Biotechnology Reports 1–2 (2014) 15–21

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Treatment strategies for high resveratrol induction in *Vitis vinifera* L. cell suspension culture

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ARTICLE INFO

Article history:
Available online 19 May 2014

Keywords:
Vitis vinifera
Cell suspension culture
Resveratrol
Stilbene
Elicitation
In situ adsorption

ABSTRACT

Bioprocesses capable of producing large scales of resveratrol at nutraceutical grade are in demand. This study herein investigated treatment strategies to induce the production of resveratrol in *Vitis vinifera* L. cell suspension cultures. Among seven investigated elicitors, jasmonic acid (JA), salicylic acid, β-glucan (GLU), and chitosan enhanced the production of intracellular resveratrol manyfold. The combined treatment of JA and GLU increased extracellular resveratrol production by up to tenfold. The application of Amberlite XAD-7 resin for *in situ* removal and artificial storage of secreted resveratrol further increased resveratrol production by up to four orders of magnitude. The level of resveratrol produced in response to the combined treatment with 200 g/L XAD-7, 10 μM JA and 1 mg/mL GLU was approximately 2400 mg/L, allowing the production of resveratrol at an industrial scale. The high yield of resveratrol is due to the involvement of a number of mechanisms working in concert.

1. Introduction

One of the most widespread stilbenes is resveratrol ($C_{14}H_{12}O_3$). The biosynthesis of resveratrol is controlled by stilbene synthase (EC 2.3.1.95). This enzyme uses the same substrates and catalyzes the same condensing-type enzyme reaction as chalcone synthase (EC. 2.3.1.74), which is involved in the biosynthesis of flavonoids including anthocyanins [1,2]. Resveratrol is then the skeleton for producing other stilbenes; for instance, a glycosylation of resveratrol can lead to piceid while an oxidative dimerization of resveratrol units can form ε-viniferin, a resveratrol dehydrodimer [3]. Resveratrol has been found to have a number of health benefits: Bradamante et al. [4] revealed that resveratrol prevented heart-artery diseases by reducing cholesterol and harmful blot clots, and hardening of the arteries. Resveratrol also showed cancer chemopreventive and therapeutic effects [5], and it can act as a neuroprotectant [6].

Due to these health benefits, there is an increasing demand for effective approaches to produce resveratrol. Although this compound can be chemically synthesized [7], the need for a safe and green product is in favor of using natural sources. However, the production of resveratrol directly from plants confronts a number of drawbacks, such as yield variation, pathogens, low purity and a long growth period. Thus, plant cell culture is preferred as this approach overcomes those obstacles while ensuring a continuous supply of products in uniform quality [8], which is important to industrial bioprocesses. Grape cell suspension cultures have been reported to accumulate stilbenes including *trans*-resveratrol, *trans*/*cis*-piceid, ε-viniferin, δ-viniferin, pterostilbene, and *trans*-astringin [9,10]. However, the accumulation of resveratrol in untreated grape cell cultures is low, less than 0.01% of dry weight or 2–3 mg/L [11].

The production of secondary metabolites in plant cell and tissue cultures can be enhanced by elicitors [12]. A number of elicitors including UV, methyl jasmonate, and indanoyl-isoleucine triggered the production of secondary metabolites, including resveratrol [10,13–16]; however, the roles of many other potential elicitors remain to be investigated. If secondary metabolites are secreted, *in situ* adsorption is considered. Amberlite XAD-7, hereafter XAD-7, surpassed other XAD adsorbents in adsorption of many antioxidants including α-tocopherol and α-tocopheryl acetate, which share several common characteristics of resveratrol [17]. *In situ* adsorption might be crucial, as exogenous resveratrol at a concentration greater than 100 μM or 22.8 mg/L inhibited cell growth of *V. vinifera* cv. 'Pinot Noir' in a dose-dependent manner [18].

In this study, the elicitation of seven compounds, including jasmonic acid (JA), salicylic acid (SA), 3-methyl-salicyclic acid (MeSA), betaine (BET), β-glucan (GLU), methyl-β-cyclodextrin (MeCD) and chitosan (CHI) was investigated in single and combined treatments for enhancing the production of resveratrol in *V. vinifera* L. cv.

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http://dx.doi.org/10.1016/j.btre.2014.04.002

Gamay Fréaux cell suspension cultures. As resveratrol was found secreted into the medium, the elicitation technology was then combined with *in situ* adsorption and artificial extracellular storage for optimizing resveratrol production, with a view toward large-scale production.

2. Materials and methods

Unless indicated, all chemicals were purchased from Sigma (Australia).

2.1. Cell suspension culture

The *V. vinifera* L. cv. Gamay Fréaux cell line was a gift from Dr. Francois Cormier (Québec, Canada). This cell line was grown in GC-2 medium pH 5.7–5.8, which is B5 medium supplemented with 30 g/L sucrose, 250 mg/L casein hydrolysate, 0.1 mg/L α-naphthaleneacetic acid, and 0.2 mg/L kinetin. Cell suspension cultures were maintained on a reciprocating shaker (Ratek Instruments, Australia) at 100 strokes/min at 27 ± 1 °C. The cultures were kept in the dark to prevent the biosynthesis of anthocyanins that complete with resveratrol and other stilbenes for the same precursors.

