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Thomas Chastang, Victor Pozzobon, Behnam Taidi, Eric Courot, Christophe Clément, et al.. Resveratrol production by grapevine cells in fed-batch bioreactor: Experiments and modelling. *Biochemical Engineering Journal*, 2018, 131, pp.9 - 16. 10.1016/j.bej.2017.12.009 . hal-01666093

**HAL Id: hal-01666093**

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Submitted on 22 Jul 2020

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# Resveratrol production by grapevine cells in fed-batch bioreactor: experiments and modelling

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## Abstract

The purpose of this work was to scale-up the culture of grapevine cells (*Vitis labrusca*) from shake-flasks (100 mL) to a 5L stirred bioreactor in order to study the bioproduction of resveratrol under controlled conditions. Biomass, resveratrol and sugar concentrations as well as pH and dissolved oxygen were monitored daily. The experiments were conducted twice for a total period of two months. The culture was elicited during the exponential growth phase with methyl jasmonate, leading the cells to exhibit a non-trivial behaviour during the resveratrol production phase. A model of the system behaviour involving simple phenomenology is proposed and validated against the experimental results. This model demonstrates that the system dynamic can be decomposed into four phases: a lag phase (cell growth slowing down), a starting phase (beginning of resveratrol production), a surge phase (significant resveratrol production, important cell death) and a stationary phase.

**Keywords:** Modelling; Resveratrol; Plant Cell Bioreactors; Fed-Batch Culture; *Vitis labrusca*

## Abbreviations:

dO <sub>2</sub> :	dissolved oxygen
DW:	dry weight
HPLC:	high performance liquid chromatography
MeJA:	methyl jasmonate
PSO:	particles swarm optimisation
RSD:	relative standard deviation

## 1. Introduction

Resveratrol, the most widely studied phytoestrogen, plays a key role in plant defense mechanisms and has potential therapeutic properties for humans, showing activities in cancer prevention and treatment (Delmas et al., 2006) as well as protection against neurodegenerative pathologies (Rocha-González et al., 2008). Additionally, resveratrol induces the Sirtuin genes involved in resistance against type-2 diabetes in higher organisms and enhances the longevity of organisms with a short life span, such as yeasts and drosophilae (Howitz et al., 2003; Wood et al., 2004). Furthermore, resveratrol has been proved to inhibit significantly advanced glycation end products formation, reducing the risk of developing diabetes diseases (Shen et al., 2017). Resveratrol's fame as a natural protective product provides a strong marketing tool for any products containing this compound (Renaud et de Lorgeril 1992) and resveratrol is mainly used in cosmetics and as a nutritional supplement (Vercauteren et al., 2003; André and Renimel 2010).

Biotechnology offers a promising alternative for the production of resveratrol and derivatives, avoiding many of the drawbacks of chemical synthesis or extraction from vine cuttings, two methods in conflict with sustainable development (André and Renimel 2010). Two biotechnological approaches are nowadays widely investigated to produce resveratrol: (i) the use of genetically-modified microorganisms (Wang et al., 2012; Wu and al., 2013) and (ii) *in vitro* plant-cell cultures under controlled conditions (Bru-Martinez and Pedreno Garcia, 2007; Donnez et al., 2009), especially from grapevine or peanut. The biosynthesis of resveratrol is often obtained through the use of elicitors. Elicitation consists of stimulating the plant-cell defense-responses through a physical, biological or chemical stress (Mulabagal et al., 2004; Zhao et al. 2005; Namdeo 2007). Cell cultures of *Vitis vinifera* cv. Gamay Fréaux var. Teinturier cultured in presence of high sucrose concentrations produced piceids and other glucoside derivatives of resveratrol (Larronde et al., 1998). L-alanine was shown to activate the expression of the stilbenes pathway in *Vitis labrusca* (Concord) (Chen et al., 2006) through the programmed cell-death response. Methyl jasmonate can also induce stilbenes synthesis in *Vitis vinifera* cv. Gamay Fréaux var. Teinturier (Vitrac et al., 2002), in *Vitis vinifera* cv. Limberger (Repka et al., 2004) and cv. Barbera (Tassoni et al., 2005) and in rootstock 41B cells (Donnez et al., 2011). Dimethyl- $\beta$ -cyclodextrin can be used as elicitor alone or combined with methyl jasmonate (Lijavetzky et al., 2008) or with coronatine (Almagro et al., 2015) in order to trigger a high production of resveratrol up to g/L. The combination of a resin (XAD-7 ; 200 g/L), jasmonic acid and  $\beta$ -glucan led for example to a resveratrol production of 2400 mg/L (Vuong et al., 2014). For a recent review, show Jeandet et al., 2016. Biosynthesis of resveratrol was also achieved by somatic embryos cultures in air-lift bioreactors (Sun et al., 2016). Transformed root cultures of peanut (*Arachis hypogaea*) (Medina-Bolivar et al., 2007; Halder et al., 2016), or grapevine cv Pinot noir (Tisserant et al., 2016) have also to be cited as a mean to produce resveratrol and derivatives as well as transgenic cell cultures of grape

(Kiselev et al., 2016). Different varieties (strains) of *Vitis* have been grown in bioreactors: *Vitis vinifera* (L.) cv. Gamay Fréaux var. Teinturier, especially one clone (GT<sub>2</sub> strain) chosen for its high anthocyanins production (Krisa et al., 1999); the variety 41B rootstock (*Vitis vinifera* cv. Chasselas×*Vitis berlandieri*) (Donnez et al., 2011) for the production of resveratrol and its oligomers, viniferins; and *Vitis vinifera* cv. Barbera for resveratrol and piceid resveratrolsides (Ferri et al., 2011).

The purpose of this study was to scale-up the culture of *Vitis labrusca* (Concord) cells from 100 mL shake-flasks to a 5L stirred bioreactor in order to model the bioproduction of resveratrol under controlled conditions. Parameters such as pH, biomass, resveratrol, sugar concentrations and dissolved oxygen were followed daily over a period of one month in order to characterize the kinetics of cell growth and resveratrol production.

