy-related fluxes

Figure 5 presents the sensitivity of the production potential to phosphate and energy-related fluxes. The fluxes of the TCA cycle (Vmax(8) and Vmax(15)) seem to have contradictory behaviours, with Vmax(8) having a positive effect on the production potential and Vmax(15) having a negative effect. However, the flux No.15 leads to oxoglutarate (OXO) which feeds the reaction No.10 (fixation of NH4). The negative effect of Vmax(15) on the production potential might thus come from the fixation of NH4. Inversely, flux No.8 will derive OXO from pathway No.10, thus competing with the fixation of nitrogen. However, the conclusions on the fluxes of the TCA cycle might be biased because of the effect that these fluxes have on the energetic metabolism. Moreover, the TCA cycle in this model is a simplified pathway in which many metabolites are not considered. A full description of the TCA cycle could yield different results in the sensitivity analysis

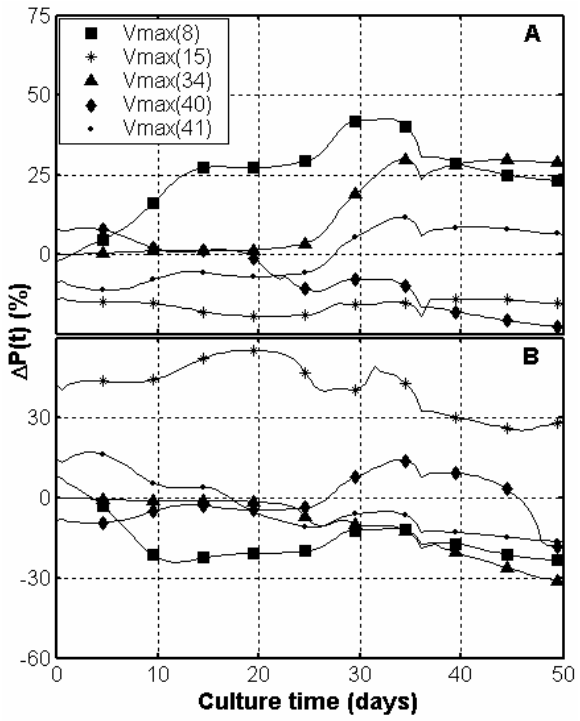

Fig. 5. Sensitivity of *P(t)* to phosphate and energetic metabolism related fluxes. A: +50% variation of v_{max}. B: -50% variation of v_{max}. Lines represent simulation results and dots are added for clarity.

An increase in Vmax(34), which describe the degradation of organic phosphate molecules (OP) to free Pi, had a positive effect on production potential. Since OP affects the simulated specific growth rate (reaction 43, Table 1), a decrease in its concentration can limit cell growth, thus leaving more metabolic resources to be channelled to the secondary metabolism. An increase in the maximum specific respiration rate (Vmax(41)) leads to an increase of ~10% in the production potential. The flux for maintenance on ATP (Vmax(40)) had a negative effect on production potential. An increase in Vmax(40) induced a decrease of 24% in production potential. These results show that the cells energetic state and fluxes could be significant factors for cell productivity.

4.4 Perturbation of initial nutritional conditions

The sensitivity of the production potential to initial nutritional conditions was simulated (Figure 6). It can be observed that the changes in initial conditions don't seem to have a sustainable effect on the production potential up to 25-30 days of culture. Extracellular initial concentration in nitrate had the highest impact on the production potential. A decrease of 50% in the initial ENO3 concentration leads to an increase of 15% in production potential. This is still in accordance with the experimental results of Lamboursain and Jolicoeur (2005) who observed the highest production potential for cells grown on low NO_3^- medium.

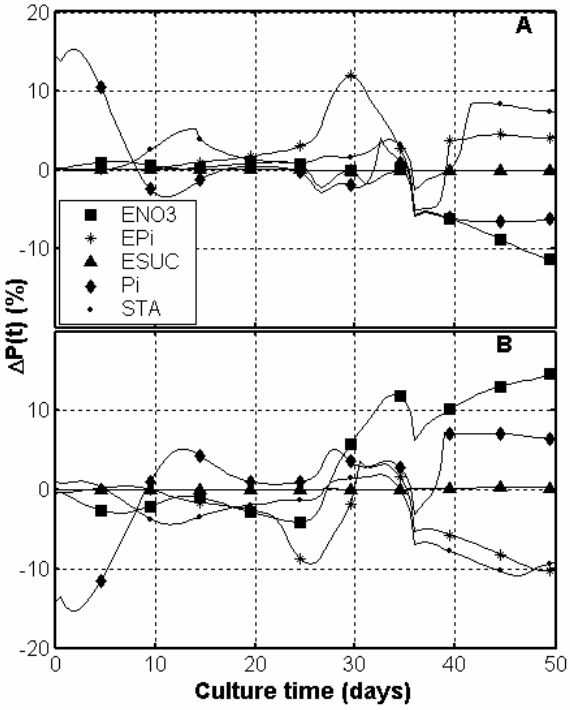

Fig. 6. Sensitivity of *P(t)* to initial nutritional conditions. A: +50% variation of X_0. B: -50% variation of X_0. Lines represent simulation results and dots are added for clarity.

The main carbon source concentration (ESUC) had no significant effect on the production potential. This is not in contradiction with the results for the carbon fluxes (Figure 3, section 4.1). An increase in extracellular sucrose concentration will not necessarily be translated into an increase in intracellular carbohydrates if the fluxes 32, 33 and 35 (carbohydrates transporters) are the same. This result suggests that the carbohydrates sources must be available at the intracellular level to have an effect on production potential. An increase in initial intracellular starch concentration (STA) leads to an increase of 7% in production potential after 50 days of culture. Inversely, a 50% decrease in initial STA

leads to a 10% drop in production potential. The availability of intracellular carbon sources is thus an important factor on plant cell production potential.

5. CONCLUSION

An extensive sensitivity analysis of the production potential of plant cells was performed using a dynamic metabolic model. The equation used to describe the production potential includes both the intracellular concentrations in precursors and the fluxes that produce the precursors. The sensitivity of the cell production potential to changes in parameters (enzyme level) and initial conditions was then tested. The suggested framework was shown to be in qualitative accordance with experimental results on the influence of cells nutrition on secondary metabolites production. The cells capacity to uptake sugars was shown to have a positive effect on the production potential. The fixation of nitrogen for cell growth had a negative effect on the production potential. The energetic metabolism was also shown to affect the production potential. The sensitivity to initial conditions in nutrients was also simulated and qualitatively compared to experimental results. The sensitivity analysis yielded valuable insight on the links between cell nutrition and production potential. The suggested framework could be extended to other plant cell species since it only includes the pathways of the primary metabolism. It could also be used to identify simple or multiple potential targets for genetic engineering or to optimize culture strategies.

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