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Different biotic and abiotic elicitors influence betalain production in hairy root cultures of *Beta vulgaris* in shake-flask and bioreactor

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Abstract

The hairy roots of *Beta vulgaris* grow on a simple medium producing good levels (1.2% or 88.4 mg L^{-1}) of betalains—a red water-soluble food colourant. In an attempt to enhance betalain productivity, the hairy roots were contacted with several biotic elicitors such as purified glycans of microbial origin (200–500 mg L^{-1}), extracts of whole microbial cultures (0.25–1.25%) and the respective culture filtrates (5–25%, v/v). Similarly, abiotic elicitors, particularly metal ions, upto 10-folds of that present in the nutrient (MS) medium, were tested. It was observed that though there was a significant suppression of biomass in almost all the treatments, a significantly high productivity of betalain was observed in *Penicillium notatum* DCP-treated cultures (158 mg L^{-1} on 7th day) among biotic elicitors, pullulan-treated cultures (202 mg L^{-1} on 10th day) among purified glycans and calcium treated cultures (127 mg L^{-1} on 7th day) among abiotic elicitors, whereas control cultures showed productivities of only 43.3 mg L^{-1} on 7th day and 88.4 mg L^{-1} on 10th day. Since most of the elicitors caused early elicitation (on 7th day) and suppressed biomass resulting in reduced overall productivity, a strategy of using elicitor at late exponential growth phase was considered and such a strategy was adoptable to scaled up process using a bubble-column bioreactor, where too the addition of elicitor at late exponential phase resulted in about 47% higher productivity of betalains. The present study is the first report where a large number of elicitors are systematically screened and used for scaled-up production of betalains.

Keywords: Betalain; Elicitation; Calcium; Culture filtrate; Bubble column reactor

1. Introduction

Plant secondary products are of immense use as potential drugs, nutraceuticals and food additives. Due to their limited availability and complexity for chemical synthesis, an alternative route such as tissue culture is receiving importance for large-scale production of desired compound. Recently, the transformed root cultures have been shown to possess rapid growth, uniformity, stability and capacity to synthesize higher levels of secondary metabolites than those found in normal roots [1–5]. Betalaines are a mixture of red-violet pigments comprising mainly of betacyanins – violet pigment and betaxanthin – yellow pigment [6] that are in demand for dairy, meat and other refrigerated food products. The advantages of beet hairy root cultures for the production of natural pigments over that of field grown material have been explained in an earlier report [4,6–9].

Enhancement of secondary metabolites by elicitation is one of the few strategies recently finding commercial application. Elicitors are compounds of mainly microbial origin or non-biological origin, which upon contact with higher plant cells, trigger the increased production of pigments, flavones, phytoalexins and other defense related compounds [10–16]. Cell cultures of *Lithospermum erythrorhizon* capable of synthesizing the red naphthaquinone pigment, shikonin and its derivatives when failed to produce the same in liquid medium, elicitors such as agaropectins acting as elicitors restored the synthesis of shikonin [17]. Further, immobilization of cells in calcium-alginate beads enhanced productivity by 2.5-folds [18]. The

Abbreviations: CF, culture filtrate; DCP, dried cell powder; FW, fresh weight

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yeast elicitor, *Saccharomyces cereviseae* increased the production of berberine by 4-folds in *Thalictrum rugosum*. Rajendran et al. [19] observed 3-fold elicitation of anthocyanin in cultured cells of *Daucus carota*. Several bacterial preparations were studied for their elicitation activities in *Tagetes patula* cultures [20,21] with as high as 4-fold increase over the control [22]. In general, the secondary metabolites that are involved in plant defense functions undergo significant elicitation as a response to external physical, chemical and biological stimuli. Nevertheless failure to elicit does not necessarily mean that the metabolic pathway cannot be triggered. A combination of inappropriate medium and elicitor as well as unsuitable concentration of the latter can result in ineffective elicitation. For example, phenyl propanoid (PP) pathway was not induced in all cultures of *Vanilla planifolia* by yeast extract [23], whereas the same elicitor was used by the same authors to induce phytoalexin in cultures of *Glycine max* and alkaloid enhancement in *Thalictrum rugosum* and *Eschiltzia calfornica*. The (PP) pathway could however be triggered in *V. planifolia* by using chitosan as an elicitor [23] indicating that a successful elicitation is a very challenging process requiring intense screening procedures.

