Sotolone Production by Hairy Root Cultures of *Trigonella foenum-graecum* in Airlift with Mesh Bioreactors

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3-Hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone) and 3-amino-4,5-dimethyl-2(5*H*)-furanone, the postulated precursor of sotolone, were detected in hairy root cultures of *Trigonella foenum-graecum* (fenugreek) by GC-MS. The hairy root cultures in both conical flasks and airlift with mesh bioreactors were achieved from hypocotyl of seedling by infection with *Agrobacterium rhizogenes*. In flasks, the mathematical relationship between hairy root growth and conductivity was established and afterward used to evaluate the biomass evolution in bioreactor cultures due to the difficulty of obtaining direct biomass samples from the bioreactor. The GC-MS analyses of ethanolic extracts from hairy roots revealed the presence of two important compounds: sotolone (1.2% of the volatile fraction) and 3-amino-4,5-dimethyl-2(5*H*)-furanone (17% of the volatile fraction). These results point out that biotechnological production of sotolone in bioreactors is possible. Additionally, these hairy root cultures offer, for the first time, an excellent biological model to study the biosynthetic pathway of sotolone in fenugreek.

Keywords: *Sotolone; 3-amino-4,5-dimethyl-2(5H)-furanone; hairy roots; airlift bioreactor; Trigonella foenum-graecum; fenugreek*

INTRODUCTION

Hairy root cultures offer great potential for the production of valuable secondary metabolites and the study of the associated secondary metabolic pathways from many plants (*1*−*3*). The advantages of using hairy roots are their independence of plant growth regulators, high growth rates, and genetic and biosynthetic stability (*4*, *5*). The main restriction for commercial exploitation of hairy root cultures has been their scaling up, as there are few specially designed bioreactors able to support the growth of the interconnected tissue and distribute the biomass consistently throughout the vessel (*6*−*9*).

On the other hand, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone), a furanone component of the characteristic aroma of *Trigonella foenum-graecum* (fenugreek) seeds (*10*, *11*), is one of the most important high-value natural flavoring substances (*12*, *13*). Because of its strong reminiscent odor, sotolone has been widely used as an aroma in the food (*14*) and tobacco industries (*15*). It is the principal flavoring compound in French flor-sherry wine (*13*) and gives the characteristic flavor to sake (*16*); it enhances the flavor of soy sauce (*17*), sugar molasses (*12*), barley malt (*18*), roasted coffee (*19*), and stewed beef (*20*). In addition to its importance as a flavoring, several furanones have been shown to have anticancer activity in mice, and they could contribute to human health as part of a complex antioxidant mixture of food-derived compounds (*21*). Besides sotolone, in fenugreek a wide range of secondary metabolites such as flavonoids and sapogenins (*22*, *23*), which might contribute to the medicinal and nutritional properties of fenugreek extracts, have been recognized (*14*, *23*). In contrast with sotolone, some of these flavonoids and sapogenins have been identified in plant cell and tissue cultures of fenugreek (*22*, *24*). In a previous study, for instance, we found that hairy roots of fenugreek produce diosgenin in airlift bioreactors and conical flasks, but they did not produce sotolone (*7*).

Several synthetic pathways for sotolone have been postulated (*10*, *25*−*29*). Early studies suggested that sotolone might originate by degradation of threonine (*30*) and from acetaldehyde and α-ketobutyric acid in sake and wine (*12*), or it might be thermally induced by oxidative deamination of 4-hydroxyisoleucine (HIL) or its corresponding lactone, 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone (*10*). Sauvaire et al. (*31*) suggested that HIL, an unusual amino acid that represents nearly 80% of free amino acids of dry fenugreek seeds, could be the natural precursor of sotolone in fenugreek. Recently, the presence of 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone was confirmed in fenugreek seeds (*27*), and afterward Rapior et al. (*29*) reported the presence of 3-amino-4,5-dimethyl-2(5*H*)-furanone in *Lactarius helvus* cultures producing sotolone. The first of the two above amino compounds has been postulated as the putative precursor of sotolone; however, the second one has been related only with the fenugreek aroma (*32*), but its possible role in the metabolic pathway of sotolone has not been investigated.