The experimental results presented in this study show an initial exponential growth phase up to the moment of elicitation. Then, the cells exhibit a non-trivial physiological behaviour during the resveratrol production phase. A model of the system behaviour involving simple phenomenology is proposed and validated against the experimental results. This model casts light onto the phenomenology underlying this new behaviour.

## 2. Materials and Methods

### 2.1. Experimental procedure

#### 2.1.1. Plant material

Stock Concord cell suspensions were grown in Erlenmeyer flasks (100 mL working volume in 300 mL total volume). They were established from calli kindly provided by Professor Vincenzo De Luca (Brock University, Canada) in 2008 and since cultured in a modified Gamborg B5 liquid culture medium (Gamborg et al., 1968) supplemented with sucrose (30 g/L),  $\alpha$ -naphthaleneacetic acid (0.1 mg/L) and kinetin (0.2 mg/L). Cell suspensions were incubated (23°C) in darkness on a rotary shaker (SHKE 8000; Thermo scientific, 110 revolutions per minute; 25.4 mm/orbit radial movement) and weekly subcultured by resuspending the cells into fresh medium at an inoculation rate of 33% (volume per volume).

#### 2.1.2. Fed batch culture

A fed-batch culture regime has been chosen as design experiment for the bioreactor. A stirred bioreactor (Sartorius Stedim, Biostat B plus) with a glass vessel (total volume 6.6 L; working volume 0.4-5.0 L) equipped with a standard helix (3 blade segment impeller, adjustable;  $\varnothing = 7$  cm) was used. The rotation speed of the stirrer was set to 100 rpm and all experiments were conducted at 23°C. Dissolved O<sub>2</sub> was measured with a polarographic (OxyFerm FDA 325; Hamilton) dO<sub>2</sub> probe. The probe was calibrated for the zero point straight after autoclaving whilst still hot and for the 100% dO<sub>2</sub> point by passing air (750 mL/min) through the attemperated medium (23°C). The culture pH was measured with a pH-probe (EasyFerm Plus K8 325; Hamilton). This was calibrated (7.0 and 4.0) before autoclaving. During the experiment, every time a sample was taken from the bioreactor, a point calibration of the bioreactor-probe was made using the pH value obtained from a freshly calibrated external pH probe (PL-700PC; Bioblock Scientific). The aeration rate immediately after inoculation was fixed at 20 NmL/min (0.01 volume of air at atmospheric pressure introduced per bioreactor working volume per minute). When the measured dO<sub>2</sub> of the culture reached zero the aeration rate was increased to 730-780 NmL/min to compensate the increased oxygen demand of the culture. The actual air-flow out of the bioreactor was measured daily by timing the volume of gas accumulated in an inverted measuring cylinder (1 or 2 L depending on the flow rate) filled with water. Samples (45 - 90 mL) of culture were removed from the bioreactor daily or twice daily (on days when medium was added) for analysis and were not replaced by fresh medium.

The medium (1.2 L) in the bioreactor was inoculated with shake-flask cultures (3X100 mL). After 7 days of culture, 0.8 L of medium was added and 3 days later (day 10) the volume of the culture was completed to 5 L. At day 15 (358 h) of the culture (batch 1) or day 14 (333 h) for batch 2, the cells were elicited with the addition of methyl jasmonate (Sigma-Aldrich 95% purity; 0.5 mM). The methyl jasmonate was first mixed with ethanol 99,9 % before addition into the culture. We have previously checked that ethanol did not affect the cells behaviour at this concentration.

#### 2.1.3. Biomass measurements

For DW measurements the cell suspension was centrifuged (5,000 g, 20 min, 18°C). The pellet was then dried at 105°C for 7 days, cooled in a desiccator in the presence of anhydrous calcium sulphate and weighed.

#### 2.1.4. Resveratrol measurements

After storage at -20°C and subsequent thawing of the sample (20 mL) the medium was washed with 40 mL of ethyl acetate and the organic phase was evaporated to dryness using a rotary evaporator (Büchi, Rotavapor RII). We have checked that more of 90% resveratrol was found in the medium of culture. The dry residue was resuspended in 1 mL of methanol and the concentration of resveratrol was measured by HPLC (Ultimate 3000, Thermofisher) with a column designed for stilbene separation (2.1 mm x 100 mm Acclaim RSLC PolarAdvantage II) and an ultraviolet detector (DAD 3000). The mobile phase consisted of water (Millipore) as elution solvent A and acetonitrile (Fisher; HPLC grade) as elution solvent B. A linear gradient elution-profile was used. The column pressure was generally adjusted between 200 and 450 bars and the oven temperature was 30°C. Resveratrol, approx. 99 % gas chromatography (Sigma-Aldrich), was used as a standard. For a sample injection volume of 10  $\mu$ L the detection limit was 0.5 mg/L and the saturation limit was 100 mg/L. In

this range of resveratrol concentration the linearity of the calibration curve was  $R^2 = 0.99994$  over 50 points. The average relative standard deviation on the linear part of the calibration curve was 0.7%.

### 2.1.5 Sugar measurements

Carbohydrates (sucrose, glucose and fructose) were measured by HPLC (Ultimate 3000, maximum pressure 620 bars; Thermofisher) with a RI detector using an isocratic elution profile (constant flow rate = 0.5 mL/min). The mobile phase consisted of water (Millipore) acidified with sulphuric acid (2 mM). The column pressure was 70 bars and the oven temperature was 30°C. The standards we used were D-(-)-sucrose (Acros Organics), D-(+)-glucose (Sigma-Aldrich) and D-(-)-fructose (Fisher Scientific). All were analytical grade chemicals. The detection limit for carbohydrates was 0.05 g/L for an injection volume of 10  $\mu$ L with saturation at 10 g/L. The linearity of the calibration curve was between  $R^2 = 0.99880$  to  $R^2 = 1.00000$  over 80 points. The average relative standard deviation on the linear part of the calibration curve was 0.6 %.