A few workers have attempted enhancement of betalain content in hairy root cultures of red beet. When copper sulphate was added to 2-week-old hairy root cultures of *Beta vulgaris* at a concentration of 5 μM, a 2-fold increase in pigment accumulation was observed in 24 h [24]. Use of polyamines, putrescine and spermidine, each at 0.75 mM, resulted in respectively 1.63- and 2-fold higher productivities of betalains in red beet hairy roots where product enhancement occurred mainly due to biomass increase [25]. The same set of polyamines (putrescine and spermidine) supplied to red beet hairy roots in bubble column bioreactor showed 1.3-fold higher betalain content on 24th day [26]. Methyl jasmonate fed at 40 μM to the hairy root cultures of red beet enhanced 1.35-fold higher betalain content on 24th day, by which time about 20% of biomass had been suppressed [26] resulting in no improvement in net productivity. Use of very high levels (100 μg mL^{-1} medium) of freeze-dried powders of *Haematococcus pluvialis* and *Spirulina platensis* resulted in, pigment increase of 2.28-fold on 15th day for *H. pluvialis* and 1.16-fold increase on 25th day for *S. platensis* [27]. In most of such cultures the control medium either received distilled water or the treatments received addition of certain nutritional factors leading to erroneous comparison indicating a need to screen elicitors in properly designed experiments. Therefore, the present report focuses on screening of a number of biotic and abiotic elicitors for further enhancement of betalain productivity.

2. Materials and methods

A hairy root clone, established by infecting the cotyledonary leaf explants obtained from aseptic seedlings of the red beet variety ''Ruby Queen'', was used throughout the present study. Induction of hairy roots and their maintenance conditions have been explained in an earlier communication [9].

2.1. Treatment with microbial polysaccharides

The microbial polysaccharides used as elicitors are bacterial glucans such as curdlan from *Alkaligenes faecalis* [28]; xanthan from *Xanthomonas campestris* [29]; Dextran from *Leuconostoc mesenteroides*, pullulan from *Aurobasidium pullulan* and chitosan from crab shell chitin (Sigma, Moribo, USA) were used in the media at concentrations of either 200 or 500 mg L^{-1}. The biomass increase and betalain content were recorded at 0, 3, 7, 10 and 13 days after treatment.

2.2. Biotic elicitors

Based on earlier reports and the availability of cultures, culture media filtrates (CF) and dry cell powders (DCP) of various fungi, yeast and bacteria were used. Fungi used were *Aspergillus niger*, *Penicillium notatum*, *Rhizopus oligosporus*, *and R. oryzae*. Yeast species used were *Rhodotorulla glutamis*, *Rhodosporidium torulloides*, *Saccharomyces cereviceae* and *Candida curvata*. Among the bacteria *Streptococcus diacetylactis*, *Lactobacillus caseii*, *L. plantarum* and *Pediococcus pentaseaus* were used. All these microbial cultures were kindly supplied by Dr. M.C. Varadaraj, Scientist, Food microbiology Department CFTRI, Mysore.

2.3. Abiotic elicitors

Different metal ions such as calcium, magnesium, manganese, zinc, copper, iron and cobalt were used at different levels like 10-, 20- and 30-fold of their respective concentrations in the normal MS medium, except for vanadium which is not a component of MS medium was used at the concentrations of 25, 50 and 100 mg L^{-1}.

2.4. Maintenance of microbial cultures

2.4.1. Fungi

The fungal cultures were maintained as slants in potato dextrose agar (PDA) medium containing 20 g of dextrose and pH adjusted to 5.5 with 10% tartaric acid and 20 g of agar was added and the final volume was made up to 1 L using distilled water and autoclaved medium was used in culture tubes as slants. Liquid medium prepared similarly without agar was used to grow fungal mycelia for treatment as elicitor.

2.4.2. Yeast

Yeast cultures were maintained on agar slants containing peptone (5 g L^{-1}), beef extract (3 g L^{-1}), sodium chloride (5 g L^{-1}) and agar 20 g L^{-1}. The pH was adjusted to 7.0,