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In the present study, on the basis of GC-MS analysis we report the identification and production of sotolone and 3-amino-4,5-dimethyl-2(5*H*)-furanone in hairy root cultures of fenugreek in mesh airlift bioreactors. The significance of the presence of these compounds in the hairy root cultures is discussed in light of the sotolone biochemical pathway and its potential biotechnological production.

MATERIALS AND METHODS

Bacterial Strain. *Agrobacterium rhizogenes* strain LBA9402 (pESC4), containing the reporter gen GUS and NPT II gene, was used in the present study. The strain was grown on YMB medium (*33*), containing kanamycin and riphampycin (50 mg/L) (Sigma Chemical Co., St. Louis, MO), and subcultured at month intervals.

Plant Material Propagation. Seeds of fenugreek from Sinclair McGill, U.K., were surface sterilized by immersion in 70% ethanol (1 min) and then in sodium hypochlorite solution (5% w/v available chlorine) for 10 min and washed with sterile distilled water. The surface sterile seeds were transferred to flask cultures containing B5 medium (*34*) supplemented with sucrose 30 g/L, pH 5.8, and Gelrite 2.0 g/L (Phytagel, Sigma Chemical Co.). The flask cultures were incubated at 24 °C and 800 lx (cool white fluorescent tubes, Osram) for seedlings.

Hairy Root Cultures. The hypocotyl of 2−3-week-old seedlings was inoculated with a 48-h-old culture of *A. rhizogenes* by puncturing the tissue with a sterile hypodermic needle as previously described in Rodríguez et al. (*7*). Two weeks after the hypocotyl infection, the profuse roots emerging from the punched site were excised and transferred into B5 liquid medium containing ampicillin 500 mg/L (Pentrexyl, Bristol, U.K.). The bacteria-free hairy root cultures were incubated on an orbital shaker (G-20, New Brunswick, NJ) at 120 rpm, 25 ± 1 °C, and 800 lx. These cultures were maintained by transferring every two weeks 0.3−0.5 g of fresh weight of biomass from the bacteria-free hairy root cultures into 250 mL conical flasks containing 50 mL of B5 fresh medium without ampicillin. The studies were carried out with a fenugreek hairy root clone, referred to as RTF5. The success for transformation of RTF5 was confirmed by applying the southern blotting technique as described by Cabrera et al. (*35*).

Inoculum. The experiments in flasks were performed in 125 mL conical flasks containing 40 mL of B5 liquid medium and inoculated with 0.1−0.2 g of fresh weight of hairy root tips (1−2 cm in length). For the bioreactor, the inoculum was a 10-day single hairy root culture grown into a 500 mL conical flask incubated at 120 rpm, 25 ± 1 °C, and 800 lx.

Culture in the Airlift with Mesh Bioreactor. A stainless steel mesh with a 3-mm pore size was fixed as concentric draught-tube along the 9-L column of an airlift bioreactor as previously reported (*7*). The bioreactor was prepared with 8 L of B5 medium, sterilized by autoclaving at 121 °C for 40 min, and inoculated with 30−35 g of apical meristematic tissue from fresh hairy roots grown in a 500 mL conical flask. The bioreactor culture was maintained at 25 ± 1 °C, 800 lx, and 0.3 vvm. Sampling from the bioreactor was performed by withdrawing 15 mL of medium through a vacuum sampling port. The growth of biomass in the bioreactor was estimated from the medium conductivity based on the respective mathematical relationship experimentally established from the conical flask cultures. After 30 days, the whole biomass from the bioreactor was collected, washed with tap water, and allowed to dry for 4 days at room temperature. The dry hairy roots were stored at 25 °C in polystyrene bags, and their oil extracts were GC-MS analyzed after 1, 3, and 6 months for their aroma composition.