## 2.2. Mathematical model

The goal of the model is to describe and to provide further understanding of the observed behaviour of the culture after elicitation. Indeed, numerous models already exist to describe cells growth in bioreactor. Among them several degrees of complexity exist from classical Monod's law (Droop 2009) to more sophisticated models taking into account light supply (Béchet et al., 2013) or nutrient shortage (Lemesle and Mailleret 2008). In this work, the model differs from classically proposed models. It aims at predicting the biomass and resveratrol concentrations in an elicited culture. The model building strategy is simple: first a phenomenology is assumed, then a model is proposed. Finally, an optimisation procedure is used to determine whether or not numerical parameters, allowing the model to properly describe the observed behaviour, could be found. This kind of approach has already been proved successful in previous studies (Packer et al., 2011, Yang et al., 2011).

### 2.2.1. Foreseen phenomenology

The model is based on simple phenomenological concepts involving biomass and resveratrol concentrations as keys for explaining the system behaviour. Considering biomass, dry weight curves allow to go step further. Indeed, after elicitation, dry weight curves switch from an upward trend to a downward trend (Fig. 1.a and 1.b). One should note that dry weight accounts for both living and dead cells. Thus this evolution indicates that after elicitation some cells die and are lysed so that the biomass dry weight decreases. In the light of these experimental observations, three different species could be considered in the model: the living cells (L), the dead cells (D) and the concentration of resveratrol (R). In this model, the impact of nutrient concentration on the cell behaviour is not taken into account because fructose concentration is thought to be high enough to prevent any shortage over a run (above 2.3 g/L, Fig. 3). Each of these three species has its own behaviour (Fig. 4). Living cells can divide and therefore increase their number and produce resveratrol. Resveratrol is a defense metabolite for plants; it seems relevant for its synthesis to be as fast as possible to face the potential exponential growth of the microbial plant aggressor. From a mechanistic point of view, resveratrol production auto-activation seems to be a way to achieve exponential resveratrol production. Thus, in the model, resveratrol triggers its own production. In addition, it inhibits living cell growth (Calderón et al., 1993). because its production is in direct competition with cell division metabolism. Finally, by its defensive role, resveratrol induces cell death. While dying, the cells release their cellular material in the medium (Calderón et al., 1993). This material is chemically active and could be able to interact with resveratrol molecules.. In this model, the dead cells are therefore considered capable of degrading resveratrol.

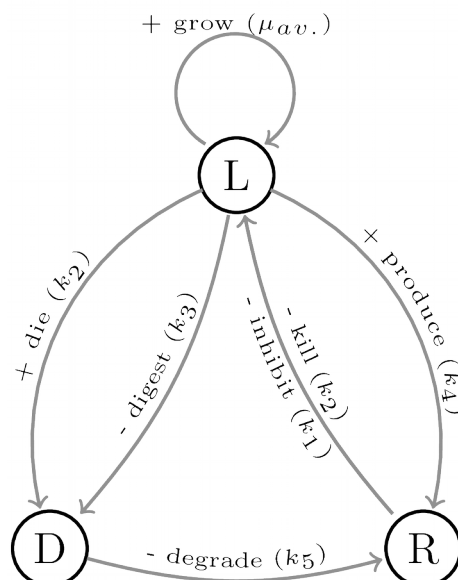


Figure 4. Phenomenology proposed in the model. L: living cells, D: dead cells, R: resveratrol. The interaction between these 3 parameters will lead to the equations described in the material and methods.

### 2.2.2. Proposed model

The model is based on conservation equations for the three species mentioned above. The bioreactor is considered as perfectly stirred. Thus, the concentrations only depend on the time variable. All of these modes of action are described using first order kinetic models. With these assumptions, it is possible to write a set of balance equations for the living cells (L, Eq. 1), the dead cells (D, Eq. 2) and the resveratrol concentration (R, Eq. 3).

$$\frac{dL}{dt} = \mu_{av} L \frac{1}{1+k_1 R} - k_2 LR \text{ Eq. 1}$$

$$\frac{dD}{dt} = k_2 LR - k_3 LD \text{ Eq. 2}$$

$$\frac{dR}{dt} = k_4 RL - k_5 RD \text{ Eq. 3}$$

This set of equations is completed by the following set of initial conditions with  $t_0$  being the elicitation time.

$$L(t=t_0) = L_0 \text{ Eq. 4}$$

$$D(t=t_0) = 0 \text{ Eq. 5}$$

$$R(t=t_0) = R_0 \text{ Eq. 6}$$

$L_0$  is known from dry weight measurement at the elicitation time.  $R_0$  could theoretically be known at the elicitation time but, due to its very low value and measurement uncertainty, the optimisation algorithm was chosen to determine its value.

### 2.2.3. Optimization procedure for parameters determination

The former model, that requires values for  $k_1, k_2, k_3, k_4, k_5$  and  $R_0 \cdot \mu_{av}$ , has already been calculated using the dry weight curves evolution before elicitation during the exponential growth phase (Section 3.2). The model parameters were determined using Particle Swarm Optimization (PSO) method. This method is derived from animal groups behaviour (Eberhart and Kennedy 1995, Reynolds et al., 1987) and nevertheless shows mathematical relevance in solving optimisation problems with numerous parameters (Andras 2012). It was successfully applied to a wide range of cases (Banks et al., 2007) including some with highly discontinuous objective functions (Wetter and Wright 2004).

In this work, the objective function of the optimization is the relative gap between the model predictions with a given set of parameters and the experimentally observed values of DW and resveratrol concentrations (numerical values of DW are obtained by Eq. 7). For every set of parameters evaluated by the optimization algorithm, the predicted DW and resveratrol concentrations were obtained by solving the system using a timestep converged explicit Euler scheme. Then, the relative gap is computed.

$$DW = L + D \text{ Eq. 7}$$

$k$  parameters values are explored in a  $10^{-10}$  to  $10^{+10}$  range,  $R_0$  is explored in a 0 to 100 mg/L range. In order to uniformly explore the wide domain of the possible values of  $k$ , these parameters are formulated as  $10^n$ ,  $n$  varying between -10 and +10. The PSO algorithm is run until the parameters are determined with three significant digits. The parameters were determined using the two batches independently.

