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Resveratrol production in bioreactor: Assessment of cell physiological states and plasmid segregational stability

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ABSTRACT

Resveratrol is a plant secondary metabolite commonly found in peanuts and grapevines with significant health benefits. Recombinant organisms can produce large amounts of resveratrol and, in this work, *Escherichia coli* BW27784 was used to produce resveratrol in bioreactors while monitoring cell physiology and plasmid stability through flow cytometry and real-time qPCR, respectively. Initially, the influence of culture conditions and precursor addition was evaluated in screening assays and the data gathered was used to perform the bioreactor assays, allowing the production of 160 μg/mL of resveratrol. Cellular physiology and plasmid instability affected the final resveratrol production, with lower viability and plasmid copy numbers associated with lower yields. In sum, this study describes new tools to monitor the bioprocess, evaluating the effect of culture conditions, and its correlation with cell physiology and plasmid segregational stability, in order to define a viable and scalable bioprocess to fulfill the need for larger quantities of resveratrol.

1. Introduction

Resveratrol (3,5,4′-*trans*-hydroxystilbene) is a phytoalexin and a polyphenolic compound that belongs to the stilbene family [1]. This natural occurring and multi-biofunctional chemical [2] exists in both *cis*- and *trans*- isomeric forms due to its two phenol rings linked by a styrene double bond [3]. Resveratrol is produced by plants in response to biotic and abiotic stress and has been used as a folk remedy to treat various ailments. Several biological properties have been associated with the use of resveratrol, namely cardio and neuroprotective effects [4,5], anticancer, and antimicrobial [6,7] as well as the ability to prolong lifespan [8]. Based on its presumed properties, the interest in resveratrol by the pharmaceutical, nutraceutical, and cosmetic industries is increasing [9]. Resveratrol used by these industries is generally chemically synthesized through several routes [10]. As chemical synthesis is a time-consuming process [10] that may be affected by the low reactivity of reagents, more sustainable alternatives to chemical synthesis are in demand for resveratrol production. In order to overcome these hurdles, new biological-based processes using plant cell systems and recombinant microorganisms are being evaluated to produce resveratro [19]. Despite the high resveratrol amounts produced by *Saccharomyces cerevisiae* [11], *Escherichia coli* is the recombinant microorganism of choice due to its ability to quickly produce this compound [9], sometimes in large amounts, as has been described in previous studies [12]. Process productivity can be severely affected by cell physiology and plasmid stability [14], due to decreased cell growth, as a result of lower cell viability, or due to lower enzyme quantities, as a result of decreased plasmid copy number or gene expression [15]. So, in order to optimize resveratrol production and to guarantee the maximal output of the process, the assessment of cultivation conditions and other process variables effect in cell physiology and plasmid segregational stability is of vital importance [13]. The present work describes resveratrol production in bioreactor using *E. coli* BW27784 transformed with pAC-4CL1 and pUC-STS plasmids while monitoring cell physiology and plasmid segregational stability through flow cytometry and real-time qPCR, respectively, in order to evaluate whole process performance.

2. Materials and methods

2.1. Plasmid, bacterial strain, and growth conditions

The bacterial host *E. coli* BW27784 (*E. coli* Genetic Stock Center, New Haven, CT, USA) was transformed with pAC-4CL1 plasmid (Addgene plasmid 35,947, Cambridge, MA, USA) encoding for 4-coumaroyl CoA ligase from *Arabidopsis thaliana* and pUC-STS plasmid (Addgene plasmid 35,949, Cambridge, MA, USA) encoding for stilbene synthase from *Arachis hypogaea* [16]. Plasmid

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pAC-4CL1 has a p15A origin with the genes coded by the plasmid being constitutively expressed. pUC-STS has a pBR322 origin of replication and the genes carried by this plasmid were also constitutively expressed from the *lac* promoter [16]. *E. coli* was genetically manipulated using transformation by the heat shock protocol. Briefly, the competent cells were generated by addition of magnesium chloride (100 mM) and calcium chloride (100 mM in the first step and 85 mM in the second step of the protocol) to *E. coli* cells obtained from a LB medium cultivated at 37 °C. Then, the suspension was incubated on ice for 25 min and the pellet was collected. The transformation was performed by addition of 1 μL of each plasmid, followed by incubation on ice for 30 min, heating at 42 °C for 30 s and subsequent transfer to ice. 200 μL of SOC medium were added to the previous suspension and incubated at 37 °C. For selection of transformants, this suspension was spread in LB plates containing 50 μg/mL chloramphenicol and 100 μg/mL ampicillin. The expression system was cultivated in M9 medium (per 1 L of water: 6.779 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 1.25 g of yeast extract, 5 g of glycerol, 2 mL of $MgSO_4{\cdot}7H_2O$ 1 M, and 0.1 mL of $CaCl_2{\cdot}2H_2O$ 1 M) [16]. All cultures were started with an OD_{600} of 0.05, grown in 250 mL shake flasks containing 62.5 mL of medium, with 50 μg/mL chloramphenicol, and 100 μg/mL ampicillin, at 250 rpm and 30 °C.

2.2. Screening assays

In order to establish working ranges for further experiments, four factors were tested in screening assays: precursor (*p*-coumaric acid) concentration (0–20 mM), OD_{600} at time of precursor addition (0.1–1), temperature (25–42 °C), and pH (5–9). *p*-Coumaric acid was dissolved in DMSO to a final concentration of 1 M and sterilized by using a 0.22 μm pore size filter. Growth was suspended after 48 h of fermentation.