Oil Extraction. The extraction of the essential oil fraction from the hairy roots was performed as described in the French Norm NF-V-03-409 (*36*), using three samples of 10 g of dry tissue and distilling the mixture at atmospheric pressure for 2 h in a Stahl's system. The extracts were stored at −20 °C pending GC-MS analysis.

Oleoresin Extraction. At least three samples of dry hairy roots (10 g) were extracted three times for 4 h with 120 mL of absolute ethanol (J. T. Baker, Philipsburg, NJ) in a reflux system (Soxhlet). The extracts were pulled and vacuum-concentrated at 35 °C in a rotary evaporator Büchi (RE111, 461), and stored at −20 °C pending for GC-MS analysis.

Dry Weight. The dry biomass from a whole flask culture was measured by oven-drying the rinsed hairy roots at 90 °C by 24 h. For each measurement, at least three repetitions were made.

Conductivity. It was measured in the biomass-free culture medium with a digital conductivity meter (ATI Orion model 170) at 25 °C using a water bath for the temperature equilibration of the sample. A relationship between conductivity and dry weight was established using the biomass from 30 hairy root conical flask cultures. The correlation obtained was used afterward to estimate the biomass concentration in the airlift bioreactor during the time course of the culture.

N-Nitrate and P-Phosphate. The brucine method and the molybdate method (*37*) were used to colorimetrically evaluate nitrogen and phosphorus, respectively. For both conical flasks and bioreactor cultures each measurement represents the average of three independent cultures.

Total Sugars. The total sugar level was determined by using the phenol−sulfuric method (*38*). The sampling and replication of the measurements were as pointed out for nitrogen and phosphorus.

GC-MS Chromatographic Analysis. Separation and identification of volatile constituents were performed with a GC-MS system, fitted with a gas chromatograph (5890-Hewlett-Packard series II) and a mass selective detector (5971-Hewlett-Packard) with a potential of 70 eV for ionization by electron impact and an emission current of 50 mA. A G1034C Chemstation controlled the mass spectrometer that scanned from m/z 38 to 300. Samples (1 μL) were injected in split mode at 250 °C (split ratio 30:1) onto a DB-1 (polydimethylsyloxane) fused silica capillary column (20 m × 0.20 mm i.d., 0.5 μm film thickness). The oven was held at 50 °C for 2 min and then heated at 3 °C/min to 250 °C. Helium was used as the carrier gas in a constant flow of 0.6 mL/min. An *n*-alkane series (C_6−C_{18}) was analyzed under the same conditions to obtain the linear retention index (LRI) value for the components of the reaction mixture. Identification was performed by comparing the mass spectra and the linear retention indices with those of true compounds and data reported in the literature (*39*, *40*).

Statistical Analyses. To estimate the coefficients of the linear equations that best predicted the value for biomass in the bioreactor cultures, the routine Linear Regression analysis of the statistical package SPSS was used (*41*).

RESULTS AND DISCUSSION

Time Course of Growth of Hairy Roots in Conical Flasks. In the case of the hairy root cultures in bioreactor there were difficulties in evaluating the biomass evolution using fresh or dry weight; hence, the values of these are not shown. Studies involving plant cell cultures in bioreactors have shown that direct sampling to evaluate the biomass evolution during the time course is invariably a problem to solve (*7*, *42*), particularly due to the formation of cellular aggregates (*43*) or to the morphology and size of the organ involved (*44*). This problem has encouraged researchers to suggest indirect methodologies (*45*), of which the conductometric method has been found to be efficient, economic, and reliable in most applications (*46*), and it is not affected by the culture system (*43*, *44*). Thus, to evaluate the relationship between growth of the hairy roots and their sotolone production in the bioreactor, the correlation between growth and conductivity during the time course of the cultures was first studied in