## 3. Results

The fed batch culture was realized in duplicate and even if some values differ, we will see that all the tendencies followed are the same, allowing the establishment of a model for the production of resveratrol in these conditions.

### 3.1 Cell growth

Biomass evolution was monitored for the two batches (Fig. 1a, 1b) and the dry weight in the bioreactor showed an exponential phase from 100 h (day 4) until elicitation time at day 13 (320 h). After elicitation the exponential phase ended but growth continued until 550 h (day 23) reaching 43 g DW of biomass in the 5L of culture. For the second batch, a short stationary phase (150-180 h) was observed immediately after the first addition of medium. In both batches, the biomass decreased after day 23 due to cell death (Fluorescein Diacetate control of the cells).

The exponential growth rate was measured for the two batches using a semi logarithmic plot (Fig. 2). In the two experiments, three exponential growth phases were detected (an intermediate stationary phase was observed for batch 2) (Table 1). The specific growth rates ( $\mu$ ) were determined by the slopes on the semi-log graphs representing the exponential correlations; they were slightly different (a 11% variation) for the two batches with the mean value of  $0.0083 \pm 0.001 \text{ h}^{-1}$  corresponding to an average doubling time of 84.5 h. In the specific case of batch 2, the intermediate stationary phase may be linked to an accidentally induced stress.

The biomass concentration reached in our experiments was 9.8-10.1 g DW/L that is comparable to other studies: 27.6 g DW/L (Aumont et al., 2004), 19.7 g DW/L (Lijavetsky et al., 2008) and 2.3 g DW/L (Ferri et al., 2011) showing a good adaptation of the suspended cells in our bioreactor conditions of culture.



Table 1. Specific growth rate of *V. labrusca* cells in bioreactor culture.

Batch	Exp. phase (h)	$\mu$ ( $\text{h}^{-1}$ )	Generation time (h)	Correlation coefficient ( $R^2$ )	No. of points used in the determination
1 <sup>st</sup>	96 – 358	0.0076	91.2	0.994	12
2 <sup>nd</sup>	22 – 141	0.0079	87.7	0.990	4
2 <sup>nd</sup>	165 – 333	0.0093	74.5	0.997	7
Average	-	0.0083	84.5	-	-
RSD	-	11%	10%	-	-

### 3.2 Sugar consumption

Figure 3 reports the carbohydrate concentration during the culture period. The sucrose concentration (the sole carbon source added to the medium) decreased rapidly and was depleted by 120 h. Each time fresh medium was added (at 141h and 212 h), the same pattern was observed with a rapid decrease in concentration and subsequent depletion of sucrose. This was always accompanied by an increase in the glucose and fructose concentrations in the medium indicating extracellular invertase activity (Copping and Street 1972, Thom et al. 1981). Glucose was then consumed in preference to fructose. It is notable that elicitation occurred when practically only fructose remained as the sole carbon source.

It was possible to calculate the rates of consumption of each sugar from the medium in the absence of the others (Table 2). The glucose consumption rate was calculated over a period with no sucrose left in the medium and fructose not consumed by the cells. Each sugar kinetic (sucrose, glucose and fructose) had a linear section during which the concentration of the other carbohydrates remained constant. This indicates that over that period of time it was the sole to be consumed; these linear sections were used to calculate the constant rates of consumption of each carbohydrate (Fig. 3). The sucrose concentration decreased at the beginning of the incubation period, while the cells could take up glucose and fructose. As sucrose is not produced from the degradation of the other carbon sources present in the medium, its consumption rate could be measured realistically. Contrary to sucrose and glucose, the consumption rate of fructose was calculated after elicitation. In fact, elicitation was carried out after the total consumption of glucose, meaning that only fructose was the carbon source for the production of resveratrol.

Table 2. Carbohydrate utilisation by *V. labrusca* cells during bioreactor culture.

Sugar	Measurement made over (h)	Rate of disappearance from the growth medium (g/L/h)	Variation in the concentration of the other sugars over the period of measurement
Sucrose	0 – 117	0.225	Glucose and fructose, both increase
Glucose	286 – 333	0.104	No sucrose, fructose concentration constant
Fructose	404 – 501	0.057	Sucrose and glucose completely depleted

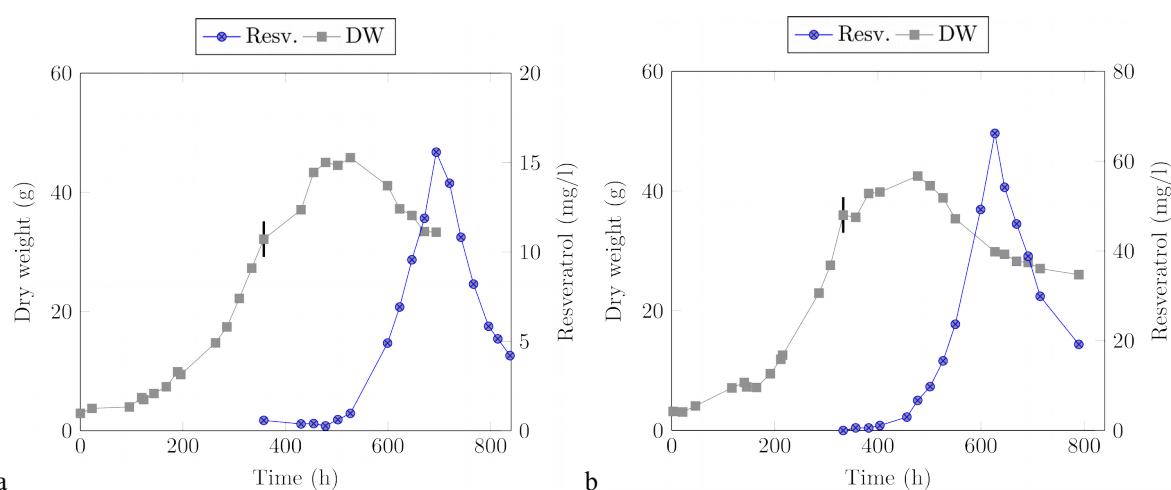


Figure 1. Concord grape cells growth and resveratrol production after elicitation by Me JA (0.5 mM) in the first batch (a) and in the second batch (b); a vertical bar at 358 h (day 15) or at 333 h (day 14) indicates the elicitation time. The total consumption of glucose was chosen as the common elicitation point between the two runs.

