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Rosmarinic acid production by *Lavandula vera* MM cell-suspension culture

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Abstract The time courses of growth and rosmarinic acid production by *Lavandula vera* MM cell suspension were investigated. The uptake of the main nutrients (sucrose, nitrogen, phosphorus, K, Ca, Mg) was followed during cultivation and the data on the physiology of the *L. vera* MM cell culture are presented. It was established that the cell culture synthesizes rosmarinic acid during the linear phase of growth for a relatively short period (between the 4th and 8th days of cultivation). The influence of sucrose concentration in the nutrient medium on cell growth and accumulation of rosmarinic acid by *L. vera* MM cell culture was investigated. The results showed that 7% sucrose in the nutrient medium ensured a steady growth of the cell suspension and increased the yield of rosmarinic acid (29.2 g/l dry biomass and 507.5 mg/l rosmarinic acid compared to 13.0 g/l dry biomass and 68.6 mg/l rosmarinic acid for the control cultivation with 3% sucrose).

Introduction

Rosmarinic acid has an antimicrobial, antiviral, and antiphlogistic effect, which makes it a valuable product for the pharmaceutical and cosmetic industries (Parham and Keserling 1985). It is also one of the efficient natural antioxidants (Shahidi et al. 1992).

Rosmarinic acid is widespread in the plants of the Lamiacaeae and Boraginaceae families (Scarpati and Oriente 1958; Mölgaard and Ravn 1988), though in insignificant quantities. It has been established that cell cultures, obtained from plants of these two families, produce considerable amounts of rosmarinic acid (Zenk et al. 1977; De-Eknamkul and Ellis 1988; Hyppolyte et al. 1992; Ilieva et al. 1995).

A number of investigations show that the amount of rosmarinic acid accumulated by all its cell producers is directly related to the concentration of sucrose in the nutrient medium (Zenk et al. 1977; Sumaryono et al. 1991; Hyppolyte et al. 1992; Martinez and Park 1993; Su et al. 1993; Park and Martinez 1994; Petersen et al. 1994).

In this note data are presented on the physiology of a new producer of rosmarinic acid – *Lavandula vera* MM cell culture, as well as on the influence of different initial concentrations of sucrose on the growth of *L. vera* MM cell culture and the accumulation of the rosmarinic acid.

Materials and methods

Cell culture

Lavandula vera MM callus cell culture was maintained in a Linsmayer-Skoog (LS) agar nutrient medium (Linsmayer and Skoog 1965), supplemented with 30 g/l sucrose and 0.2 mg/l 2,4-dichlor phenoxyacetic acid. It was grown for 2 weeks in a thermostat (28 °C), in the dark, and could be stored for 2 months in a refrigerator. The cell suspension of *L.vera* MM was grown in LS medium of the same composition. It was cultivated in conical flasks with 1/5 net volume, on a shaker (11.6 rad/s), in the dark, at 28 °C. The inoculation was performed with 20% (v/v) cell suspension, cultivated under the same conditions for 7 days.

The effect of sucrose

Cultivation was performed in a LS nutrient medium, supplemented with 50 g/l, 60 g/l, 70 g/l and 80 g/l sucrose, under the conditions described above. The results were compared with those obtained from cultivation on standard LS medium (Linsmayer and Skoog 1965) with 3% sucrose (control).

Extraction of rosmarinic acid

The rosmarinic acid was extracted from the cell biomass according to Hyppolyte et al. (1992).

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Analysis

The growth of the *L. vera* MM cell suspension was followed by determining the dry biomass (Dixon 1985).

The rosmarinic acid content in the methanol extracts was determined by HPLC analysis on a Perkin-Elmer (series 4) 250 × 4.6-mm PE column (C18, 10 μm). The solvent system was 2% acetic acid (A) and 2% acetic acid/acetonitrile 7:3 (B), and a linear gradient from 70% to 30% A in 40 min was applied; the flow rate was 1 ml/min and the column effluent was monitored by UV detection at 320 nm. Sucrose, glucose, nitrate and ammonium ions in the culture medium were determined by means of enzyme test combinations (Boehringer-Mannheim, Germany) and phosphorus ions by a chemical test combination (Merck, Germany). Calcium and magnesium ions in the culture medium were determined colorimetrically (Karageorgiev 1972), and the potassium ions by means of a flame photometer (Flavo 4, Carl Zeiss, Germany).

