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BETALAIN PRODUCING CELL CULTURES OF *BETA VULGARIS* L. VAR. BIKORES MONOGERM (RED BEET)[1]

ROLAND R. LEATHERS. CATHERINE DAVIN, AND JEAN PIERRE ZRYD

Laboratoire de Phytogénétique Cellulaire, Université de Lausanne, Lausanne CH 1015, Switzerland (R. R. L.. C. D., J. P. Z.); and Génie Biologique, Swiss Federal Institute of Technology, Lausanne CH 1015, Switzerland (R. R. L., C, D.)

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SUMMARY

The betalains are a class of natural pigments comprising the yellow betaxanthins and the violet betacyanins. Callus lines developed from *Beta vulgaris* L. var. bikores monogerm exhibited cell colors ranging from white/green (non pigmented) through yellow, orange, red, and violet and were representative of all betalain pigments found in the whole plant. The betalains have gained particular interest from the food industry as potential natural alternatives to synthetic food colorants in use today. Red beet extracts (E162), which contain significant amounts of the betacyanins, are currently used in products such as yogurts and ice creams. We describe here the characteristics of culture growth and betalain production for cell suspensions derived from the orange (predominantly betaxanthin-producing) and violet (betacyanin producing) callus lines. The major factors affecting betalain biosynthesis in both cultured and whole plant tissues are reviewed.

Key words: Beta vulgaris; betalain(s); betaxanthin(s); betacyanin(s); cell culture(s): food colorant(s).

INTRODUCTION

The betalains are a class of natural pigments comprising the yellow betaxanthins and the violet betacyanins. These pigments are of particular academic interest because of their limited biological distribution, occurring only in those plant species confined to the order *Caryophylalles*, notably the red beet *(Chenopodaceae)*, and certain fungi such as the fly-agaric mushroom *(Amanita muscaria).* The majority of pigment-producing plants accumulate the more diverse family of phenylalanine-derived anthocyanins (Strack and Wray, 1989). More recently, the betalains have gained interest because of their potential role as natural replacements for some currently used synthetic food colorants.

The pathway of betalain biosynthesis is described in Fig. 1. These tyrosine-derived pigments result from the conjugation of betalamic acid (the chromophore), by means of an imino linkage, with either cyclo-dopa (which may be glycosylated), giving rise to the betacyanins, or with an amine, resulting in the betaxanthins. It has been demonstrated that a range of naturally occurring and semisynthetic betaxanthins may result from the condensation of betalamic acid with either protein or nonprotein amino acids (Trezzini and Zryd, 1991) and other amines such as EDTA, under conditions of acid pH. Conjugation of the chromophore with either an amine or cyclo-dopa substituent is believed to occur spontaneously in vivo under conditions of acid pH, as found in the plant cell vacuole (Renaudin and Guern, 1982). The stoichiometry of the pathway requires two molecules of tyrosine for the biosynthesis of one molecule of betacyanin. Biosynthesis of the chromophore, betalamic acid, from dopa was shown recently to proceed via the intermediate, seco-dopa (Terradas and Wyler. 1991).

In the past we have used three model systems to study various aspects of the biochemistry, enzymology, genetics, molecular genetics, and biotechnology of betalain biosynthesis. *Portulaca grandiflora* Hook (large-flowered purslane) has been used for numerous genetic studies of the betalain pathway and has played a major role in the elucidation of the number of genes involved in the regulation of betaxanthin and betacyanin biosynthesis (Trezzini, 1990). The red capped, fly agaric mushroom *(Amanita muscaria)* has been the preferred tissue for studies pertaining to the enzymology of the betalain pathway, this tissue providing the most stable form of the enzyme, dopa 4,5-dioxygenase (Girod and Zryd, 1991), which catalyses the biosynthesis of the chromophore (Fig. 1) from dopa. Cell lines developed from *Beta vulgaris* L. var. bikores monogerm. which exhibit a variety of colored phenotypes. have been used as visual markers of cellular differentiation during studies of the regulation of betaxanthin and betacyanin biosynthesis (Girod and Zryd. 1987; Girod and Zryd. 1991).

Betalains and the food industry. These same *B. vulgaris* cell lines have also stimulated interest from the biotechnology section of the food industry because of their high endogenous levels of betalain pigments (Schwitzguébel et al.. 1991). Tightening government restrictions and bans on many synthetic food colorants have pushed the food industry to seek alternative pigment sources, with particular interest in naturally occurring compounds such as the betalains and the anthocyanins. Growing concerns over the adverse health effects of certain synthetic color additives such as tartrazine (El02). which has been associated with a certain brain disorder, various forms of cancer, and has been shown to cause hyperactivity in chil-