2.2. Elicitor treatment procedure

Pre-cultured 7-day-old cell suspensions were filtered through a 50 μm stainless mesh (Endecotts Ltd. London, England), and the cells were transferred in 20 mL fresh GC-2 medium to reach the concentration of 50 g fresh cells/L. The flasks in triplicate were incubated on a reciprocal shaker (Ratek Instruments, Australia) at 100 strokes/min in a dark, temperature-controlled room at 27 ± 1 °C. Elicitors were added after 4 days of culture when cells begin their log phase of growth [19].

The stock solutions of JA, SA, MeSA (Aldrich, Australia), MeCD (Aldrich, Australia), CHI, BET and GLU were filter-sterilized through a 0.22 μm Millipore filter (Minisart®, Sartorius, Germany). Less than 50 μL of elicitor solutions (or 1/400 of the final culture volume) were added to avoid any adverse effects of the solvents. Samples in triplicate were taken on day 4, and on every three days after the addition of elicitors.

2.3. XAD-7 pre-treatment

XAD-7 with an average pore diameter of 90 Å and surface area of 450 m^2/g was used. XAD-7 beads were first soaked in 100% methanol for 30 min at room temperature (RT). They were then washed 3 times with MilliQ water on a filter unit with Whatman#1 filter paper (Whatman International Ltd., England) to remove traces of methanol, and left at RT to dry. XAD-7 beads were weighed and placed (20 g/L and 200 g/L XAD-7) in each flask before the medium GC-2 was added.

2.4. Elicitation and sampling in XAD-7 experiments

Ten mL GC-2 containing 1 g of fresh cells was transferred to 100 mL Erlenmeyer flasks containing 10 mL medium with the desired concentration of XAD-7. Thus, cells were grown with XAD-7 before the treatment of elicitors. At every sampling point, mixture of cells and XAD-7 from each flask was centrifuged at 2500 × *g* for 5 min at 4 °C using an IEC Centra-8R centrifuge (International Equipment Company, USA). Then, 200 μL medium from each tube was taken for the total extracellular phenolics analysis and 10 mL medium was for the analysis of extracellular stilbene. The cell and bead samples were filtered through a Whatman#1 filter paper (Whatman International Ltd., England) and dried in the oven for dry weight measurements.

For extraction of stilbenes from XAD-7 beads, samples were transferred into 20% sucrose solution, and gently stirred at the liquid surface to promote bead separation. Grape cells, which remain suspended, were removed by pipetting and the settled bead phase was vacuum filtered. Dried beads were weighed and then extracted for 1 h in 100% methanol with a volume equivalent to 20-fold of bead weight. The liquid phase was collected for HPLC analysis. All procedures were conducted in dim light to avoid photochemical alterations of stilbenes.

2.5. Cell growth measurement

During a culture cycle, approximately 2–3 mL volume of cell suspension from each flask was taken and centrifuged at 2000 × *g* for 5 min at 4 °C (IEC Centra-8R centrifuge, USA). The fresh cells were taken and weighed on pieces of aluminum foil, which were predried at least 30 min in 70–80 °C oven. The remaining cells were dried for 2 days in a 70–80 °C oven to calculate the dry cell weight (DCW).

2.6. Phenolics extraction and measurement

The phenolics concentrations were measured using a modification of the Folin–Ciocalteu technique described by Singleton and Rossi [20]. About 40 mg of fresh cells was homogenized in a 20-fold volume of 100% ethanol (Merck, Australia) containing 0.1% HCl for 1 min at 22100–24500 rpm by using a homogenizer (CAT X120, Germany). The homogenate was left for 30 min at RT for extraction. After being centrifuged for 10 min at 7500 × *g* (Mikro 12-24, Hettich, Germany), the supernatant was collected. The supernatant (for intracellular phenolics) or the medium (for extracellular phenolics) were added with a sufficient amount of the Folin–Ciocalteu reagent, vortexed and incubated for 7 min at RT. The chemical reaction was terminated by 20% sodium carbonate solution (Aldrich, Australia). The absorbance at 760 nm was measured in a UV mini-1240 spectrophotometer (Shimadzu, Japan) to calculate the concentration of phenolics, using gallic acid (3,4,5-trihydroxybenzoic acid) as the standard.

2.7. Anthocyanin extraction and measurement

Procedures were carried out in dim light as a portion of extract was also used for stilbene analysis. Fifty to sixty mg of fresh cells was extracted with an acidified methanol solution (0.1% HCl) of 20-fold volume equivalent to the fresh cell weight. The resultant suspension was vortexed and incubated overnight at 4 °C for a complete extraction, and then microcentrifuged at 12000 × *g* for 5 min (Mikro 12-24, Hettich, Germany). A portion of the supernatant was measured at $A_{530\,nm}$ (UV mini-1240, Shimadzu, Japan) for quantification of anthocyanins using cyanidin-3-monoglucoside, one of the major anthocyanins in *V. vinifera* L. grape extracts [21], as the standard. The remaining supernatant was for analysis of stilbenes by HPLC.

2.8. Extracellular stilbene extraction

The culture was centrifuged at 2500 × *g* for 10 min at 4 °C in an IEC Centra-8R centrifuge (International Equipment Company, USA). 10 mL of the supernatant was added to 10 mL of 100% ethyl acetate (Aldrich, Australia), and mixed thoroughly for 5 min. The mixture was left at RT for 30 min to allow phases to settle, and then the upper phase was collected. The extraction was repeated to completely extract all the stilbenes in the medium. The upper phase was vacuum dried in a concentrator system (Centrivap, Labconco, USA)