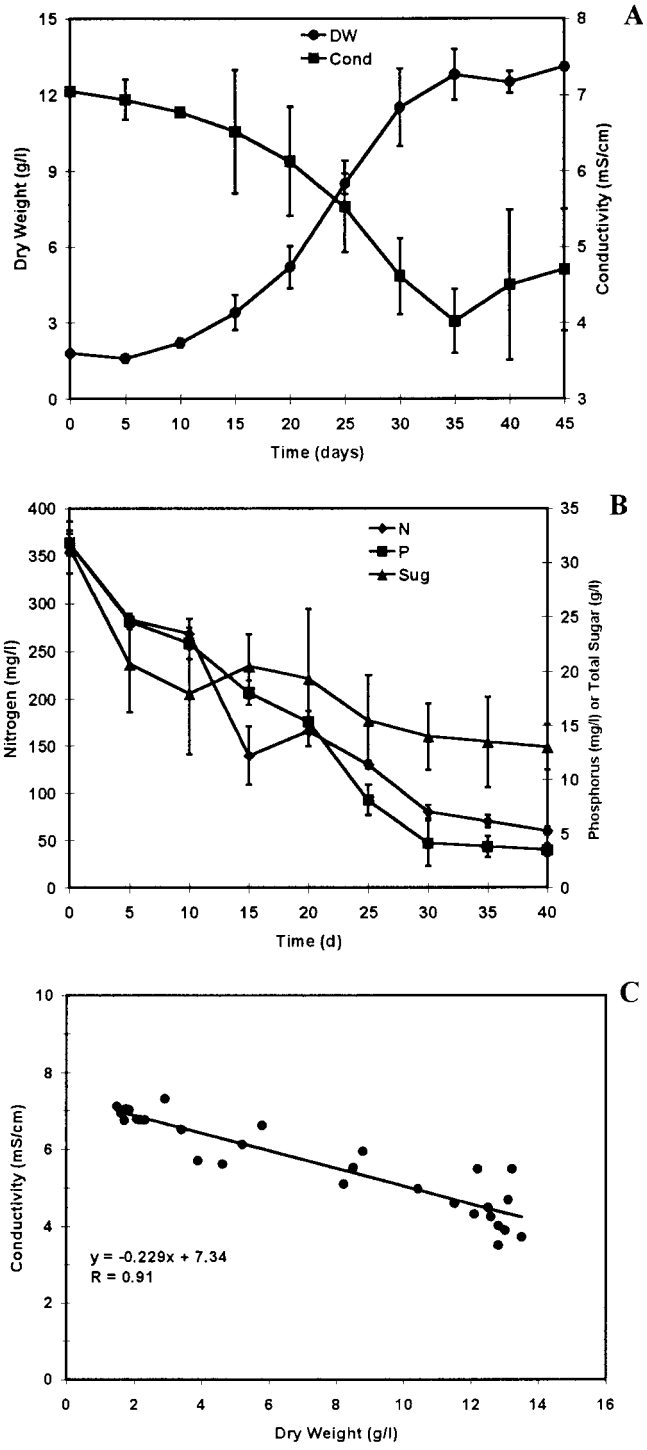

Figure 1. Time course (A) and macronutrient uptake (B) of hairy root cultures in conical flasks, and correlation (C) of the dry weight with the changes in the conductivity of the culture medium. The error bars represent the standard deviation of at least three determinations.

conical flasks. An inverse relationship between biomass production and conductivity was clearly observed (Figure 1). Conductivity declined gradually while the biomass was increasing. Both parameters, weight and conductivity, showed that at the beginning of the time course the hairy root growth was very slow, entering into the exponential phase after 5 days and into the stationary phase after 35 days (Figure 1A). The conductivity decline in both microbial and plant cell cultures has been directly associated with the cellular uptake of ionic components from the culture medium

Table 1. Estimated Regression Statistics[a] for the Linear Relationship of Conductivity, Nitrogen, Phosphorus, and Nonionic Sugar Profile in the Hairy Root Cultures with the Dry Weights and the Conductivity of the Medium Culture