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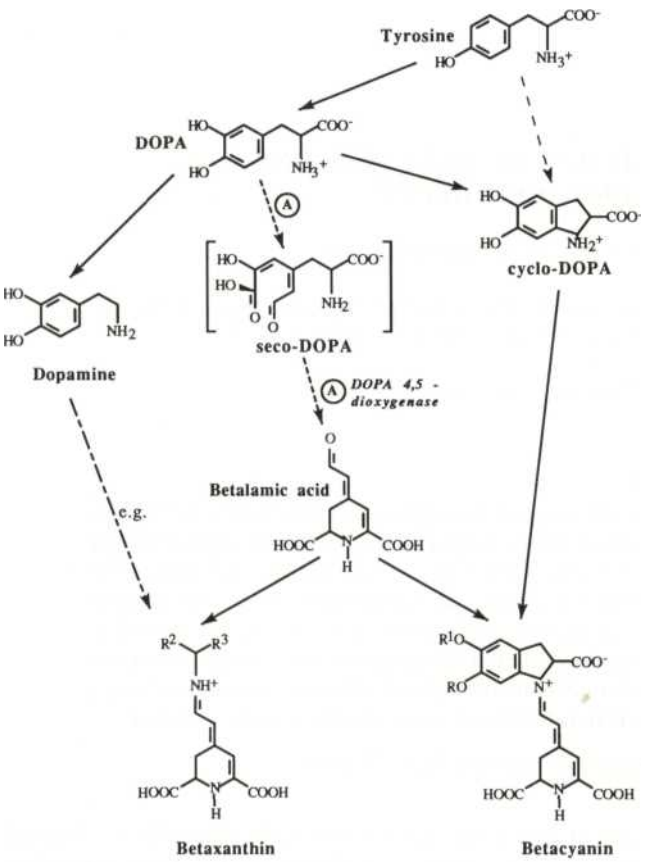

FIG. 1. Structurally, the betalains are composed of a chromophore (betalamic acid), conjugated via an imino linkage to either an amino acid or other amine (e.g., dopamine) to give the betaxanthins, or to a cyclo-dopa residue (which may be glycosylated at position R or R^1) giving the betacyanins. Conjugation of betalamic acid with glutamine, dopa, or dopamine gives rise to the betaxanthins, vulgaxanthin I, dopaxanthin, and miraxanthin V, respectively. Betanin (Rl = glucose) and betanidin (Rl = H) are two commonly occurring betacyanins.

dren, require that foods be formulated with more natural ingredients (Spears, 1988). The use of tartrazine in foodstuffs has now been banned by several European nations and American health agencies. A few nations, such as Norway, have gone one step further and banned the use of synthetic food colorants altogether.

So why do we need food colorants? The use of colorings as additives in foods and drinks is a significant factor to food manufacturers and consumers alike in determining the acceptability of processed foods (Spears, 1988). It is known that color plays a major psychologic role in determining both our choice of food and our idea of food quality and has a recognized effect on our perception of food flavor and aroma (Timberlake and Henry, 1986). "For the manufacturer, added colorings assure batch to batch uniformity and help reinforce colors already present which are less intense than the consumer would expect". "For the consumer, added colorings help to restore the natural color of foods lost during processing, while helping to provide appealing and readily identifiable products" (Spears, 1988). The betalains represent a safe, natural alternative to some synthetic color additives in current use, there is no upper limit to the recommended daily intake and they are readily available (elongated cells) could undergo the transformation to the red phenotype (spherical cells). The rare occurrence and in the form of red beet extracts. Other betalain properties include; a) high solubility under aqueous conditions, b) high molar extinction coefficient, and c) stability under conditions of low pH and temperature. The principle disadvantage of these molecules is their instability at high temperature and alkaline pH. Red beet extracts (E162), which contain significant amounts of the betacyanins, are currently used in products such as yogurts and ice creams.

FACTORS AFFECTING BETALAIN BIOSYNTHESIS IN CULTURED TISSUES

A variety of physical and chemical factors have been implicated in the regulation of pigment biosynthesis in betalain accumulating plants. Those believed to be the most important are discussed below with particular reference, where possible, to cell cultures of *B. vulgaris* L.

Growth regulators. Cell lines developed from *Beta vulgaris* L. var. Bikores monogerm exhibited cell colors ranging from white/ green (nonpigmented) through to yellow, orange, red, and violet (Girod and Zryd, 1991). The basic inherited cell phenotypes exhibited colors representative of all betalain pigments found in the whole plant. Development of the different colored cell lines from whole plant tissues was dependent on the use of specific callus induction sequences, but once established the individual phenotypes could be perpetuated on a maintenance medium (Girod and Zryd, 1991). An important factor in establishing and stabilizing these cell lines was the growth regulator composition of the medium, particularly the ratio of auxin 2,4-dichlorophenoxy acetic acid (2,4-D) to cytokinin (6-BAP). Interestingly, all colored phenotypes could be classified into two groups according to their cellular morphology. White, orange, and violet callus cultures were composed of small cell aggregates and many elongated cells, up to 200+ μm in length. The green, yellow and red phenotypes were characterized by the presence of clusters of small spherical cells. Under maintenance conditions, phenotypic stability of these cell lines was high. In the event of a phenotypic conversion, the direction of the transformation was dependant on the initial state of differentiation. Only a limited number of spontaneous phenotypic transformations were possible; those proceeding in one direction only (e.g., white to green or green to red); those proceeding more frequently in one direction than the other (e.g., yellow to red or orange to violet); and those which occurred only rarely (e.g., violet to red or orange to yellow). Changes in the growth regulator composition of the culture medium could be used to modulate the direction and frequency of the interconversion events, resulting in chimeric phenotypes. For example, red sectors appeared on yellow calli transferred to media containing a reduced 2,4-D concentration. The rapidity of this response (one to two cell generations) indicated that the hormone-induced phenotypic transformation was associated with the replication of cellular DNA and was therefore likely to be a function of the cell proliferation process (Girod and Zryd, 1991). Hormone-induced and spontaneous phenotypic transformations were generally limited to interconversions between those colored phenotypes having the same morphology. However, occasional transdifferentiation events were also observed in which a spontaneous or a hormone-induced transformation would result in a change of both the cell's phenotype and morphology. For example, the orange cell phenotype (elongated cells) could undergo the transformation to the yellow phenotype (spherical cells), while the violet cell line

