Batch and Fed-Batch Production of Betalains by Red Beet (*Beta vulgaris*) Hairy Roots in a Bubble Column Reactor◊

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Hairy root cultures from red beet (*Beta vulgaris* L.), which could be used for the commercial production of biologically active betalain pigments, were cultivated in a 3 L bubble column bioreactor in batch mode with various rates of air supply. Both the growth of the roots and betalain volumetric yields were highest (12.7 g accumulated dry biomass/L and 330.5 mg/L, respectively) with a 10 L/h (0.083 vvm) air supply. The air flow rate also influenced the betacyanins/betaxanthins ratios in the cultures. Growth and betalains production were then examined in two fed-batch regimes (with a 10 L/h air supply), in which nutrient medium was fed just once or on five occasions, designated FBI and FBII, respectively. The root mass accumulation was increased in the FBI feeding regime (to 13.3 g accumulated dry biomass/L), while in FBII the betalains content was ca. 11% higher (15.1 mg betacyanins/g dry weight and 14.0 mg betaxanthins/g dry weight) than in the most productive batch regime. Data on the time course of the utilization of major components in the medium during both operational modes were also collected. The implications of the information acquired are discussed, and the performance of the hairy roots (in terms of both growth and betalains production) in the bubble column reactor and previously investigated cultivation systems is compared.

Key words: Betalains, *Beta vulgaris*, Bubble Column Bioreactor, Hairy Roots

Introduction

Betalains are water-soluble, nitrogen-containing pigments that include red-violet betacyanins and yellow betaxanthins, which are considered to be immonium conjugates of betalamic acid with cyclo-dopa and amino acids (or amines), respectively (Strack *et al.*, 2003). In recent years there has been an increasing tendency to use plant-derived colorants in food systems, since use of many synthetic dyes (especially red and yellow ones) has been restricted, or even banned, by the US Food and Drug Administration and corresponding authorities in other parts of the world, because of their potential risks for consumers (Jimenes-Aparicio and Gutierrez-Lopez, 1999). Betalains are effective scavengers of reactive oxygen species (Pavlov *et al.*, 2002), and thus help prevent oxidative stress-related disorders (Kanner *et al.*, 2001). Red beet (*Beta vulgaris*) betalains have also been found recently to provide protection against lung and skin cancer (Kapadia *et al.*, 2003). Their utility as colorants, safety (beetroot extract E162 is approved by EU legislation) and important biological activities make betalain pigments attractive targets for commercial production. However, the production and use of betalains are limited, due to their complexity, the difficulties involved in synthesizing them chemically, and their sensitivity to extreme conditions (Strack *et al.*, 1993).

In vitro hairy root cultures, obtained through genetic transformation of red beet with *Agrobacterium rhizogenes*, have been shown to be capable of accumulating relatively high amounts of betalain pigments during their cultivation in shake flasks (Pavlov *et al.*, 2002, 2005), stirred tank reactors (Georgiev *et al.*, 2006) and RITA® temporary immersion systems (Pavlov and Bley, 2006). Al-

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though hairy root cultures have several advantages (including high biochemical and genetic stability, together with relatively fast growth in growth regulator-free media; Georgiev *et al.*, 2007) they are extremely sensitive to a number of stress factors that reduce their growth and/or biosynthetic potential. Thus, commercial exploitation of the hairy root culture metabolism requires the development of suitable bioreactor systems and operational regimes, in which key physical and chemical parameters are optimized. Bubble column reactors with appropriate bubble parameters are considered to be suitable for cultivating plant organ cultures (*e.g.* hairy roots), because they provide low stress environments, mainly due to the absence of mechanical agitation (Paek *et al.*, 2005). Furthermore, the ease of recovering root tissues from unbaffled bubble columns outweighs the advantages of more complex designs (Curtis, 2000).

The aim of the study presented here was to investigate the betalains production by transformed *Beta vulgaris* root cultures in a bubble column bioreactor operating in both batch and fed-batch modes.