	R^2	df	F_0	P	$b0^b$	m^b
Independent: Dry Weight						
conductivity	0.844	28	151.94	0.000	7.3	−0.23
nitrogen	0.768	25	82.75	0.000	304.4	−19.8
phosphate	0.809	16	67.62	0.000	26.6	−1.98
sugars	0.504	25	25.43	0.000	24.6	−0.9
Independent: Conductivity						
nitrogen	0.669	25	50.59	0.000	−238.4	71.2
phosphate	0.801	25	100.85	0.000	−28.9	7.5
sugars	0.382	25	15.45	0.001	0.4	3.1

[a] R^2, coefficient of determination; df, degrees of freedom; P, P value for significance ($P > 0.05$) of regression. [b] The regression coefficients, $b0$, intercept; and m, slope.

(*45*, *47*). However, in the present study the pattern of uptake of nitrogen and phosphate (Figure 1B), as well as the nonionic sugar nutrients, showed doubtful correlation ($R < 0.9$) with both dry weight and conductivity (Table 1). Even though the P values point out that there is a linear effect between the pattern of uptake for these nutrients with the dry weight and conductivity, the low coefficient of determination (R^2) values suggest that the true relationship between these factors with the evolution of biomass and conductivity was not a straight line model. Therefore, the decline of any of these three major nutrients was not quantitatively proportional to the biomass production. In contrast, a good linear relationship ($n = 30$, $R = 0.91$) between dry weight and conductivity through the time course of the hairy root cultures in flasks, proper to predict the biomass evolution, was achieved (Figure 1C). Thus, the statistical parameters from this lineal correlation were used further to estimate the time course of hairy root growth in the bioreactor cultures. This method facilitated the evaluation of the growth of the hairy roots in the airlift bioreactor without the risk of microbial contamination or loss of biomass.

Estimation of the Time Course of Hairy Root Culture in the Airlift with Mesh Bioreactor. As a rule, applications of airlift bioreactors have allowed the development of several systems specific for plant cell and tissue cultures (*48*, *49*). In this study, it was necessary to modify the typical airlift design to achieve a homogeneous distribution of the roots all over the reactor. Obviously, such distribution is determined by the configuration of the reactor and its operation (*48*). Basically, the traditional airlift bioreactor has three zones with different flow standards (*49*): a drive or lower zone, where gas is injected and the liquid flow has predominantly vertical sense upward, producing a gradient of densities; a separation or upper zone, where the turbulence facilitates the liquid and gas phases to be mixed; and, finally, a depletion zone, where the liquid is degasified and recycled to the drive zone. The global behavior of the reactor is the sum of the interrelationship of the mentioned zones. In the airlift with mesh bioreactor used in this work, the air bubbles rising produced a soft traffic of the fluids and conferred gentle movement to the roots, which at the time become anchored randomly in a homogeneous distribution over the space of the mesh. In previous work, an airlift with central arms was used to grow hairy roots of *Trigonella foenum-graecum* (*7*); however, the growth reached in the reactor with mesh was favored by the better anchorage