2.3. Bioreactor assays

E. coli was cultivated in four 0.5 L working volume parallel bioreactor (Infors HT, Bottmingen, Switzerland) containing 250 mL of M9 medium. The bioreactors were operated with strictly controlled parameters including pH, temperature, airflow, agitation (250 rpm) and dissolved oxygen (30%). The pH was maintained through the automatic addition of 1 M NaOH and 1 M H_2SO_4. All the parameters were monitored continuously using the IRIS software (Infors HT, Bottmingen, Switzerland) and all cultures were performed under subdued light in order to avoid *trans*-resveratrol isomerization to *cis*-resveratrol. Fermentations were carried out for 30 h and samples were taken aseptically at 22 and 30 h of fermentation to control growth and to evaluate resveratrol production, cell physiology and plasmid stability. The dry cell weight was calculated based on the previous established relation between OD_{600} and dry cell weight where one unit of OD_{600} was found to correspond to a dry cell weight of 0.25 g/L [17].

2.4. Analytical chromatography

Prior to injection, resveratrol was extracted from cell-free culture supernatant using a liquid–liquid extraction with ethyl acetate. Briefly, 1 mL of culture broth was centrifuged at 13,000 rpm for 5 min. The resulting supernatant was mixed with 50 μL of hydrochloric acid and carbamazepine (internal standard (IS), 100 μg/mL final concentration) and extracted with 1 mL of ethyl acetate. The extraction mixture was dried at 30 °C under a nitrogen gas stream, dissolved in 100 μL of mobile phase [18] and filtered through a 0.22 μm pore size filter. Each sample was analyzed in triplicate and all samples were stored at −20 °C prior to HPLC analysis to prevent resveratrol degradation. Ten μL of extract were applied to a Zorbax 300SB-C18 reverse-phase analytical column (4.6 mm ID × 150 mm, Agilent Technologies, Santa Clara, CA, USA) using an Agilent 1200 UPLC system equipped with a diode array detector. The process was performed as described in Paulo et al. [18], with a flow rate of 1 mL/min. Standard curves were constructed by plotting the area ratio between resveratrol and IS versus resveratrol concentration. All resveratrol analyses were performed in triplicate at each fermentation time.

2.5. Flow cytometry

Samples were analyzed on a CyAn ADP (Beckman Coulter, Brea, CA, USA) flow cytometer equipped with a 20 mW semiconductor laser at 488 nm. Fluorescence (FL1 and FL3 bandpass filters) and light scatter (FSC and SSC) signals were acquired logarithmically. Acquisition was performed with Summit 4.3 (Beckman Coulter, Brea, CA, USA) software. To reduce electronic and small particle noise, threshold levels were set on SSC. For the evaluation of cell viability, a bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX, 2.5 μg/mL final concentration) and propidium iodide (PI, 10 μg/mL final concentration) dual staining was performed as previously described [13]. The fluorescence signals were collected by FL1 (BOX) and FL3 (PI) bandpass filters and 5000 events/cells were acquired for each sample.

2.6. Real-time qPCR

Fermentation samples for real-time qPCR were prepared as previously described [13]. Specific primers (Stab Vida, Lisboa, Portugal) for chloramphenicol resistance gene (forward: 5′-ACCGTAACACGCCACATCTT-3′; reverse: 5′-TTCTTGCCCGCCTGATGAAT-3′) and ampicillin resistance gene (forward: 5′-TCCTTGAGAGTTTTCGCCCC-3′; reverse: 5′-TTCATTCAGCTCCGGTTCCC-3′) were used to amplify fragments in each of the two plasmids used. Real-time qPCR efficiency was determined for this primer set using standard solutions of known plasmid copy number. Real-time qPCR (IQ5 Biorad, Hercules, CA, USA) reactions were performed using 3 μL of sample for a 20 μL reaction containing 10 μL of Maxima™ SYBR Green qPCR Master Mix (Fermentas, Burlington, ON, Canada) and, 400 nM of pAC-4CL1 or 200 nM of pUC-STS primer set. Regarding pUC-STS, reactions were incubated at 95 °C for 3 min, followed by 30 cycles of 10 s at 95 °C and 30 s at 58 °C. For pAC-4CL1, reactions were incubated at 95 °C for 3 min, followed by 30 cycles of 10 s at 95 °C and 30 s at 60 °C. The amplified PCR fragments were checked by melting curves: reactions were heated from 55 to 95 °C with 10 s holds at each temperature (0.05 °C/s). Bacterial cell concentration was kept constant at 3×10^4 cells/reaction and for each fermentation sample, triplicate measurements were performed. PCN standards for calibration curve were made according to a previously described method [13]. Acquisition and analysis were performed in BioRad IQ 5 Software, Hercules, CA, USA.

3. Results and discussion

3.1. Screening assays

In order to determine the most relevant parameters and their ranges for resveratrol production in bioreactors, a set of screening assays was performed and the results are summarized in Fig. 1. In these assays, four parameters were evaluated: concentration of *p*-coumaric acid added, optical density (OD_{600}) of the culture when this addition was performed, incubation temperature, and pH.

The strategy used in these screening assays was based on a selection of baseline set of levels for each factor (1 mM of precursor