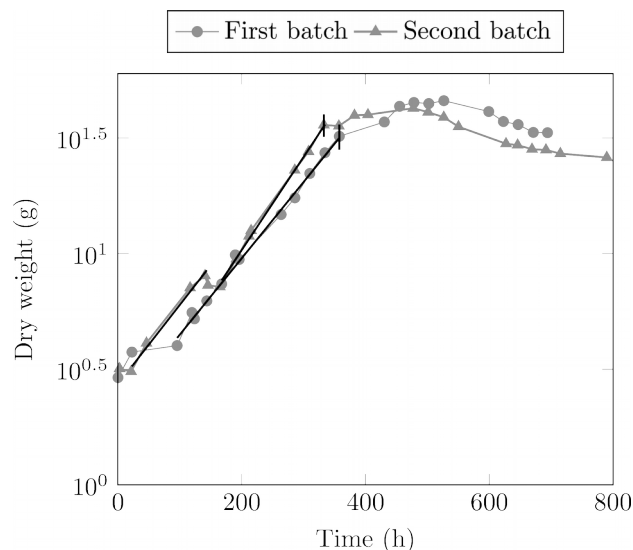


Figure 2. Semi-log plot of the Concord grapevine dry weight in the 5L bioreactor. Two vertical bars indicate the elicitation time for each batch. The three straight lines indicate the exponential fits.

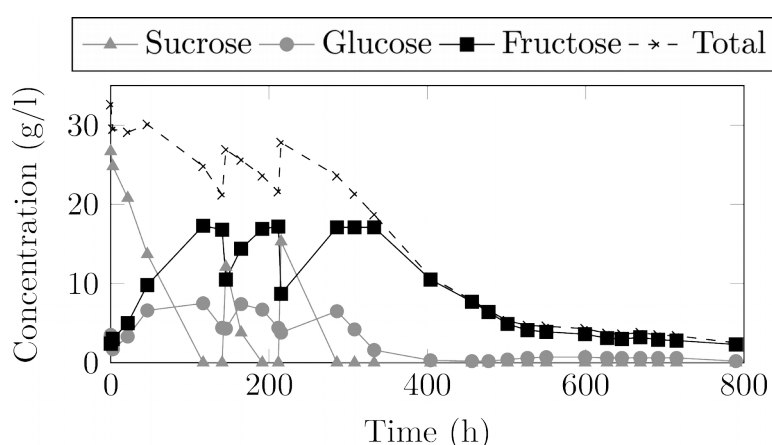


Figure 3. Carbohydrate concentration in the 5L bioreactor over time for the first batch. As it can be observed, elicitation with MeJA occurred when fructose remains the sole source of sugar in the medium (358 h). The same kinetics were obtained for the second batch.

### 3.3 Resveratrol production

The resveratrol concentration in the medium remained at approximately zero for the first 71-169 h (3-7 days) after elicitation (Fig. 1.a and 1.b). Then the resveratrol concentration in the medium increased with a non-linear progression up to a maximum: 16 mg/L at 336 h (day 14) after elicitation for batch 1 and 66 mg/L at 294 h (day 12) after elicitation for batch 2. After reaching this peak, the concentration of resveratrol decreased non-linearly on both the bioreactor batches. These maximum concentrations are comparable with others results obtained in bioreactor with 209 mg/L resveratrol by rootstock 41B cells elicited with methyljasmonate (Donnez et al., 2011) or Barbera cells elicited with chitosan (48 mg/L)(Ferri et al., 2011). Overproduction has been described but only in presence of cyclodextrins associated to MeJA with a production of 7 g/L of resveratrol in a stirred bioreactor with Gamay cells (Vera-Urbina et al., 2013).

Resveratrol production in the second batch was higher than in the first. The conditions used for the two batches were identical but each culture, with a few differences in term of growth has to be considered as independent in term of resveratrol production, as it appeared impossible to obtain drastically the same quantity of resveratrol in two subsequent cultures of 1 month each. Our purpose is to propose a model for the production of resveratrol following a MeJA elicitation, the important here is not to reach the maximal yield of production, but to analyse the curves of production. Growth of *Vitis labrusca* and subsequent resveratrol production were therefore successfully transferred from shake-flask scale to 5L bioreactor culture. Thanks to extensive measurements, these results paved the way to resveratrol production modelling.

### 3.4 Numerical results

Figures 5.a and 5.b report experimentally observed and numerically predicted DW concentrations versus time after elicitation for both runs. In both cases, the agreement between the model predictions and the experimental observations is good. The model captures the two successive trends, i.e. a moderate increase followed by a decrease. The model also predicts living and dead cells populations inside the reactor. In the first phase (0-150 h after elicitation), the DW concentration increases with the multiplication of the living cells. Then, in a second phase, living cells die and the dead cells population increases. In parallel to their number augmentation, dead cells are digested, leading to a decrease in the DW concentration. Figures 5.a and 5.b reports experimentally observed and numerically predicted resveratrol concentrations in the reactor versus time. Here again, the model predictions are in good agreement with experimental observations.

Nevertheless, the model fails to properly capture the sharp peak shape of the resveratrol concentration profile. The discrepancy surely arises from the actual complexity of the biological system. Our model only considers the interactions between 3 species (living cells, dead cells and resveratrol). This description is accurate enough to capture the experimentally observed behaviour. Yet, it fails to precisely describe with high precision “second order” features such as the peak, which is predicted as a bell shaped curve.

Table 3 reports the parameters values determined by the PSO algorithm for the two runs. It is important to note that the values are quite close (less than one order of magnitude of difference). The fact that the coefficients are close even though the two batches are different provides proof of the model quality. It is therefore possible to conclude that the proposed model accounts for the main phenomena behind the experimentally observed behaviour.