long periods (several weeks) required to invoke such transformation events was indicative of a process of redifferentiation.

Light. Light has also been shown to be an important regulator of betacyanin formation in betalain-producing cell cultures and whole plants from a number of species. Intensively pigmented cell cultures of *B. vulgaris* (Girod and Zryd, 1987) and *P. grandiflora* (Böhm et al., 1991; Kishima et al., 1991) became colorless when maintained in the dark. *Chenopodium rubrum* callus cultures accumulated higher levels of betacyanins in the dark than in the light, but when maintained as liquid cultures in the dark betacyanin levels became markedly reduced when compared with the light grown controls (Berlin et al., 1986). Dark grown *C. rubrum* cell suspensions continued to accumulate low levels of betacyanins for several, months. However, when returned to the light, dark-grown cultures (resumed betacyanin accumulation, within two to three growth cycles, at levels found in the light-maintained controls. The accumulation of betacyanins in dark-grown cultures indicates that light is not a prerequisite for betacyanin formation in *C. rubrum,* but is rather a powerful stimulant of betacyanin biosynthesis. In *Amaranthus tricolor* seedlings, betacyanin synthesis may be stimulated either by exposure to light or by treatment with cytokinins (Colomas, 1975; Stobart and Kinsman, 1977; Piattelli, 1981; Bianco-Colomas, 1986). The synergistic effect of light and kinetin on betacyanin production in *A. tricolor* seedlings was also observed in cultured tissues from the same species (Bianco-Colomas and Hugues, 1990). High betacyanin (amaranthin + isoamaranthin) yielding callus lines derived from *A. tricolor* seedlings could not be maintained in the dark for longer than one growth cycle (Bianco-Colomas and Hugues, 1990). During this period, betacyanin levels became greatly reduced (0.53 µmole•g dry weight $(DW)^{-1}$). The application of either kinetin (1.2 *µM)* or fluorescent light (10 W • m^{-2}) to dark-grown cultures resulted in an increased betacyanin biosynthesis, 7.82 and 11.35 µmole • g DW^{-1}, respectively. When light and kinetin treatments were applied simultaneously, the total betacyanin accumulation was greater than the sum of the two individual treatments (28.80 µmole•g DW^{-1}). When the above light and kinetin treatments were repeated in the presence of 5,6-dichloro-l-b-D-ribofuranosylbenzimidazole (DRB; a specific inhibitor of the physiologic responses to cytokinin), a pronounced inhibition of the kinetin-dependent betacyanin synthesis was observed (45% inhibition at 40 *µM* DRB), whereas the light-induced synthesis remained unaffected by the presence of DRB (Bianco-Colomas et al., 1988). This result led the authors to suggest a possible independent action of the two inducing factors on betacyanin biosynthesis. The mechanism by which cytokinin stimulates betalain biosynthesis is not fully understood. One hypothesis states that cytokinin may regulate the availability of tyrosine to the pathway, probably by inducing the synthesis or activation of tyrosine- and dopa-oxidases (Stobart and Kinsman, 1977). Other workers have rejected this hypothesis on the basis of data obtained from radiotracer and enzymology studies, where no such correlation between cytokinin and enzyme activity or precursor levels could be found (Elliott, 1983). In terms of culture growth, the effect of light seems to be species, and even variety, dependent. Light- and dark-grown cultures of *C. rubrum* exhibited similar growth profiles (Berlin et al., 1986), whereas *A. tricolor* cultures could not be maintained for longer than one growth cycle in the dark (Bianco-Colomas and Hugues, 1990). In *P. grandiflora* calli, betalain biosynthesis and accumulation are light dependent (Böhm et al., 1991; Kishima et al., 1991). However, in terms of culture growth, reports indicate that some *P. grandiflora* calli will grow in either light or dark (Kishima et al., 1991) whereas other callus lines will not grow in the dark (Böhm et al., 1991). This of course raises the question as to whether light is an absolute requirement for betalain biosynthesis in dark-grown cultures that exhibit no growth. In our own work with *B. vulgaris,* the growth of high betacyanin-yielding cell suspension cultures became reduced with repeated subculture when maintained in the dark and cultures became bleached within two cycles. This result indicates that light is a prerequisite for betalain formation in cultured *B. vulgaris* cells.