The data presented are the averages from two independent experiments, which were repeated twice for each factor under study.

Results

The basic physiological characteristics of *L. vera* MM cell culture

L. vera MM plant cell culture was maintained for 5 years as a callus on the agar medium and was subcultured every 20 days. It grew as a homogeneous friable callus with a white to beige colour. A cell suspension from *L. vera* MM was comparatively easy to obtain and during the second passage of submerged cultivation it was already a homogeneous bluish-white suspension with steady characteristics of growth and biosynthesis. At the beginning of the growth cycle the cell aggregates comprised 4–6 cells. During the linear phase of growth of the cell suspension, especially during intensive biosynthesis of rosmarinic acid (6–8th day), the shape of the plant cells changed from oval to ovoid and the number of constituent cells in an aggregate increased to 10–12. Vacuolation of the plant cells is also worth noting.

The cell suspension grew intensively and the maximum accumulated biomass was read on the 8th day of cultivation (13 g/l dry biomass) (Fig. 1A). The biosynthesis of rosmarinic acid took place between the 4th and the 8th day, being especially intensive between the 6th and the 8th day, when the maximum amount of rosmarinic acid accumulated in the cells was determined (about 68 mg/l) (Fig. 1A).

The peculiarities of the carbohydrate metabolism of the *L. vera* MM cell suspension are presented in Fig. 1B. The process of fast hydrolysis of sucrose to glucose and fructose, which was completed on the 4th