Materials and Methods

Transformed root culture

The *Beta vulgaris* hairy root clone used in the experiments was established by infecting red beet (*Beta vulgaris* variety Detroit Dark Red) leaf explants with *A. rhizogenes* ATCC 15834 using the co-cultivation method described by Pavlov *et al.* (2002). The hairy roots obtained were maintained on solid MS medium (Murashige and Skoog, 1962), supplemented with 30 g/L sucrose, with three-week subcultivation intervals. Two-week-old cultures grown in 500 mL Erlenmeyer flasks (with 100 mL liquid MS medium), on a shaker (110 rpm, 26 °C), in the dark were used as inocula for the bioreactor experiments.

Bioreactor experimental scheme

Experiments were performed in a bubble column bioreactor of 3 L capacity, equipped with an ADI 1030 controller (Applikon®, Schiedam, the Netherlands). For each cultivation, the bioreactor was filled with 2 L MS medium and sterilized at 121 °C for 30 min. After cooling to 26 °C it was then inoculated with ~1.0–1.2 g (dry weight) *Beta vulgaris* hairy roots per litre medium, which were cultivated under the following operational regimes:

- batch process at three different air flow rates (5, 10 and 15 L/h);
- fed-batch process I (FBI), in which 1 L MS medium was added 13 d after the start of the cultivation;
- fed-batch process II (FBII), in which 0.2 L MS medium was added on each of five occasions (from day 13 to day 17 after the start of cultivation).

In both fed-batch regimes the air flow rate was 10 L/h.

Hairy root growth

The hairy roots were separated from the culture medium through filtration and the culture medium was analyzed using pH and conductivity meters. The roots were harvested, washed with distilled water, weighed (fresh biomass, FB) and dried (at 60 °C) for about 24 h (to constant weight) for determination of dry biomass (DB). Accumulated dry biomass and effective doubling times were calculated using the formulas (Curtis, 2000; Pavlov and Bley, 2006)

$$ADB = FDB - IDB \quad (1)$$

and

$$t_d = \ln 2 \cdot \Delta t / \ln (X_f / X_i), \quad (2)$$

where ADB is the accumulated dry biomass (in g/L), FDB the final dry biomass, IDB the initial dry biomass, t_d the doubling time (in d), Δt the total culture interval, X_f and X_i are the final and initial biomasses, respectively.

To obtain time courses of the estimated root growth during the bioreactor cultivations the following relationship between increases in biomass and reductions in medium conductivity was applied:

$$\Delta X = \Delta C \, \beta, \quad (3)$$

where ΔX is the total culture growth, ΔC the total change in conductivity and β represents the proportional coefficient, which needs to be determined.

Extraction and determination of betalains

The frozen hairy root samples were ground with sea sand using a mortar and pestle, and extracted three times with 50% (v/v) aqueous ethanol solution (solid : liquid ratio 1 : 10). The pooled extracts were then centrifuged at 10000 rpm for 10 min and

their betalain contents were determined by measuring the absorbance using a Beckmann DU520 UV/vis spectrometer, at wavelengths of 476 nm, 537 nm and 600 nm, following the method of Nilsson (1970).

Determination of sugars and inorganic salts

Sucrose, glucose and fructose levels in the culture medium were determined using an enzyme test kit (R-Pharm, Germany, Cat. No. 10716260035), while nitrate, ammonium and phosphate ions were determined using test kits supplied by Merck (Germany, Cat. Nos. 1.09713.0001, 1.00683.0001, 1.00798.0001, respectively).

Statistical analysis of the data

The data presented are averages from two independent experiments. All measurements were done in triplicate. Differences in betalains accumulation and betacyanins/betaxanthins ratios between the regimes were analyzed by One-way ANOVA and Turkey HSD tests with $\alpha = 0.05$.