The effects of light quality and quantity on betalain biosynthesis have been studied to a limited extent and are not fully understood. Exposure of *A. tricolor* and *C. plumosa* seedlings to either white or red light resulted in an increased production of betacyanin and betaxanthin pigments (Giudici de Nicola et al., 1974). When short light-exposure times were used (4 h for white light or 15 min for red light), the stimulating response on betacyanin biosynthesis could be inhibited by brief (5 min) terminal exposure to far-red (FR), indicating the operation of a phytochrome-dependent system. Prolonged exposure to white light also resulted in both an increase in cellular chlorophyll and betalain accumulation, but was not reversed by brief FR treatment. Administration of levulonic acid (an inhibitor of chlorophyll synthesis) to seedlings during extended white light treatments resulted in an inhibition of chlorophyll and betalain biosynthesis, indicating that the photosynthetic system may play an important role in the regulation of betalain biosynthesis. Interestingly, continuous exposure to FR also resulted in an increased synthesis of betalain pigments and chlorophyll. FR-induced pigment biosynthesis was strongly reduced by 2,4-dinitrophenol (DNP; an inhibitor of cyclic photophosphorylation) and levulonic acid, but remained unaffected by 3-(3,4-dichlorophenyl)-l,l-dimethylurea (DCMU; an inhibitor of noncyclic photophosphorylation). This result suggests that the continuous FR stimulus is perceived by the photosynthetic system acting only through cyclic photophosphorylation. Because residual betalain synthesis occurred when chlorophyll formation had been completely suppressed, indicates that the photosynthetic system was not the only active photoreceptor under conditions of continuous FR irradiation. A short (15 min) terminal exposure to red light during a 24-h continuous FR exposure resulted in a stimulation of betalain biosynthesis which could be reversed by subsequent brief exposure to FR. This result demonstrates the involvement of phytochrome in the continuous FR-mediated induction of betalain biosynthesis. *C. rubrum* cell suspension cultures grown under blue light (16 h photoperiod) exhibited a 30% increase in culture betacyanin content when compared with the white light grown controls (Berlin et al., 1986). Blue and red light treatments were both shown to stimulate betacyanin biosynthesis in *A. caudatus* seedlings (Obrenovic, 1990). The optimal blue light effect was attained after an exposure of just 10 min. The effect of a terminal FR exposure (5 min) on betacyanin biosynthesis in red-light-treated seedlings was dependent on the duration of the red light treatment, changing from a stimulatory effect with brief red light treatments of up to 5 s, becoming inhibitory with increasing red light exposure times. A terminal FR pulse (5 min) after exposure to blue light (5 min) did not result in a significant inhibition of betacyanin biosynthesis. Unfortunately, the majority of reports pertaining to the effects of light on betalain biosynthesis give very few or no precise details about the light quality, intensity, or fluence used during experiments.

In the Laboratoire de Phytogénétique Cellulaire (LPC), red beet

cell lines are maintained under a 16/8 h light/dark photoperiod. Illumination is from Sylvania high intensity, daylight fluorescent tubes giving a fluence of 55.0 μmol • m^{-2} • s^{-1} for callus cultures (red_{630}/far-red_{73O} = (7.71/3.72 μmol • m^{-2} • s^{-1}) = 1.69; PAR = 52.7 μmol • m^{-2} •s^{-1}). For cell suspension cultures, a higher fluence of 81.5 μmol•m^{-2}•s^{-1} was used (red_{630}/far-red_{730} = (11.05/5.23 μmol•m^{-2}•s^{-1}) = 1.99; PAR = 71.6 μmol • m^{-2}•s^{-1}). The daylight fluorescent tubes give important emission bands at red and blue spectral wavelengths. These conditions have been found to give good culture growth rates and high betalain yields, but the optimal lighting conditions (photoperiod, light intensity, fluence, and wavelength) for these cultures have not yet been determined. It is not yet known whether the different colored callus phenotypes exhibit differences in their requirement for light.