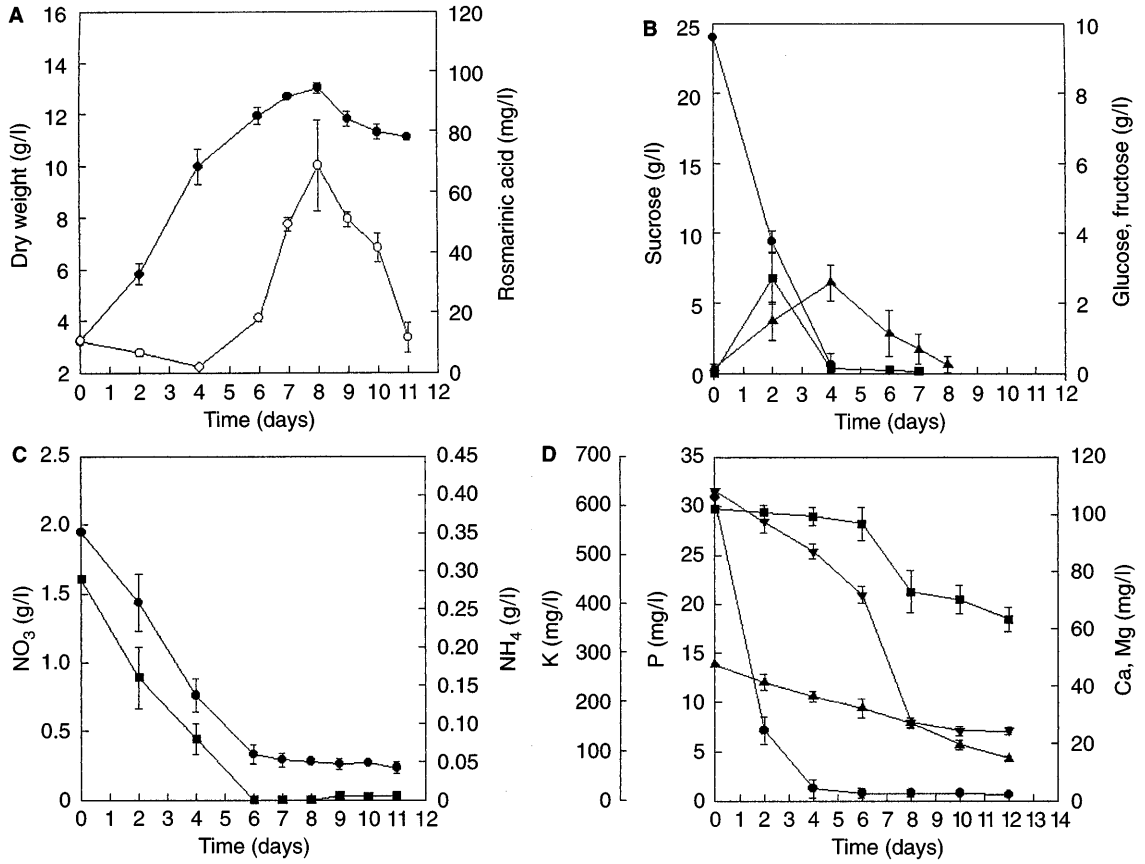

Fig. 1A–D The basic physiological characteristics of the *Lavandula vera* MM cell suspension. **A** Growth and rosmarinic acid production: ● dry weight, ○ rosmarinic acid. **B** Carbohydrate utilisation: ● sucrose, ■ glucose, ▲ fructose. **C** Uptake of the nitrogen sources: ● NO_3, ■ NH_4. **D** P, Ca, Mg, and K uptake: ● P, ■ Ca, ▲ Mg, ▼ K

day of cultivation, is clearly outlined. It should be noted that intensive consumption of the monosaccharides obtained was observed simultaneously with the inversion of sucrose. The cell culture of *L. vera* MM tended to have a slight preference for glucose, which was completely exhausted on the 4th day. Consumption of fructose was slower and determinable amounts of it were found even on the 8th day of cultivation.

The time course of nitrogen uptake by the *L. vera* MM cell suspension (Fig. 1C) showed that intensive consumption of both nitrogen forms (NH_4 and NO_3) was observed at the beginning of cultivation. By the 6th day of cultivation the ammonium ions were exhausted and the nitrate ion concentration in the culture medium reached 0.35–0.40 g/l, remaining constant until the end of the growth cycle.

The time courses of consumption of phosphorus, potassium, magnesium, and calcium are shown in Fig. 1D. Phosphorus was taken up most intensively and was almost completely consumed by the 4th day of cultivation. The utilisation of the potassium ions by the *L. vera* MM cell suspension correlated with the process of growth of the culture and about 100 mg/l was determined in the medium by the 8th day of cultivation. This concentration remained constant until the end of cultivation. The concentrations of calcium and magnesium changed to a lesser degree – their amounts decreased by about 40% towards the end of the growth cycle of the *L. vera* MM cell suspension.

The influence of sucrose on the growth and rosmarinic acid production of *L. vera* MM cell culture

The results given in Fig. 2 show that, with the increase in sucrose concentration in the nutrient medium, the amount of synthesised cell biomass increased and reached 29.2 g/l at 7% and 33.5 g/l at 8% sucrose. It should be pointed out that a concentration of sucrose over 5% retarded the development in the early stage and prolonged the growth cycle. For instance, the maximum amount of biomass for cultivation in media with 5% sucrose (Fig. 2A) was synthesised until the 8th day of cultivation, while for the 6%, 7% and 8% concentrations of sucrose the maximum of the cell growth was on the 12th day (Fig. 2B–D).