Results and Discussion

The *B. vulgaris* hairy root clone was obtained more than five years ago. The roots grew with many lateral branches, and in active growth stages their tips were orange colored, while their other parts were red (Pavlov *et al.*, 2002). The growth of this hairy root clone has been shown to be stable in submerged shake flask cultivation and to be accompanied by high betalains biosynthesis capacity. The linear dependence between roots growth and conductivity of the medium was revealed at the shake flasks stage as well (Pavlov *et al.*, 2005).

Batch process

The *B. vulgaris* roots were initially cultivated in the bubble column reactor in the batch operation mode with three different flow rates of inlet air (5, 10 and 15 L/h), and their growth (which can only be monitored indirectly; Georgiev *et al.*, 2007) was followed by monitoring changes in the conductivity of the medium (currently the most commonly used method; Mukundan *et al.*, 1998; Heyon and Yoo, 2003; Pavlov and Bley, 2006) and applying equation (3). The growth curves obtained show that the cultures were sensitive to the rate of oxygen supply, although there were strong similarities between the curves obtained with the three different air supply rates (Fig. 1), all of which showed a lag phase of ~4–5 days, followed by a period of intensive growth (exponential phase) over the next ~9–12 days and then an early stationary growth phase. The maximum amounts of biomass (12.7 g/L) were accumulated with an air flow rate of 10 L/h (0.083 vvm), at which the effective doubling time was calculated to be ~4.5 days. However, it should be noted that the accumulated biomasses and doubling times obtained with the other

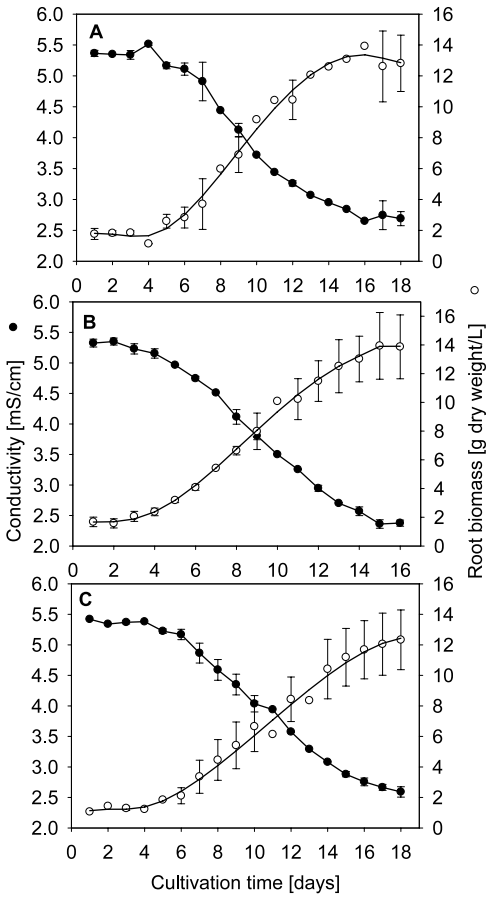

Fig. 1. Estimated time courses of growth of *B. vulgaris* hairy root cultures and conductivity changes during batch operation in the bubble column reactor (A, B and C – air flow rates of 5, 10 and 15 L/h, respectively). Error bars represent standard deviations.

Table I. Accumulated dry biomass (ADB), doubling time and biomass productivity during the cultivation of *B. vulgaris* hairy roots in the bubble column bioreactor in batch and fed-batch cultivation regimes.

	ADB [g/L]	Effective doubling time [d][a]	Productivity [g dry weight/(L d)]
Batch process:			
Air flow rate 5 L/h	11.41 ± 1.71	5.66 ± 0.16	0.63 ± 0.10
Air flow rate 10 L/h	12.71 ± 2.34	4.47 ± 0.43	0.79 ± 0.15
Air flow rate 15 L/h	11.37 ± 2.00	4.91 ± 0.39	0.63 ± 0.11
Stirred tank reactor (air flow rate 60 L/h)[b]	11.91 ± 1.25	5.11 ± 0.84	0.63 ± 0.09
Temporary immersion system (air flow rate 60 L/h)[c]	14.5	–	0.60
Fed-batch process:			
FBI, air flow rate 10 L/h[d]	13.31 ± 0.80	6.28 ± 0.12	0.61 ± 0.04
FBII, air flow rate 10 L/h[d]	11.50 ± 1.75	5.78 ± 0.23	0.57 ± 0.09