Other factors. Other macro factors such as the medium nitrogen supply and phosphate concentration have been shown to greatly influence the production of secondary metabolites in cultured plant tissues (Knobloch and Berlin, 1981) and may also play a role in the regulation of betalain biosynthesis. Increasing the nitrate and ammonium concentration to levels above those of the original B5 or MS media resulted in increased culture growth rates, but no increase in cellular betacyanin content was observed in either *B. vulgaris* (Constabel and Nassif-Makki, 1971) or *C. rubrum* cultures (Berlin et al., 1986). However, replacement of the ammonium content of MS medium by nitrate resulted in a marked increase in cellular betacyanin content in suspension cultures of *P. grandiflora* (Böhm and Rink, 1988). In *A. tricolor* cell suspensions, maximal betacyanin accumulation occurred at nitrogen levels 10-fold higher than that required for optimal culture growth (Bianco-Colomas and Hugues, 1990). In our own work, the nitrogen supply (concentration and salt) played a role in the derivation and stabilization of *B. vulgaris* L. cell lines exhibiting the different colored phenotypes (Girod and Zryd, 1991). Green, yellow, orange, and red phenotypes were maintained on media containing $KN0_3$ (1.9 g-$liter^{-1}$) + NH_4N0_3 (1.65 g• $liter^{-1}$), whereas media for the violet phenotype contained double the KNO_3 content. Generally, phosphate levels had a marked effect on culture growth, but affected betalain accumulation in a species-dependent manner. In A. *tricolor* cell suspensions, phosphate supply had no apparent effect on pigment accumulation (Bianco-Colomas and Hugues, 1990). In cultures of *C. rubrum* (Berlin et al., 1986) and *Phytolacca americana* (Sakuta et al., 1986) cellular betacyanin levels increased with increasing phosphate concentration to a maximum at 1.25 *mM* phosphate, whereas elimination of phosphate from the culture medium resulted in an increased accumulation of betacyanins in *B. vulgaris* L. (Constabel and Nassif-Makki, 1971). Initial medium carbon concentration may also affect betalain biosynthesis. Maximum cellular betacyanin levels were obtained at 2% sucrose in *C. rubrum* cell suspension cultures, and maximum biomass levels were obtained at 6% sucrose (Berlin et al., 1986). In cell suspensions of A. *tricolor*, maximal amaranthin accumulation occurred at sucrose concentrations 2 to 3 times lower than those permitting maximal growth (Bianco-Colomas and Hugues, 1990; the actual sucrose levels required for maximal growth and betalain production were not stated by the author). The sucrose concentration of the culture medium also played a role in the derivation and stabilization of *B. vulgaris* L. cell lines exhibiting different colored phenotypes (Girod and Zryd, 1991). Green, orange, red, and violet phenotypes were maintained on media containing sucrose at 30 g • $liter^{-1}$, whereas media for the violet phenotype contained only 10 g • $liter^{-1}$. Other micro constituents of the culture media may also affect betalain biosynthesis. Elements such as copper are considered to be cofactors rather than nutrients (Endress, 1976) and have been shown to have different effects in different culture systems. Increasing the cupric (Cu^{2+}) ion concentration of the culture medium inhibited betacyanin accumulation in *P. grandiflora* calli (Endress, 1976). Monovalent, divalent, and free copper ions were examined for their effects on phytochrome-mediated betacyanin biosynthesis in A. *caudatus* seedlings (Obrenovic, 1990). Induction of betacyanin biosynthesis by brief exposure to far-red, red, and blue light and prolonged exposure to red and blue light was inhibited, to different extents, by the presence of a divalent copper chelate (Cu (II) D-penicillamine). The inhibition of light-induced betacyanin biosynthesis by the divalent copper chelate could be reversed by EDTA. These results suggest that copper chelates may act as superoxide scavengers in vivo and may also affect phytochrome phototransduction (Obrenovic, 1990).

Growth and Betalain Accumulation in *B. vulgaris* L. Cell Suspension Cultures Exhibiting the Orange (BvO) and the Violet (BvV) Phenotype

Of the five differently colored *B. vulgaris* L. callus phenotypes, two (the orange and violet, referred to as cell lines BvO and BvV, respectively) have been maintained as cell suspensions in liquid culture media. The characteristics of culture growth and betalain accumulation for these two cell lines maintained in a shake flask culture system are described below.

Orange phenotype. Cell suspension cultures were readily initiated from friable *B. vulgaris* calli exhibiting the orange phenotype (Girod and Zryd, 1991). The liquid growth medium was the same as the maintenance media used for the orange callus line (Girod and Zryd, 1991) except that no agar was included in the formulation. Empirical modification of the inoculum density and the frequency of cell transfer resulted in a subculture protocol that yielded suspension cultures of highly reproducible growth performance. Cell suspensions were maintained as 130-ml cultures in 300-ml conical shake flasks at 26° C on an orbital shaker (2.5-cm stroke diameter) operated at 130 rpm. Suspension cultures were subcultured by volume during the mid-exponential phase of growth, every 5 days at a ratio of 1:4.3. The newly initiated cultures were of consistent inoculum density (approximately 2.0 g•DW•$liter^{-1}$), both within and between subcultures, and exhibited a lag phase duration of 0 to 1 day upon subculturing. Culture maintenance was described as semicontinuous. Typical growth and betalain accumulation profiles are shown in Fig. 2. Specific growth rates were typically of the order 0.23 to 0.20 $days^{-1}$, corresponding to doubling times of 3.0 to 3.5 days, respectively. Culture growth was carbon limited after a culture period of 9 to 11 days and yielded maximal biomass levels of approximately 16 to 17 g • DW • $liter^{-1}$ with 3% sucrose as the carbon source. The orange pigmentation of this cell line was due to the accumulation of both betaxanthins and betacyanins within the cell vacuole. The profile of total cellular betaxanthin accumulation was particularly interesting because of its apparent biphasicness (Fig. 2 a). During the first 2 days of culture there was a decline in the total cellular betalain content, returning to the inoculum levels within 5 days (the day of subculture). A similar cyclic evolution of betacyanin accumulation was observed in *C. rubrum* cell suspensions (Berlin et