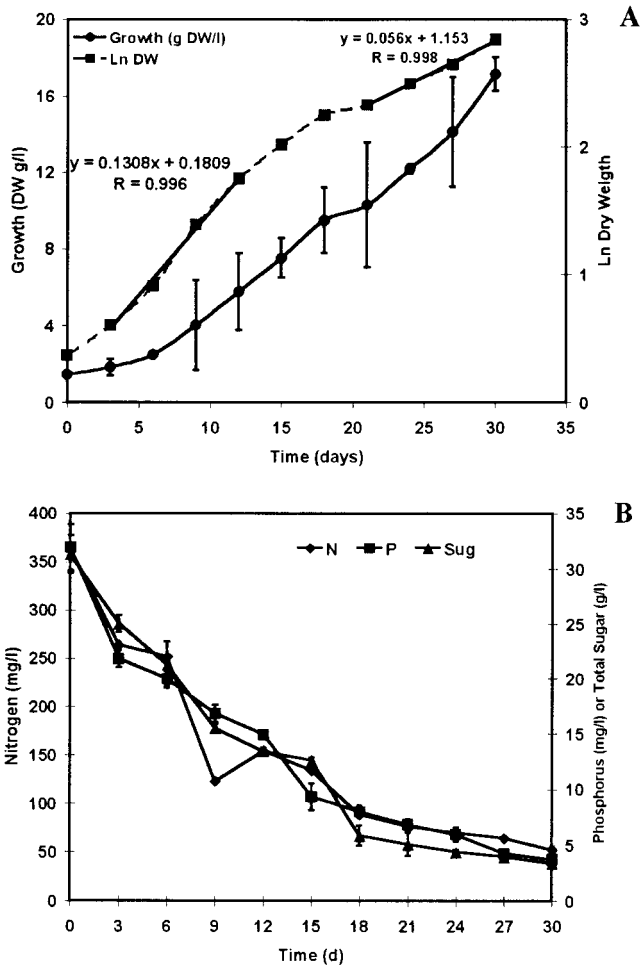

Figure 2. Time course of growth (A) and macronutrient uptake (B) of hairy root cultures of *T. foenum-graecum* grown in the airlift with mesh bioreactor. The biomass was estimated from the conductivity of the culture medium using the mathematical relationship shown in Figure 1C. The error bars represent the standard deviation of three determinations.

and homogeneous distribution of the roots through the jar. On the other hand, in the present work, the mesh facilitated the harvest of biomass, because the roots took a structure like a nodule over the mesh that could be removed as a single piece. Results with other hairy root cultures using mesh suggest that this is a practical alternative for the biomass production in reactors (*50*, *51*).

Although the phenotypic characteristics, viability, and global growth rate of the hairy roots grown in the airlift with mesh bioreactor were similar to those from conical flasks (Figures 1 and 2), the cultures in the bioreactor showed some special features that demand further discussion. In contrast with cultures in flasks, the pattern for the time course of growth in bioreactor (Figure 2A), estimated on the basis of the relationship established between conductivity and dry weight previously established in flasks (Figure 1), showed that the evolution of biomass followed a diauxic curve. Two phases of each acceleration and exponential growth were clearly observed from a semilogarithmic representation of the data (Figure 2A). The specific growth rates (μ) for both periods, the early period ($\mu = 0.130$ day^{-1}; $R^2 = 0.98$) and that after 18 days of culture ($\mu = 0.056$ day^{-1}; $R^2 = 0.98$), were similar to that exhibited by the cultures in flask ($\mu = 0.081$ day^{-1}). As for microbial cultures (*52*), diauxic growth in plant cell and tissue

Table 2. GC-MS-Identified Volatile Compounds and Their Contents (Percent of the Total Volatile Constituents) in Oleoresins[a] of Hairy Roots of *T. foenum-graecum* Kept at 25 °C for 1, 3, and 6 Months

			biomass stored for		
peak[b]	RT[c]	compound[d]	1 month	3 months	6 months
I	4.25	hexanal	2	3	5
II	7.57	γ-butyrolactone	2	5	10
III	8.80	cyclopenta-1,2-dieno	2	3	5
IV	11.53	valeric acid	2	4	6
V	14.60	3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone)	–	0.5	1.2
VI	15.05	2-(4-methylthiazo-5-yl)-ethanol	3	3	8
VII	16.70	6-methyl-2,3-dihydroxy-5,6-dihydropyran-4-one	20	22	30
VIII	19.25	3-amino-4,5-dimethyl-2(5*H*)-furanone	3	11	17
IX	20.40	5-(hydroxymethyl)furan-2-carboxaldehyde	2	3	5

[a] Oleoresin extraction was performed with ethanol from 10 g of dry hairy roots. [b] Peak numbers correspond to those in the chromatogram shown in Figure 3. [c] Retention time for the GC DB1 polydimethylsiloxane fused silica column used. [d] Main volatile compounds identified by comparison with the reference compound on the basis of retention time and GC-MS. Other compounds were too small for verification by GC-MS. –, not detected. The data represent the average from three cultures.