[a] Effective doubling time calculated as $t_d = \ln 2 \cdot \Delta t / \ln(X_f/X_i)$, not indicative of maximum doubling time.
[b] Data from Georgiev *et al.* (2006).
[c] Data from Pavlov and Bley (2006).
[d] FBI, fed-batch process involving feeding the culture with 1 L of medium on day 13; FBII, fed-batch process involving feeding with 0.2 L medium five times consecutively (from day 13 to day 17).

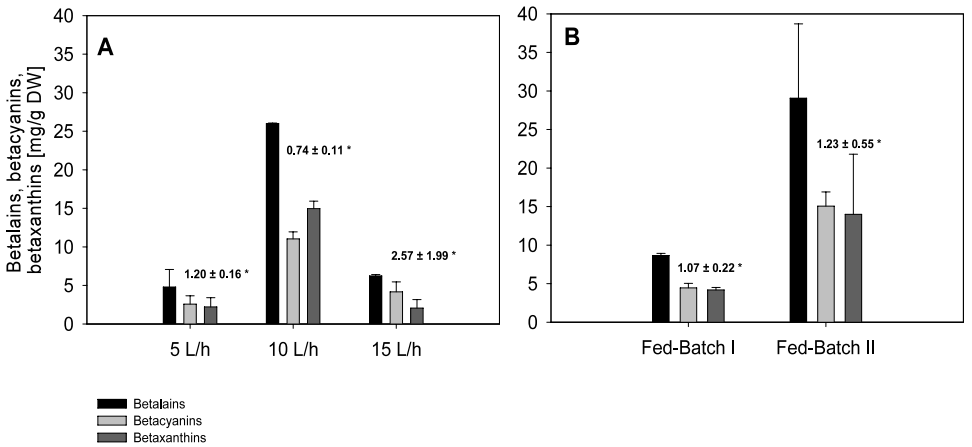

Fig. 2. Betalain, betacyanin and betaxanthin contents in the *Beta vulgaris* hairy root mass at the end of batch (A) and fed-batch (B) cultivations. The asterisked values (*) are betacyanin/betaxanthin ratios. Data obtained at different air flow rates and fed-batch operation algorithms are significantly different at $P < 0.05$. Error bars represent standard deviations.

air flow rates were not significantly lower (Table I). The calculated biomass productivity ranged between 0.63–0.79 g dry weight/(L d). Comparison of the growth parameters obtained (using the same clonal material) in this study and previously in other cultivation systems (Table I) shows that the ADB in the bubble column reactor (with the optimal air flow rate) was higher than the ADB obtained in a 5 L stirred tank reactor (Georgiev *et al.*, 2006), but ~13% lower than the ADB in RITA® temporary immersion system cultivation (Pavlov and Bley, 2006). However, overall process times in RITA® systems are longer, and thus their overall biomass productivity is lower too.

Betalains pigment production was found to be extremely sensitive to the rate of air supply, and the highest contents were also obtained at 10 L/h (26.0 mg betalains/g dry weight; Fig. 2A). The volumetric yield of the betalains at the optimal air flow rate reached 330.5 mg/L; one of the highest values reported to date. Increasing and decreasing the rate of air supply to 15 and 5 L/h, respectively, resulted in 76% and 81.5% reductions in betalain contents, clearly illustrating the importance of op-

