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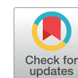
Resveratrol production by grapevine cells in fed-batch bioreactor: Experiments and modelling



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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 develop a model able to describe the bioproduction of resveratrol under the controlled conditions of a fed-batch culture. For this study, the biomass, resveratrol and sugar concentrations as well as pH and dissolved oxygen were monitored daily. The experiments were conducted twice over a three month period. The culture was elicited during the exponential growth phase with methyl jasmonate, leading the cells to exhibit a complex behaviour during the resveratrol production phase. A model of the system behaviour involving simple mechanisms is proposed and successfully confronted to 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 accompanied by significant cell death) and a stationary phase. Thus, we were able to successfully scale-up resveratrol production from 100 mL flasks to 5L bioreactors.

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1. Introduction

Resveratrol, the most widely studied phytostilbene, plays a key role in plant defense mechanisms and has potential therapeutic properties for humans, showing activities in cancer prevention and treatment [1], as well as, protection against neurodegenerative pathologies [2]. Furthermore, resveratrol has been proven to reduce the risk of developing diabetes diseases, through inhibiting advanced glycation end products formation [3]. 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) [4,5]. Resveratrol's reputation as a natural protective product provides a strong marketing tool for any products containing this compound [6]. Nowadays, resveratrol is mainly used in cosmetics and as a nutritional supplement [7,8]. Yet, its production at large bioreactor scale remains a challenge.

Advances in biotechnology offer promising alternatives for the production of resveratrol and derivatives. These approaches avoid many of the drawbacks of chemical synthesis or extraction of resveratrol from vine cuttings; two methods that conflict with sustainable development due to their high energy footprint and the organic solvents required [8]. Two biotechnological approaches are nowadays widely investigated to produce resveratrol: (i) the use of genetically-modified microorganisms [9,10] and (ii) *in vitro* plant-cell cultures under controlled conditions [11,12], 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 [13–15]. 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 [16]. L-alanine was shown to activate the expression of the stilbenes pathway in *Vitis labrusca* (Concord) [17] through the programmed cell-death response. Methyl jasmonate can also induce stilbenes synthesis in *Vitis vinifera* cv. Gamay Fréaux var. Teinturier [18], in *Vitis vinifera* cv. Limberger [19]

Abbreviations: dO_2, dissolved oxygen; DW, dry weight; HPLC, high performance liquid chromatography; MeJA, methyl jasmonate; PSO, particles swarm optimisation; RSD, relative standard deviation.

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and cv. Barbera [20] and in rootstock 41 B cells [21]. Dimethyl-β-cyclodextrin can be used as elicitor alone or combined with methyl jasmonate [22] or with coronatine [23] 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 β-glucan led for example to a resveratrol production of 2400 mg/L [24]. For a recent review on resveratrol bioproduction by grapevine cell cultures, see Jeandet et al. [25]. Biosynthesis of resveratrol was also achieved by somatic embryos cultures in air-lift bioreactors [26]. Transformed root cultures of peanut (*Arachis hypogaea*) [27,28], or grapevine cv Pinot noir [29] have also to be cited as a mean to produce resveratrol and derivatives as well as transgenic cell cultures of grape [30]. Different varieties (strains) of *Vitis* have been grown in bioreactors: *Vitis vinifera* (L.) cv. Gamay Fréaux var. Teinturier, especially one clone (GT_2 strain) chosen for its high anthocyanins production [31]; the variety 41 B rootstock (*Vitis vinifera* cv. *Chasselas* × *Vitis berlandieri*) [21] for the production of resveratrol and its oligomers, viniferins; and *Vitis vinifera* cv. Barbera for resveratrol and piceid resveratroloside [32].

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 monitored daily over a period of one month in order to characterize the kinetics of cell growth and resveratrol production. Relatively few papers describe resveratrol bioproduction in bioreactors compared to flask culture experiments [25] although the scale-up challenge is of importance.

The experimental results presented in this study show an initial exponential growth phase up to the moment of elicitation. Then, the cells exhibit a complex behaviour during the resveratrol production phase. A model of the system behaviour involving simple physiological mechanisms is proposed and successfully confronted to the experimental results. This model casts light onto the couplings 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 which were kindly provided by Professor Vincenzo De Luca (Brock University, Canada) in 2008. Since then, cells were cultured in a modified Gamborg B5 liquid culture medium [33] supplemented with sucrose (30 g/L), α-naphthaleneacetic acid (0.1 mg/L) and kinetin (0.2 mg/L). Cell suspensions were incubated (23 °C) without light 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). The medium in the bioreactor was inoculated with shake-flask cultures (3 × 100 mL). The viability measurement of cells was accomplished using fluorescein diacetate (FDA) stain as described in Donnez et al. [21].

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 marine impeller (3 blade segment impeller, adjustable; Ø = 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_2 was measured with a polarographic (OxyFerm FDA 325; Hamilton) dO_2 probe. The probe was calibrated for the zero point straight after autoclaving whilst still hot and for the 100% dO_2 point by passing air (750 mL/min) through the attempered 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 vol of air at atmospheric pressure introduced per bioreactor working volume per minute). When the measured dO_2 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 for analysis. This resulted in culture volume decreases. This is was later compensated with the addition of growth medium after 7 days and a further 3 days of incubation to a final volume of 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 suspensions were centrifuged (5000 g, 20 min, 18 °C). The pellets were then dried at 105 °C for a minimum of 24 h, 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 × 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 bar 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 μL 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. Sugars measurements

Sugars (sucrose, glucose and fructose) were measured by HPLC (Ultimate 3000, maximum pressure 620 bar; 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 bar and the oven temperature was 30 °C. The standards we used were D-(−)-sucrose (Acros Organics), D-(+)-glucose (Sigma-

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