The rosmarinic acid content also increased with the increase in the concentration of sucrose in the nutrient medium. Quantities of 507.5 mg/l for cultivation in a medium with 7% sucrose (Fig. 2C) and 624.2 mg/l for

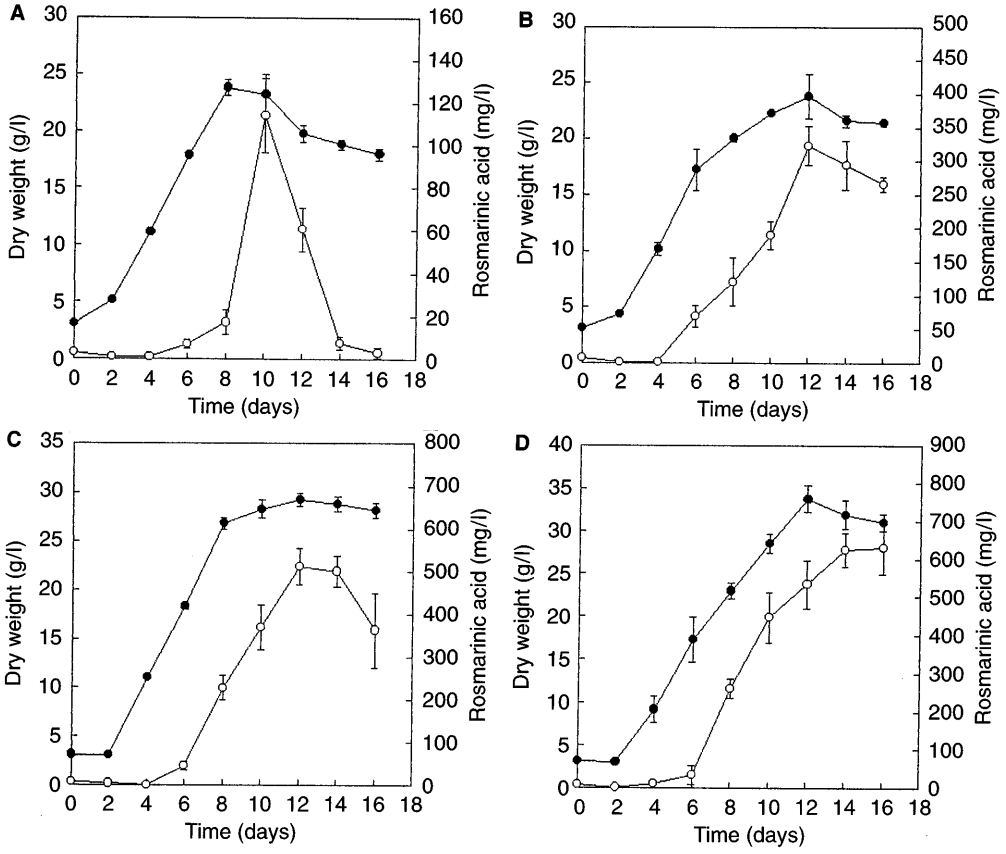

Fig. 2 Time course of growth and rosmarinic acid accumulation by *L. vera* MM cell suspension culture, cultivated in media with 5% sucrose (**A**), 6% sucrose (**B**), 7% sucrose (**C**) or 8% sucrose (**D**). ● Dry weight, ○ rosmarinic acid