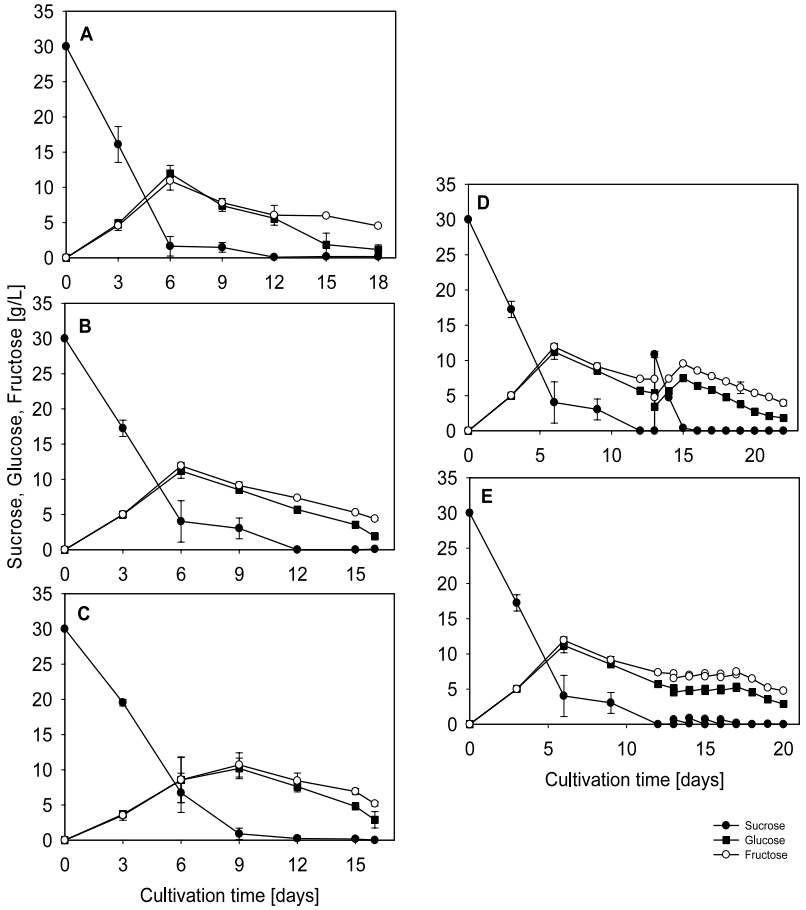

Fig. 3. Time course of carbon source utilization of *Beta vulgaris* hairy roots in batch (A, B and C – air flow rates of 5, 10 and 15 L/h, respectively) and fed-batch (D - FBI; E - FBII) culture. Error bars represent standard deviations.

timizing the air supply. Interestingly, increasing and decreasing the air flow rate also changed the betacyanins/betaxanthins ratio, in both cases in favour of betacyanins (from 0.7 to 2.6 and 1.2, respectively; Fig. 2A), which are known to have stronger antiradical activities than betaxanthin pigments (Escribano *et al.*, 1998).

To further characterize the behaviour of the hairy roots in the bubble column reactor, time courses of the utilization of the carbon source (Figs. 3A–C) and uptake of the main nutrients (Figs. 4A–C) were monitored. The sucrose consumption began with rapid hydrolysis in the culture medium, catalyzed by cell wall invertase (Shin *et al.*, 2003). The rate of sucrose hydrolysis between days 6 and 12 depended on the rate of air supply, but in all cases the sucrose in the medium was completely exhausted by day 12 (Figs. 3A–C). The products of the sucrose hydrolysis, glucose and fructose, were utilized in similar temporal patterns, and by the end of the process insignificant amounts of both monosaccharides were detected in the culture medium. Nitrogen was supplied in the growth media (as it often is) as both NH_4^+ and NO_3^-. These forms were utilized at different rates, and the ammonium was completely exhausted at the end of the process, while ~1.0–1.1 g nitrate/L remained in the media (Figs. 4A–C). This is consistent with energetic considerations (Bloom *et al.*, 1992), since nitrate anions have to be reduced to nitrite anions and then to ammonium ions via catalysis by nitrate reductase and nitrite reductase, respectively, before they can be used metabolically. The rapid uptake of the ammonia from the medium during the initial cultivation period led to a reduction in pH values (to ~4.5), which should