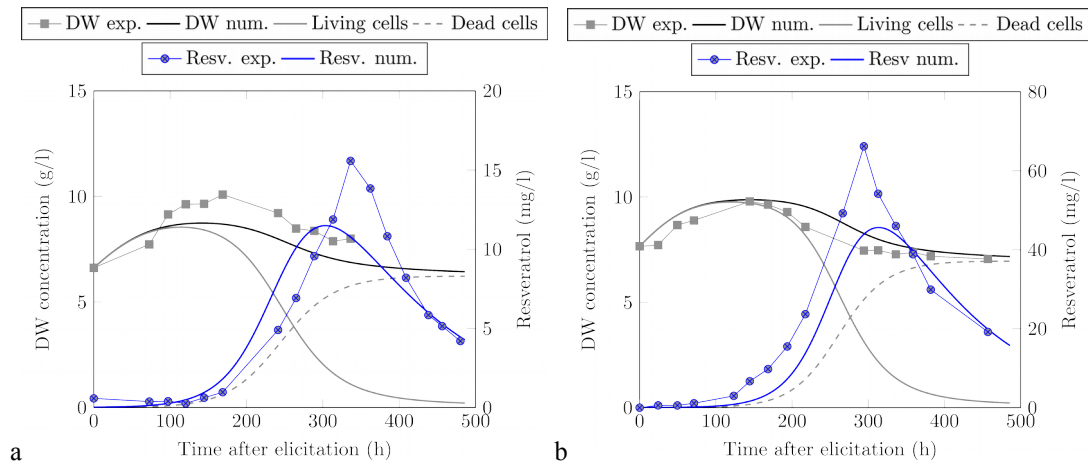


Figure 5. Experimental observations (square and circle marks) and numerical predictions (continuous lines) for the first (a) and second (b) batch after elicitation. Numerical predictions for the DW are broken down into its two components: living cell population (continuous gray line) and dead cells population (dashed gray line).

Table 3. Values of the parameters determined by the PSO algorithm for the two batches.

	Batch 1	Batch 2
$k_1$ (L/mg/h)	$10^{1.46}$	$10^{0.87}$
$k_2$ (L/mg/h)	$10^{-3.34}$	$10^{-3.65}$
$k_3$ (L/g/h)	$10^{-2.97}$	$10^{-3.08}$
$k_4$ (L/g/h)	$10^{-2.56}$	$10^{-2.55}$
$k_5$ (L/g/h)	$10^{-2.21}$	$10^{-2.67}$
$R_0$ (mg/L)	0.025	0.18

#### 4. Discussion

According to the model, it is possible to go one step further and explain the experimentally observed behaviour. It seems obvious that this behaviour is the consequence of the coupling of the phenomena taken into account by the model.

The system behaviour can be divided into four phases each having its own characteristics in term of system dynamic (Fig. 6).

The first phase (0-80 h after elicitation, readying phase) exhibits a moderate growth rate of the living cell population. During this phase, both resveratrol and dead cell concentrations are low. Physically, this phase might correspond to a period during which cell growth is inhibited in favour of the preparation of massive resveratrol production. The competition between the primary metabolism and the production of the enzymes involved in resveratrol synthesis is the most probable explanation for this inhibition.

During the second phase (80-200 h after elicitation, starting phase), resveratrol production starts. It leads to an increase of the resveratrol concentration in the bioreactor. At the end of the phase, a significant concentration of resveratrol is reached. Living cells start to die and, resveratrol auto-activates its production. This combination leads to a surge in resveratrol production that takes place in the third phase.

The third phase (200-300 h after elicitation, surge phase) exhibits strong variations within the reactor. Resveratrol is massively produced, living cells die in large number and a significant population of dead cells appears. Such a drastic behaviour comes to an end when not enough living cells remain to maintain such a high level of resveratrol production.

During the fourth phase (more than 300 h (13 days) after elicitation, stabilization phase) the system stabilizes itself. Resveratrol production decreases because too few cells remain alive. At the same time, resveratrol is degraded, leading to a decrease of its concentration in the bioreactor. With a decreasing resveratrol concentration, living cells stop to dye and reduce their resveratrol production, hence the system stabilizes.

To summarise, the cells behaviour comes down to an overreaction to the elicitation: in order to react to a simulated aggression, the living cells overproduce resveratrol which leads to their own death. Finally, after a massive production of resveratrol and the death of the major part of the cell-population, the system stabilizes with few surviving cells and a dwindling resveratrol concentration.



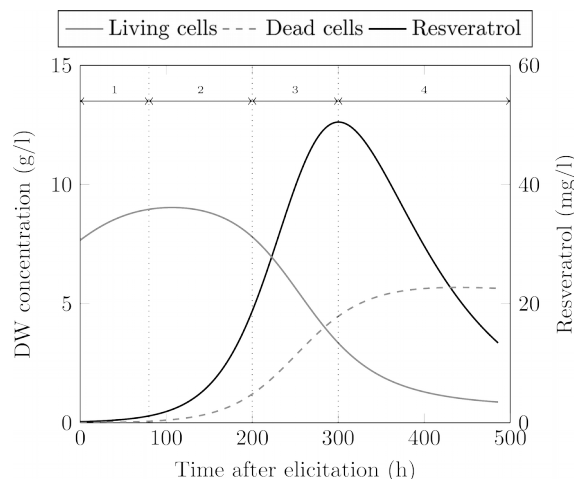


Figure 6. Decomposition into 4 phases of the system dynamic in terms of DW and resveratrol concentrations predicted by the model. (1) readying phase, (2) starting phase, (3) surge phase, (4) stabilization phase

## 5. Conclusion

In this study, growth of *Vitis labrusca* suspended cells and subsequent resveratrol production were successfully transferred from shaken-flask scale to a 5L bioreactor culture. After several additions of culture medium, resveratrol production was triggered through methyl jasmonate elicitation. Over two one-month runs, carbohydrates, dry weight and resveratrol concentrations were monitored daily. The cells behaviour after elicitation exhibited distinct patterns. Resveratrol peaked before decreasing while dry weight concentration slightly increased before dwindling. Explaining this behaviour solely using experimental observations seemed to be difficult.