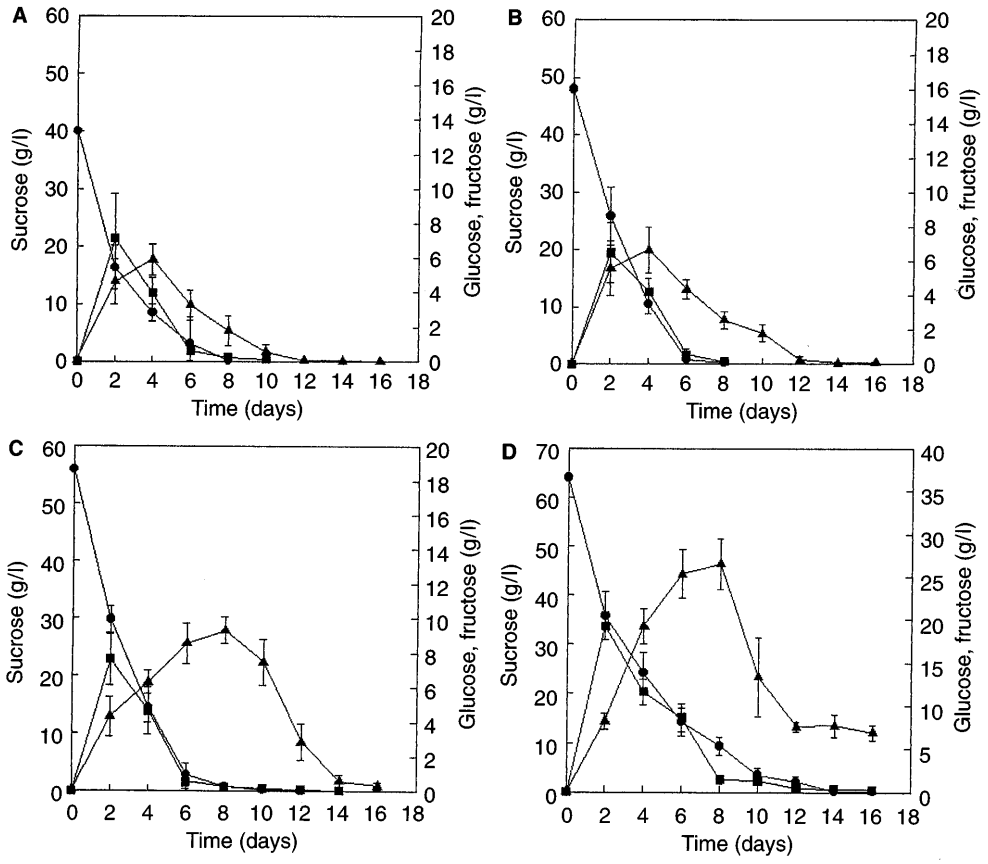

Fig. 3 Time course of carbohydrate utilisation by the *L. vera* MM cell suspension culture, cultivated in media with 5% sucrose (**A**), 6% sucrose (**B**), 7% sucrose (**C**) or 8% sucrose (**D**). ● sucrose, ■ glucose, ▲ fructose

cultivation in a medium with 8% sucrose were found (Fig. 2D). The maxima in the accumulation of rosmarinic acid by *L. vera* MM occurred on the 10th day (5% sucrose), on the 12th day (6% and 7% sucrose) and on the 14–16th day of cultivation (8% sucrose).

The *L. vera* MM cell suspension completely hydrolysed sucrose to glucose and fructose at the very beginning of the growth cycle (Fig. 3). When cultivation was performed in media with 5%–7% sucrose (Fig. 3A–C), amounts of sucrose were detected on the 6th and 8th days of cultivation, and on the 16th day for medium with 8% sucrose (Fig. 3D). In parallel with the hydrolysis of sucrose, intensive consumption of glucose and fructose by *L. vera* MM, proceeding at a different rate, was observed. In all cases glucose was consumed by the 6–8th day while fructose was consumed more slowly (by the 8–14th day respectively). An exception was the case with 8% sucrose, where glucose could be found until the 10th day, and at the end of cultivation there remained about 7 g/l fructose not consumed (Fig. 3D).

The effectiveness of the biosynthesis of dry biomass by *L. vera* MM was not considerably affected by sucrose concentration in the medium and, in all cases under study, 0.5 g dry biomass was synthesised from 1 g sucrose added (Fig. 4). With the increase in sucrose concentration, the yield of rosmarinic acid increased three times to reach 9 mg rosmarinic acid from 1 g sucrose (cultivation of *L. vera* MM in a medium with 7% sucrose) (Fig. 4).