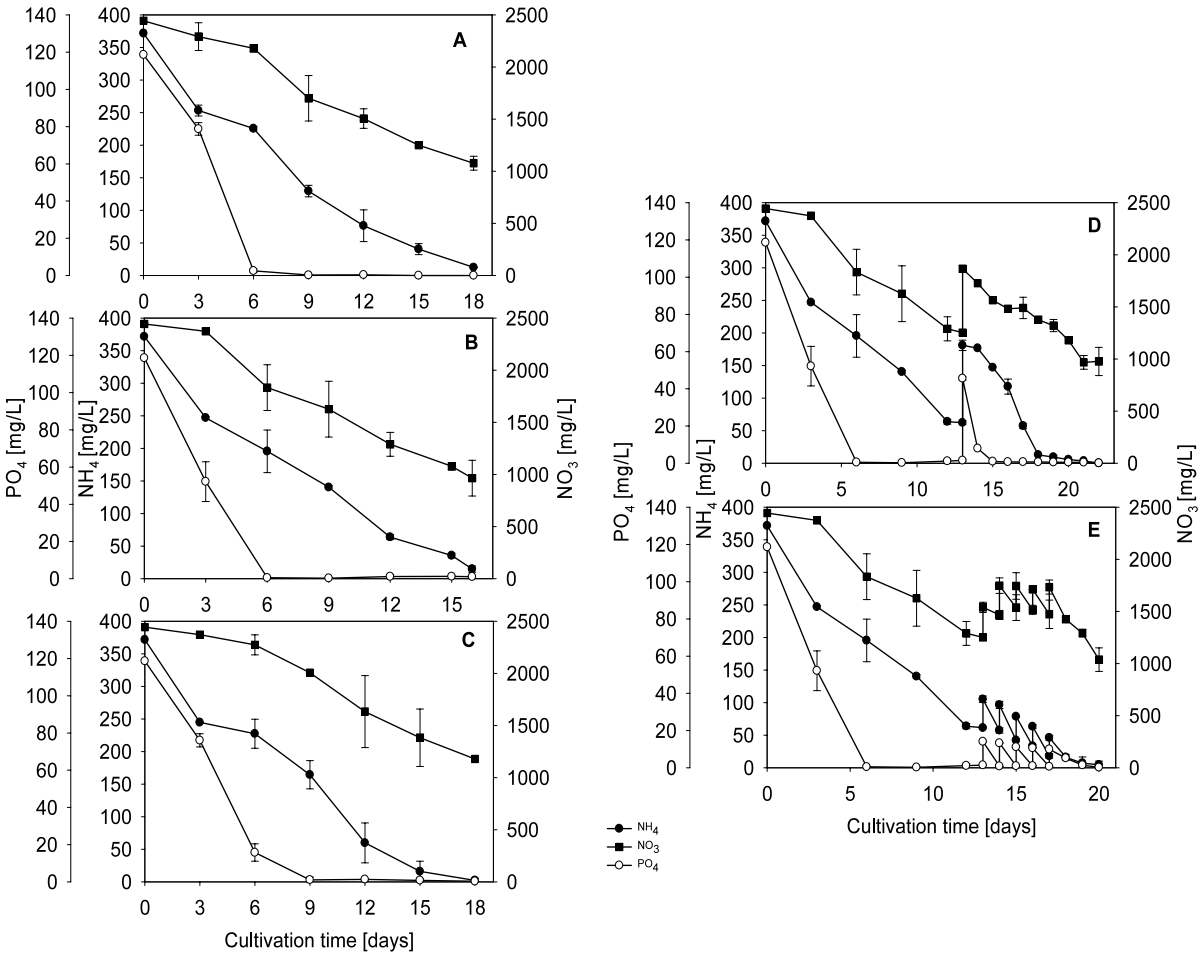

Fig. 4. Time course of nitrogen and phosphate utilization by *Beta vulgaris* hairy roots in batch (A, B and C – air flow rates of 5, 10 and 15 L/h, respectively) and fed-batch (D - FBI; E - FBII) culture. Error bars represent standard deviations.

have promoted the hydrolysis of sucrose according to Amino and Tazawa (1988). The phosphorus supplied in the media (as PO_4^{3-}) was utilized rapidly, being almost completely depleted from the medium between the 6th and 9th day (Figs. 4A–C). Similar phosphorus uptake rates have been observed in *Catharanthus roseus* hairy root cultures cultivated in stirred tank reactors (Moreno-Valenzuela *et al.*, 1999).