A model taking into account simple phenomena was proposed to describe the system. The parameters used by this model were determined on two separates runs using Particle Swarm Optimization method. The obtained parameters are close from one batch to the other, which indicates that they are intrinsic to the system. Using the model predictions, it was possible to go one step further and explain the couplings at stake behind the observed behaviour. The observed resveratrol surge and dry weight decrease are the consequences of the cells overreaction to the elicitor: living cells overproduce resveratrol leading to their own death.

We have developed a fed-batch process that would enable to start the culture with a reduced mass of inoculum, opening the way toward the production of resveratrol in bioreactor culture.

As a first step, the proposed model sheds light onto the phenomena involved in the resveratrol production by elicited grapevine cells.

## Acknowledgements

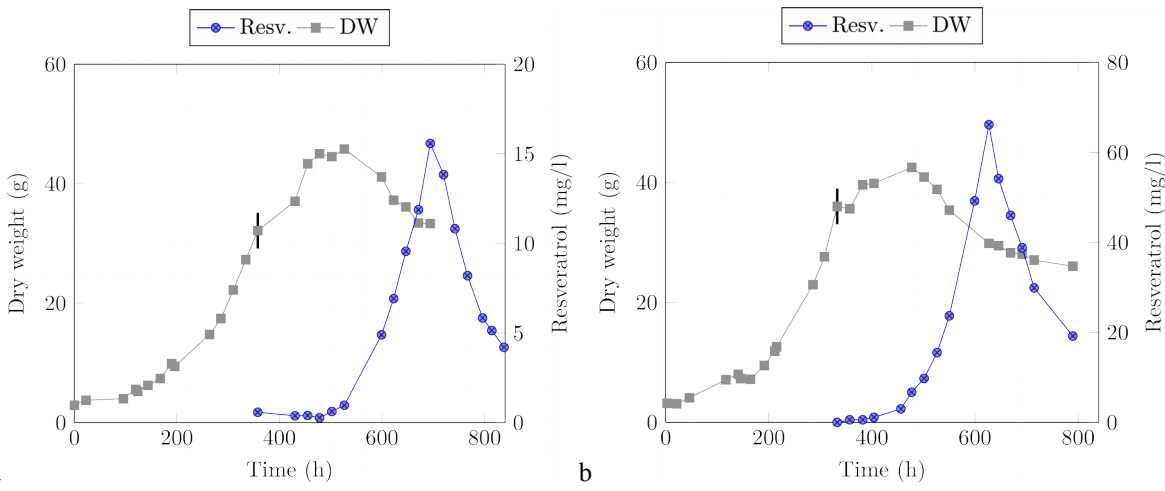
The authors wish to thank the LGPM Chair of Biotechnology (CentraleSupélec, France) for full funding of this work and the URVVC (University of Reims Champagne-Ardenne, France) for their full participation in this project including the supply of the plant cell line used in this study. Additionally, the authors wish to thank Professor Vincenzo De Luca (Brock University, Canada) for kindly providing the Concord calli to URVVC.

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**a** **b** Figure 1. Concord grape cells growth and resveratrol production after elicitation by Me JA (0.5 mM) in the first batch (a) and in the second batch (b); a vertical bar at 358 h (day 15) or at 333 h (day 14) indicates the elicitation time. The total consumption of glucose was chosen as the common elicitation point between the two runs.

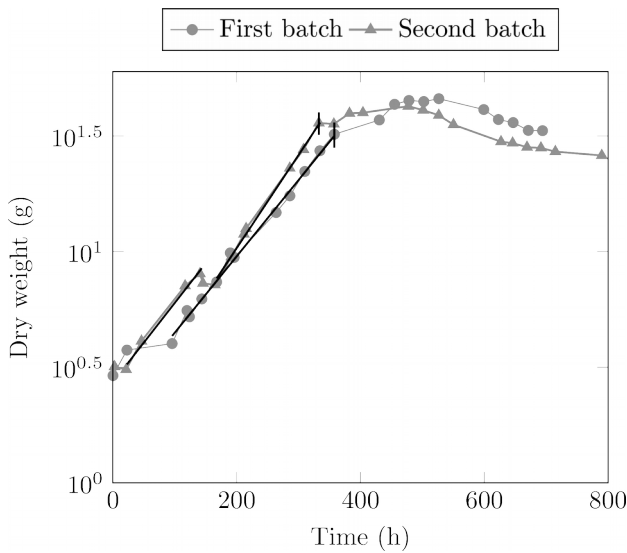


Figure 2. Semi-log plot of the Concord grapevine dry weight in the 5L bioreactor. Two vertical bars indicate the elicitation time for each batch. The three straight lines indicate the exponential fits.

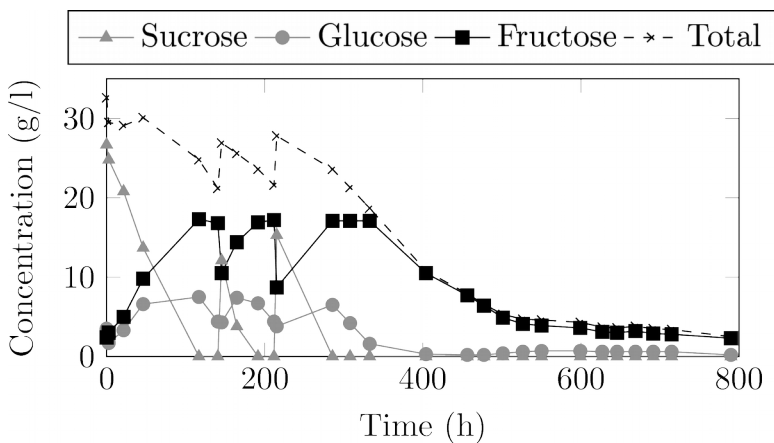


Figure 3. Carbohydrate concentration in the 5L bioreactor over time for the first batch. As it can be observed, elicitation with MeJA occurred when fructose remains the sole source of sugar in the medium (358 h). The same kinetics were obtained for the second batch.