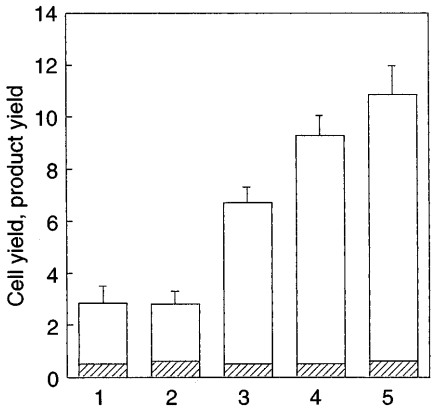

Fig. 4 Effect of sucrose concentration in the nutrient medium on yields of the cell biomass (g dry biomass/g sucrose) and rosmarinic acid (mg rosmarinic acid/g sucrose) by *L. vera* MM cultivated in media with 3% sucrose (control, *1*), 5% sucrose (*2*), 6% sucrose (*3*), 7% sucrose (*4*) or 8% sucrose (*5*). *White bars* product yield, *hatched bars* cell yield

Discussion

Biosynthesis of rosmarinic acid by *Coleus blumei* plant cell culture (Razzaque and Ellis 1977; Zenk et al. 1977) was the first such process to be announced. Later, it was established that it is also synthesised by other cell

cultures such as *Anchusa officinalis* (De-Eknamcul and Ellis 1984), *Lithospermum erythrorhizon* (Fukui et al. 1984), *Orthosiphon aristatus* (Sumaryono et al. 1991) and *Salvia officinalis* (Hyppolyte et al. 1992). We have established that the cell culture we have obtained, *L. vera* MM, also biosynthesises rosmarinic acid (Ilieva et al. 1995), despite the fact that only cell strains of *L. vera* synthesising a blue pigment, having a structure similar to that of the rosmarinic acid, have been reported so far (Banthorpe et al. 1985).

The *L. vera* MM cell suspension grew fast, hence there was no clearly outlined lag phase in its cell growth cycle (Fig. 1A) and this was mainly due to the intensive consumption of sucrose, particularly its inversion products (glucose and fructose) (Fig. 1B). On the other hand, this was probably due to the relatively large inoculum (about 3 g/l) necessary for the normal growth cycle.

Comparing the time courses of biosynthesis of rosmarinic acid by *L. vera* MM and by another cell culture, *C. blumei*, which also produces rosmarinic acid (Petersen et al. 1994), it should be noted that both cell cultures synthesise rosmarinic acid during the linear phase of growth [between days 5 and 10 in the case of *C. blumei* (Petersen et al. 1994) and between days 4 and 8 in the case of *L. vera* MM (Fig. 1A)]. It is worth noting that rosmarinic acid was synthesised for a relatively short period (5 days for *C. blumei* and 4 days for *L. vera* MM), which is indicative of the great potential of these cells for the biosynthesis of rosmarinic acid.

It has been established that, of all carbohydrates used as carbon sources, sucrose is energetically the most advantageous for the cultivation of plant cell cultures, particularly with regard to biosynthesis of secondary metabolites (Kanabus et al. 1986; Amino and Tazava 1988; Su 1995) but the cell cultures differ in the sequence in which they consume the inversion products of sucrose. The *L. vera* MM cell culture consumed glucose and fructose simultaneously (Fig. 1B), though glucose was taken up at a higher rate. *Anchusa officinalis* cell suspension, for example, which also produces rosmarinic acid, consumes glucose with a slight preference for fructose during batch cultivation, and, when cultivated in the presence of dimethylsulphoxide, it consumes glucose considerably faster than fructose (Su et al. 1993). It should be noted, however, that the period of intensive biosynthesis of rosmarinic acid by the *L. vera* MM plant cell culture always began with the uptake of glucose by the medium, regardless of the increased sucrose concentrations (Fig. 1B, Fig. 3).