Fed-batch process

Some of the main problems limiting the industrial application of hairy root cultures seem to be related to difficulties involved in inoculating the cultivation systems. We postulated that a fed-batch approach could solve some of these problems, so investigated the growth and yield parameters under two fed-batch regimes using the same batch bioreactor: FBI, feeding with 1 L MS medium on day 13 after the start of the cultivation; and FBII, feeding with 0.2 L MS medium on five occasions from day 13 to day 17 after the start of the cultivation. In both of these cases the air flow was set to the rate that gave maximal yields in the batch process tests; 10 L/h. At the time of the feeding/first feeding (day 13) the sucrose in the culture medium was completely depleted. FBI yielded the highest accumulated root mass of all of the tested regimes, while FBII yielded lower masses even than the batch processes (Table I). The higher

doubling times and lower biomass productivity values of the fed-batch processes are due to the prolonged cultivation times (between 20–22 days) and probable oxygen limitation of the biomass in the centre of hairy root clumps.

Switching from batch to fed-batch mode also affected the pigment biosynthesis in the cultures; most notably in the FBII regime betalains production (29.1 mg/g dry weight) was about 11% higher than in the batch process at the same flow rate (Fig. 2B). This finding (together with the lower ADB) suggests that the nutrients in the media added in this feeding regime were used in the roots' secondary metabolism. During the fed-batch processes the betacyanins/betaxanthins ratio was also changed, in favour of the betacyanins, from 0.7 in the batch process to 1.1–1.2 in the fed-batch processes. The same phenomenon has been observed during the fed-batch cultivation of a B. vulgaris hairy root clone in a stirred tank reactor (Georgiev *et al*., 2006), although during cultivation in shake flasks the betacyanins/betaxanthins ratio has been found to remain quite constant at ~0.6 (Pavlov *et al*., 2005).

The measured physiological parameters of the *B. vulgaris* cultures in the fed-batch regimes generally showed similar temporal patterns to those observed in the batch regimes (Figs. 3 and 4). In both cases the added sucrose was completely utilized by the end of the process, and the levels of unconsumed glucose and fructose were not significantly different from those in the batch process (Figs. 3D, E). Uptake of the main nutrients also showed similar time courses; by the end of the process the ammonia and phosphorus were completely depleted, while about 1.0 g nitrate/L remained in the culture medium. The latter phenomenon is a specific physiological feature of this *B. vulgaris* hairy root clone. In contrast, other cultures of the same species (obtained by *A. rhizogenes* transformation with the A4 strain) have been found to utilize nitrate in their growth media completely (Shin *et al*., 2003). In previous shake flask cultivations with the same media, ~300 mg/L nitrate anions remained unutilized in the stationary phase (Pavlov *et al*., 2005), indicating that the nitrate content in the MS media could probably be reduced. However, this possibility should be confirmed experimentally.

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Amino S. and Tazawa M. (1988), Uptake and utilization of sugars in cultured rice cells. Plant Cell Physiol. **29**, 483–487.

Bloom A. J., Sukrapanna S. S., and Warner R. L. (1992), Root respiration associated with ammonium and nitrate adsorption and assimilation by barley. Plant Physiol. **99**, 1294–1301.

Curtis W. R. (2000), Hairy roots, bioreactor growth. In: Encyclopaedia of Cell Technology (Spier R. E., ed.). John Wiley and Sons, New York, pp. 827–841.

Escribano J., Pedreno M. A., Garcia-Carmona F., and Munoz R. (1998), Characterization of the antiradical activity of betalains from *Beta vulgaris* L. roots. Phytochem. Anal. **9**, 124–127.