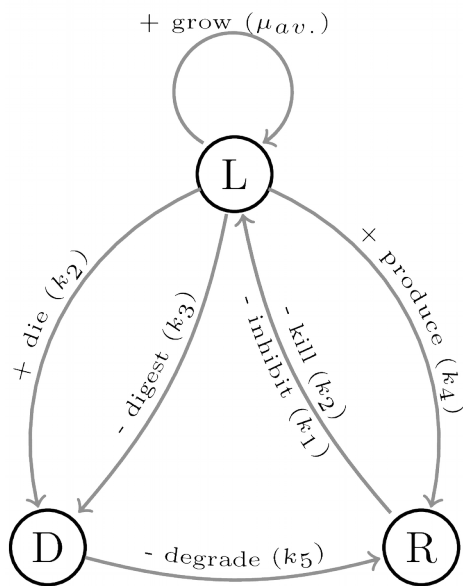


Figure 4. Phenomenology proposed in the model. L: living cells, D: dead cells, R: resveratrol. The interaction between these 3 parameters will lead to the equations described in the material and methods.

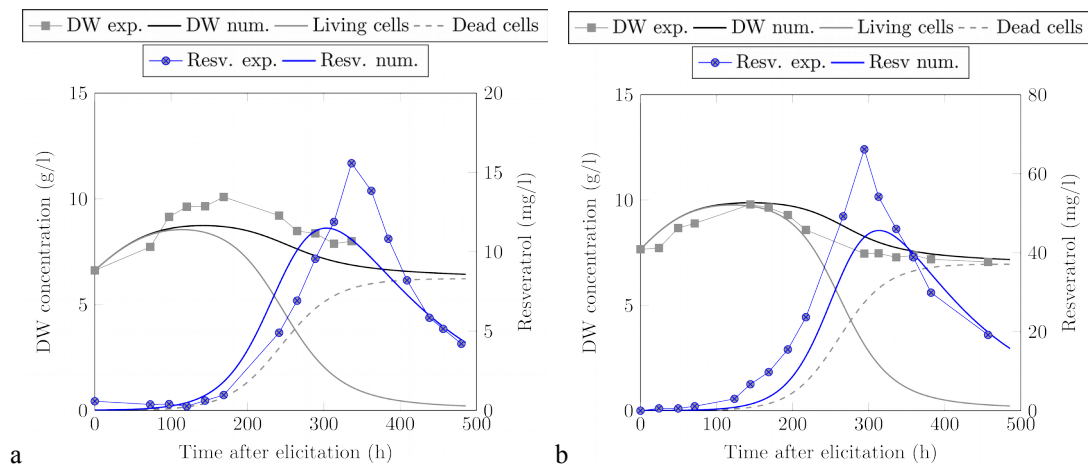


Figure 5. Experimental observations (square and circle marks) and numerical predictions (continuous lines) for the first (a) and second (b) batch after elicitation. Numerical predictions for the DW are broken down into its two components: living cell population (continuous gray line) and dead cells population (dashed gray line).

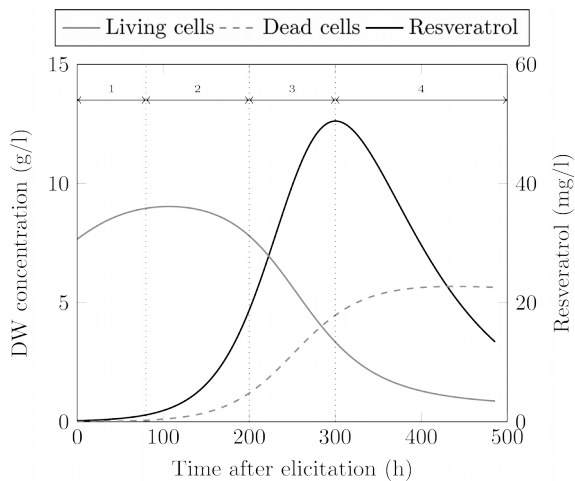


Figure 6. Decomposition into 4 phases of the system dynamic in terms of DW and resveratrol concentrations predicted by the model. (1) readying phase, (2) starting phase, (3) surge phase, (4) stabilization phase



**Table 1. Specific growth rate of *V. labrusca* cells in bioreactor culture.**

Batch	Exp. phase (h)	$\mu$ (h <sup>-1</sup> )	doubling time (h)	Correlation coefficient (R <sup>2</sup> )	No. of points used in the determination
1 <sup>st</sup>	96 – 358	0.0076	91.2	0.994	12
2 <sup>nd</sup>	22 – 141	0.0079	87.7	0.990	4
2 <sup>nd</sup>	165 – 333	0.0093	74.5	0.997	7
Average	-	0.0083	84.5	-	-
RSD	-	11%	10%	-	-

**Table 2. Carbohydrate utilisation by *V. labrusca* cells during bioreactor culture.**

Sugar	Measurement made over (h)	Rate of disappearance from the growth medium (g/L/h)	Variation in the concentration of the other sugars over the period of measurement
Sucrose	0 – 117	0.225	Glucose and fructose, both increase
Glucose	286 – 333	0.104	No sucrose, fructose concentration constant
Fructose	404 – 501	0.057	Sucrose and glucose completely depleted

**Table 3. Values of the parameters determined by the PSO algorithm for the two batches.**

	Batch 1	Batch 2
$k_1$ (L/mg/h)	$10^{1.46}$	$10^{0.87}$
$k_2$ (L/mg/h)	$10^{-3.34}$	$10^{-3.65}$
$k_3$ (L/g/h)	$10^{-2.97}$	$10^{-3.08}$
$k_4$ (L/g/h)	$10^{-2.56}$	$10^{-2.55}$
$k_5$ (L/g/h)	$10^{-2.21}$	$10^{-2.67}$
$R_0$ (mg/L)	0.025	0.18