The role of the nitrogen source in secondary metabolism has not been thoroughly clarified, though its influence on the yields of secondary metabolites for some cell cultures has been reported (Dougall 1980; Do and Corumer 1991; Srinivasan and Ryn 1993). At this point it should be noted that the relevance of the nitrogen source for secondary metabolism is a specificity of the species. What is common is that the beginning of biosynthesis of secondary metabolites is related to comparatively low levels of the nitrogen source (Dougall 1980; Do and Cormier 1991; Hirasuna et al. 1991; Srinivasan and Ryn 1993). The nitrogen source, introduced to the nutrient medium as ammonium and nitrate ions, is important both for the growth of the cell culture and for the biosynthesis of rosmarinic acid, as follows from the time courses of growth of *L. vera* MM and biosynthesis of rosmarinic acid (Fig. 1A) as well as from the time courses of consumption of ammonium and nitrate ions (Fig. 1C). Ammonium ions are especially needed in the initial stage of culture growth and it is not until they have been consumed to a certain extent that nitrate reductases are activated, reducing nitrate ions to ammonium ions and thus making possible their consumption (Hirasuna et al. 1991). On the other hand, it is manifest that the beginning of intensive biosynthesis of rosmarinic acid by *L. vera* MM plant cell culture was connected with ammonium ion limitation in the culture and a low level of nitrate ions in the medium (Fig. 1C).

There have been reports on the higher biosynthesis of rosmarinic acid in cases of phosphorus limitation (Gertlowski and Petersen 1993), as well as on biosynthetic processes unaffected by the concentration of phosphorus in the culture medium (De-Eknamkul and Ellis 1985). Intensive biosynthesis of rosmarinic acid by the *L. vera* MM cell culture commenced after phosphorus had almost completely been consumed by the medium (Fig. 1D), so in this case phosphorus plays a certain regulatory role in the process of biosynthesis of rosmarinic acid.

Regarding the uptake of calcium, potassium and magnesium from the nutrient medium (Fig. 1D), it may be noted that no direct relation between their uptake and the process of biosynthesis of rosmarinic acid by *L. vera* MM was detected.

The *L. vera* MM cell culture is distinguished by certain physiological and biochemical properties, connected with the intensive biosynthesis of rosmarinic acid. As follows from the time course of utilisation of the major nutrient components during the control cultivation with 3% sucrose (Fig. 1), the intensive biosynthesis of rosmarinic acid began after the complete consumption of glucose, ammonium and phosphate ions and after a relatively low level of nitrate ions in the medium had been reached.

The biosynthesis of rosmarinic acid by any of its plant cell producers is considerably affected by the amount of sucrose added to the nutrient medium, but the sucrose concentration in the nutrient medium needed for obtaining the maximum yield of rosmarinic acid is different for the different plant cell producers. For *C. blumei*, for instance, it varies from 5% (Ulbrich et al. 1985) to 7% (Zenk et al. 1977), while for *Salvia officinalis* it is 5% (Hyppolyte et al. 1992). The results obtained from investigations of the effect of sucrose concentration in the medium on the biosynthesis of rosmarinic acid by the *L. vera* MM cell culture showed that 7% sucrose was most effective (Fig. 2C). The intensive biosynthesis of rosmarinic acid took place for a

relatively short period (days 6–12 of cultivation, i.e. during the linear and early stationary phase) (Fig. 2C). The latter is a favourable prerequisite for an effective biotechnological method for its production. The high yield in this case, as well as in the other cases of increased sucrose concentration is, to some extent, due to the increased amount of cell biomass (in this case twice that of the control) increasing the amount of rosmarinic acid, which is an intracellular component. The more important fact, however, is that the added 7% sucrose is converted to rosmarinic acid more effectively compared with the control or with the other cases under study. In this case the rosmarinic acid achieved a threefold higher yield of sucrose in the medium (9 mg/g sucrose compared to 3 mg/g sucrose for the control cultivation; Fig. 4). At this point it should be noted that the cultivation of *L. vera* MM at sucrose concentrations over 7% was inexpedient. In the case with 8% sucrose (Fig. 2D), as a result of the substantial amount of synthesised biomass (34 g/l dry biomass at day 12), an increase in the viscosity of the medium ensued and the mass-exchange properties of the cultivation system deteriorated. This made the effective consumption of nutrient components impossible and the performance of the cultivation very difficult.

The results from the studies on the effect of different initial concentrations of sucrose on the biosynthesis of rosmarinic acid by plant cell culture *L. vera* MM revealed certain possibilities that can underlie a consequent optimisation of the composition of the nutrient medium for enhanced rosmarinic acid biosynthesis.

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