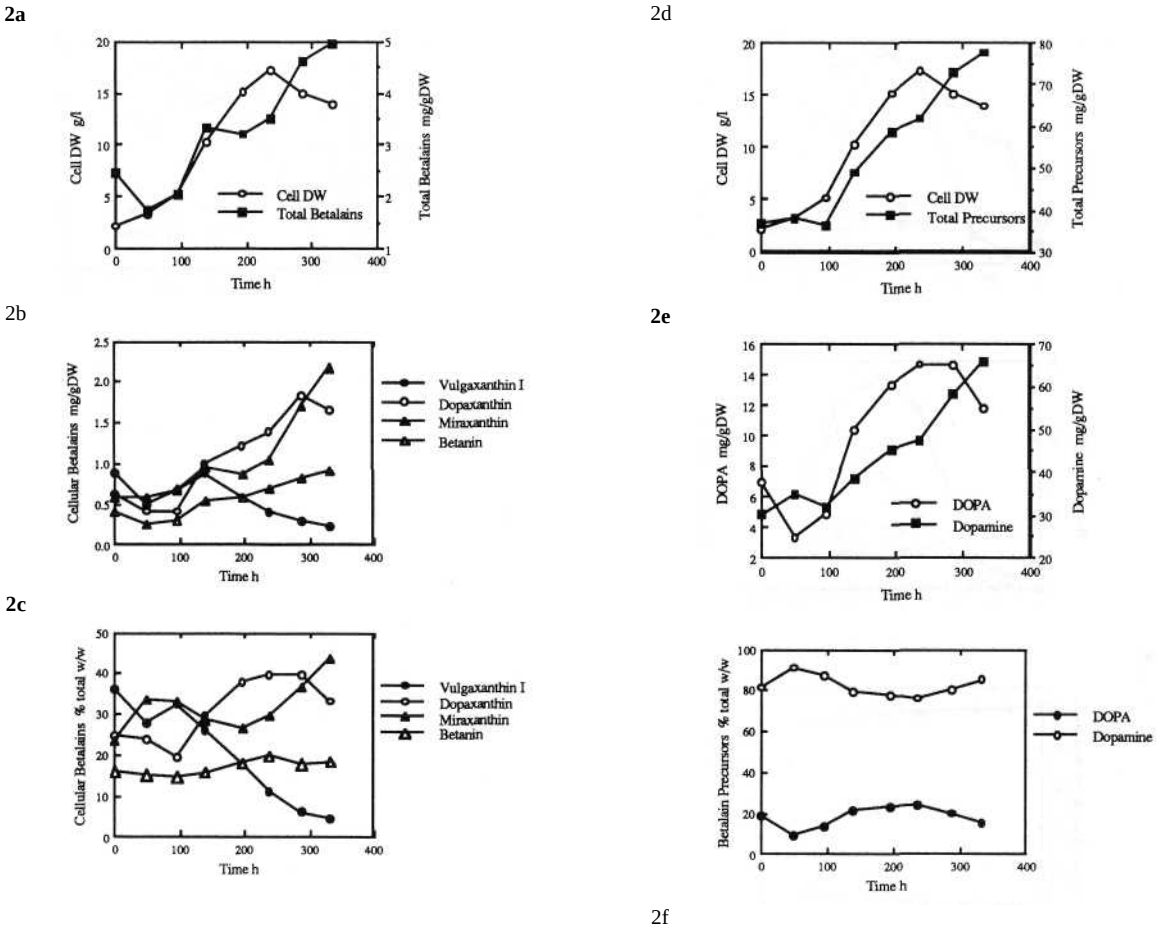

FIG. 2. Typical biomass (a) and betalain accumulation *(b)* profiles for *Beta vulgaris* L. cell suspensions (orange phenotype; BvO). Accumulation of the betalain precursors, dopa and dopamine, are also presented *(d-f);* 130-ml cultures were maintained in 300-ml shake flasks as described in the text.

al., 1986) and was attributed to a possible dilution of betalain-producing cells by newly synthesized cells lacking betacyanins. The apparent reduction in cellular betacyanin content could also have resulted from a rapid uptake of macro and micro elements from the fresh growth medium, giving an apparent increase biomass density, during the first few days of culture. Inasmuch as cellular pigment contents are generally expressed on a wt/wt basis, an increase in cell DW would result in an artificial reduction in cellular betacyanin content. The true nature of this observation can only be determined by expressing the data in terms of mass of betalain per 10^6 cells. A third mechanism, which cannot be discounted, is that of betalain degradation. In BvO cell suspensions, the initial decline in cellular betalain levels was followed by a progressive accumulation, from Day 2 onward, of vulgaxanthin I, miraxanthin V, and betanin (the betaxanthins, vulgaxanthin I, dopaxanthin, and miraxanthin V are the glutamine, DOPA and dopamine conjugates of betalamic acid, respectively). Dopaxanthin accumulation commenced only 2 days later on Day 4. The peak in betalain accumulation on Day 6 was followed by a rapid decline in cellular vulgaxanthin I levels. The accumulation of dopaxanthin, miraxanthin V, and betanin exhibited mixed growth-associated kinetics, closely following the increase in culture biomass, but then continuing, at a markedly higher rate, during the stationary phase (Fig. 2 *b).* After Day 6 of culture the hitherto stable betaxanthin composition of the total cellular betalains (Fig. 2 *c)* underwent significant changes in accordance with the increased rates of dopaxanthin and miraxanthin V accumulation, coupled with the decline in cellular vulgaxanthin I levels. The observed shift in betaxanthin composition may have resulted from changes in cellular amino acid metabolism during the course of the growth cycle. The proportion of betacyanin (betanin) in total cellular betalain extracts remained stable throughout the entire culture period (Fig. 2 *c).*