Georgiev M., Pavlov A., and Bley Th. (2006), Betalains by transformed *Beta vulgaris* roots in stirred tank bioreactor: batch and fed-batch processes. In: Proceedings of the Second International Congress on Bioreactor Technology in Cell, Tissue Cultures and Biomedical Applications (Sorvari S. and Toldi O., eds.). Turku, pp. 22–28.

Georgiev M., Pavlov A., and Bley Th. (2007), Hairy root type plant *in vitro* systems as sources of bioactive substances. Appl. Microbiol. Biotechnol. **74**, 1175–1185.

Heyon K. J. I. and Yoo Y. J. E. (2003), Adaptive estimation of hairy root mass using conductometry. J. Microbiol. Biotechnol. **13**, 617–621.

Jimenes-Aparicio A. and Gutierrez-Lopez G. (1999), Production of food related colorants by culture of plant cells. The case of betalains. Adv. Exp. Med. Biol. **464**, 195–210.

Kanner J., Harel S., and Granit R. (2001), Betalains – a new class of dietary cationized antioxidants. J. Agric. Food Chem. **49**, 5178–5185.

Kapadia G., Azuine M. A., Sridhar R., Okuda Y., Tsurata A., Ichiishi E., Mukainake T., Takasaki M., Konoshima T., Nishino H., and Tokuda H. (2003), Chemoprevention of DMBA-induced UV-B promoted, NOR-1-induced TPA promoted skin cancerogenesis, and DEN-induced phenobarbital promoted liver tumors in mice by extract of beetroot. Pharmacol. Res. **47**, 141–148.

Moreno-Valenzuela O., Coello-Coello J., Loyola-Vargas V. M., and Vazquez-Flota F. (1999), Nutrient consumption and alkaloid accumulation in a hairy root line of *Catharanthus roseus*. Biotechnol. Lett. **21**, 1017–1021.

Mukundan U., Carvalho E. B., and Curtis W. R. (1998), Growth and pigment production by hairy root cul-

tures of *Beta vulgaris* L. in a bubble column reactor. Biotechnol. Lett. **20**, 469–474.
Murashige T. and Skoog F. (1962), A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473–497.
Nilsson T. (1970), Studies into the pigments in beetroot (*Beta vulgaris* L. ssp. *vulgaris* var. *rubra* L.). Lantbrukshoegsk. Ann. **36**, 179–219.
Paek K. Y., Chakrabarty D., and Hahn E. J. (2005), Application of bioreactor systems for large scale production of horticular and medicinal plants. Plant Cell Tiss. Org. Cult. **81**, 287–300.
Pavlov A. and Bley Th. (2006), Betalains biosynthesis by *Beta vulgaris* L. hairy root culture in a temporary immersion cultivation system. Process Biochem. **41**, 848–852.
Pavlov A., Kovatcheva P., Georgiev V., Koleva I., and Ilieva M. (2002), Biosynthesis and radical scavenging activity of betalains during the cultivation of red beet (*Beta vulgaris*) hairy root cultures. Z. Naturforsch. **57c**, 640–644.
Pavlov A., Georgiev V., and Ilieva M. (2005), Betalain biosynthesis by red beet (*Beta vulgaris* L.) hairy root culture. Process Biochem. **40**, 1531–1533.
Shin K.-S., Chakrabarty D., Ko J.-Y., Han S.-S., and Paek K.-Y. (2003), Sucrose utilization and mineral nutrients uptake during hairy root growth of red beet (*Beta vulgaris* L.) in liquid culture. Plant Growth Regul. **39**, 187–193.
Strack D., Steglich W., and Vray V. (1993), Betalains. Methods in Plant Biochemistry, Vol. 8. Academic Press, Orlando, Florida, pp. 421–450.
Strack D., Vogt T., and Schliemann W. (2003), Recent advances in betalain research. Photochemistry **62**, 247–269.