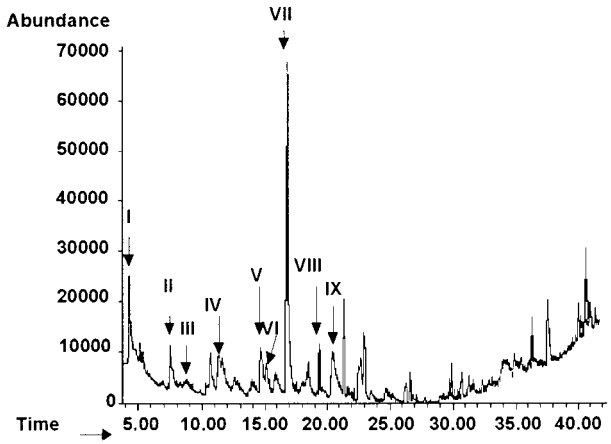

Figure 3. GC chromatogram for an ethanolic extract from dry hairy roots of *T. foenum-graecum* kept at 25 °C for 3 months. Numbers indicate the retention time (minutes) for which volatile compounds were identified and correlated with Table 2.

cultures may occur when the organism is offered a catabolizable energy source in the presence of a more readily catabolizable energy source (*53*, *54*). The diauxic growth pattern might be also a consequence of biochemical changes in the cells produced by switching the uptake of a growth-limiting nutrient to another one, usually the carbon, nitrogen, or phosphate sources. If the two nutrients are present in the medium at the same time, in diauxic growth, the organism grows first on one energy source and then there is a temporary cessation before growth is resumed on the other nutrient (*52*). In those cases, usually there is a clear inverse relationship between the growth pattern and the uptake pattern of the limiting nutrient involved. However, as for conical flasks, the correlation between the diauxic growth pattern and the uptake pattern of the major nutrients in the bioreactor cultures was uncertain (Figure 2B). It should be noted that the B5 medium used