Cultures attained maximum cellular betalain levels of 5 to 10 mg• g •DW^{-1} within 14 days, representing productivities of 0.5 to 1.0 mg betaxanthin • $liter^{-1}$ • day^{-1}. Interestingly, the orange cell line also accumulated high cellular levels of the betalain precursors, DOPA and dopamine (Fig. 2 *a,b),* attaining maximum values of 15 and 66 mg•g DW^{-1}, respectively. The accumulation of cellular DOPA and dopamine closely paralleled increases in culture biomass, with that of dopamine continuing into the stationary phase. The composition of accumulated cellular precursors remained constant throughout the growth cycle (Fig. 2*f*).

Culture volumes for cell suspensions of the orange phenotype have been successfully scaled-up into 21 shake flasks and subsequently into a 13-liter air-lift bioreactor (Chemap AG, Switzerland). Antifoam was a necessity during mass cultivations because of the

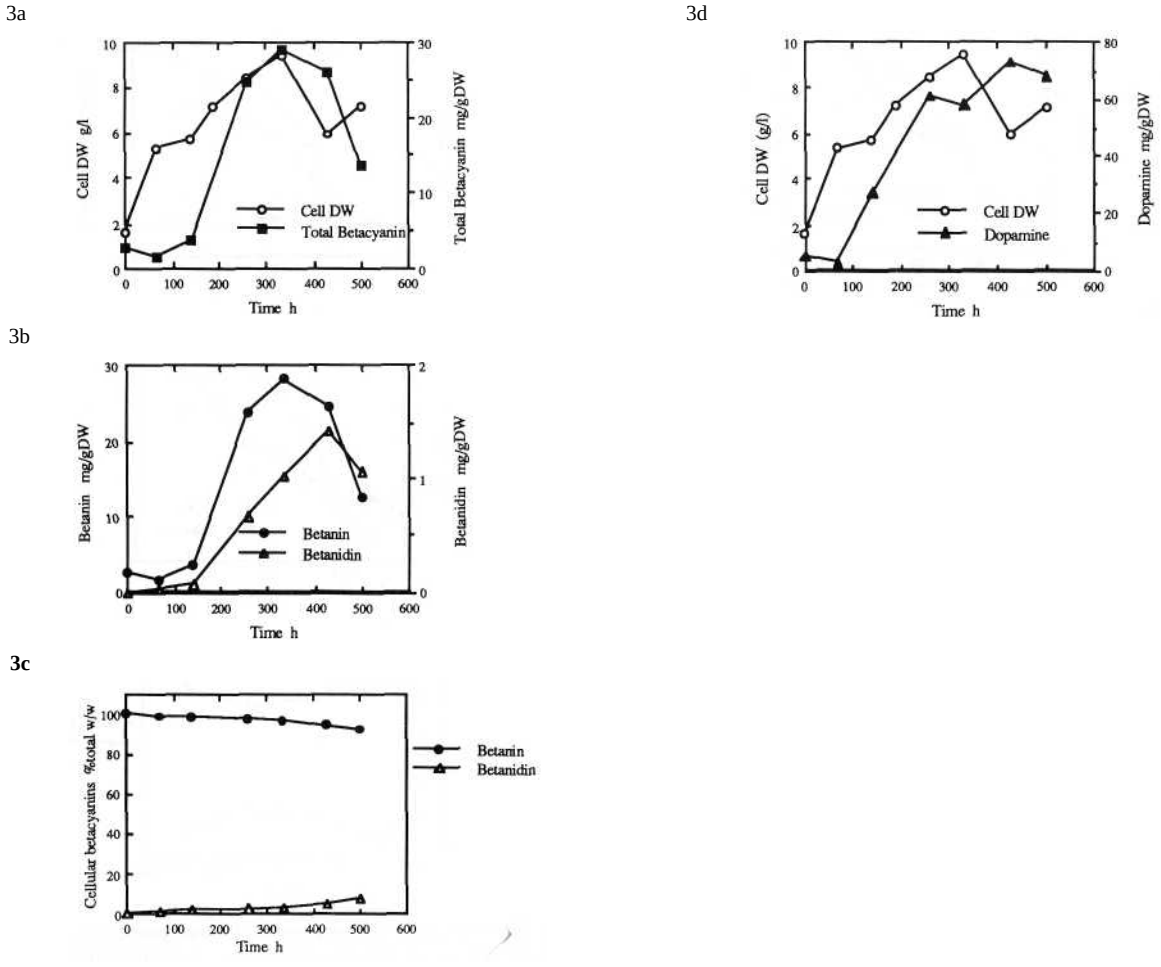

FIG. 3. Typical biomass (a) and betalain accumulation *(b)* profiles for *Beta vulgaris* L. cell suspensions (violet phenotype; BvV). Accumulation of the betalain precursor, dopamine is also presented *(d);* 130-ml cultures were maintained in 300-ml shake flasks as described in the text.

highly foaming nature of these cultures, further compounded by the air-lift bioreactor configuration. Silicon-based antifoams were found to be the most suitable because of their compatibility with culture growth, cell viability, and betalain accumulation. Operating conditions and cellular requirements for optimal culture growth and betalain productivity during mass cultivation processes are currently being determined.

Violet phenotype. Cell suspensions initiated from the violet callus phenotype continued to accumulate high levels of betacyanin. The liquid growth medium was the same as the maintenance media used for the violet callus line (Girod and Zryd, 1991) except that no agar was included in the formulation. Cultures were maintained as described for the orange phenotype, except that a 7-day subculture routine was used because of the lower culture growth rates. Typical growth and betacyanin accumulation profiles are shown in Fig. 3 *a,b.* Culture growth was characterized by specific growth rates of 0.20 to 0.13 $days^{-1}$, representing doubling times of 3.4 to 5.2 days, respectively. Culture growth was carbon-limited after 11 to 14 days, yielding maximum biomass levels of 8 to 12 g • DW • $liter^{-1}$ with 3% sucrose as the carbon source. Cellular betalains were characterized by the presence of two betacyanins, betanin and betanidin, of which betanin accounted for at least 90% of the total (Fig. 3 c). Depending on the particular cell line, cellular betacyanins attained maximal levels ranging 9 to 28 mg • g • DW^{-1}, representing productivities of 6 to 18 mg•$liter^{-1}$ • day^{-1}. Dopamine, a betalain precursor, was also accumulated in the violet cell lines, attaining maximum cellular levels of 70 to 80 mg • g^{-1} •DW^{-1} (Fig. 3d).

Betalain production using culture Beta vulgaris *L. cell suspensions.* Under the experimental conditions described here, the cellular levels of total betacyanin (wt/wt) in *B. vulgaris* L. suspension cultures (BvV) were markedly higher than those found in the whole plant (Girod and Zryd, 1991). High betacyanin levels coupled with high culture growth rates represent large increases in betacyanin productivity in the in vitro system over that of the whole plant. In addition to these interesting production characteristics, the in vitro production system offers several distinct advantages to the commercial scale producer of betalains over the conventional techniques of beet cropping and extraction:

- Rapid culture growth permits numerous fermentations in the time required to raise one crop of red beet.
- Elimination of seasonal cropping Production as and when re quired.
- Carbon-limited culture growth eases downstream processing

by eliminating the requirement for secondary fermentations, which are otherwise used in conventional betalain extraction protocols to reduce the beet sugar and nitrate levels, and as a means to eliminate the characteristic beetroot odor (geosmin) from the final product.

- No geosmin formation.
- Production of either yellow or violet betalain pigments.
- Large world markets for natural colorants.
- Chemical synthesis is still not cost effective because of low product yields.

In conclusion, despite the numerous advantages offered by the in vitro betacyanin production system, the low market price of red-beet extracts obtained by traditional cropping and extraction methods is likely to render a high cost, biotechnological process economically uncompetitive unless the betacyanin content of cultures can be further increased. Further improvements in betacyanin productivity will most likely arise from a) further optimization of culture conditions; b) increasing the biomass density of the suspension cultures; or c) by redirecting accumulated precursors into betalain biosynthesis. High betalain-producing callus lines have been visually selected for their intensity of pigmentation every 7 to 10 days at subculture for the past 6 yr. It is unlikely that selection will further improve the quality of existing cell lines unless techniques permitting a more efficient selection, such as laser cell sorting or image analysis, can be employed. In view of the economics of producing red-beet extracts by traditional means, a biotechnological process is most likely to succeed with cell lines capable of producing high levels of those pigments that are only accumulated at low levels in the whole plant. A good example of this would be the in vitro production of beta-xanthins using *B. vulgaris* L. cell suspensions (yellow or orange phenotype) because these pigments are only accumulated at nominal levels in the whole plant and there is no known naturally occurring, abundant supply. The betaxanthins, which constitute a minor part of red-beet extracts, could be used as yellow food colorants in their own right. Through application of relatively simple techniques of semisynthesis (Trezzini and Zryd, 1991), it could be envisaged that the yellow betaxanthins (betalamic acid/amino acid conjugates; Fig. 1), in addition to their potential role as food colorants, be used as a means of introducing essential dietary amino acids into foodstuffs, giving rise to the "essential dietary colorant".

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