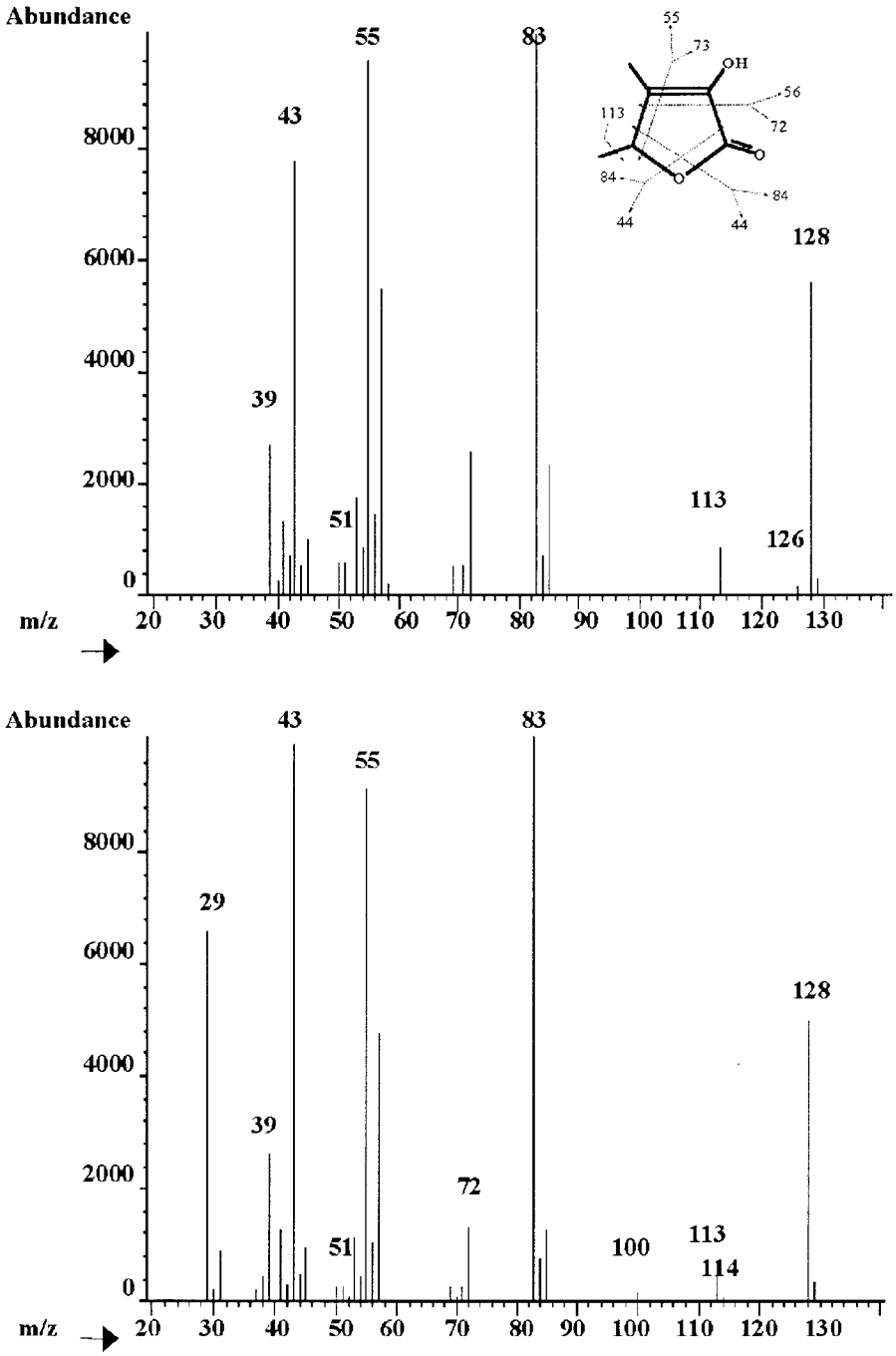

Figure 4. EI mass spectra and fragmentation pattern of sotolone, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone, present in the ethanolic extracts from hairy root cultures of *T. foenum-graecum* (upper), and the authentic compound (lower).

in this work contains two nitrogen sources, nitrate and ammonium ions (*34*), and the nitrogen uptake interruption observed in Figure 2B, just days before the biomass had reached the first plate, was also observed in several experiments performed in both bioreactor and flask cultures (data not shown). This repetitive pattern of the nitrogen uptake suggests that a relationship between this nutrient and the diauxic growth might exist, but this hypothesis requires further research.

Production of Sotolone by a 10-L Airlift with Mesh Bioreactor of Hairy Root Culture from *T. foenum-graecum*. Sotolone, the major component of the typical fenugreek aroma, was detected during the time course of the hairy root cultures grown in the airlift with mesh bioreactor. Although the fenugreek aroma was not immediately detected in fresh biomass, its presence was evident after the roots were dried and kept in the dark for some time (Table 2). The aroma was gradually generated during the drying process and reached its maximum intensity throughout the storage (Figure 3; Table 2). These results agree with those from Shankarackarya et al. (*55*), who reported that fresh seeds of fenugreek are odorless but that they gradually produce their typical aroma during the drying process. To investigate the composition of the aroma in the hairy roots, two extraction procedures for the recovery from biomass were assayed: steam distillation and extraction with ethanol. The yield of the essential oil obtained in the steam distillation was 0.06% of the dry biomass, whereas the yield of oleoresin from ethanolic extraction was 2% of the dry biomass. Interestingly, only the oleoresin extracts showed permanently the fenugreek

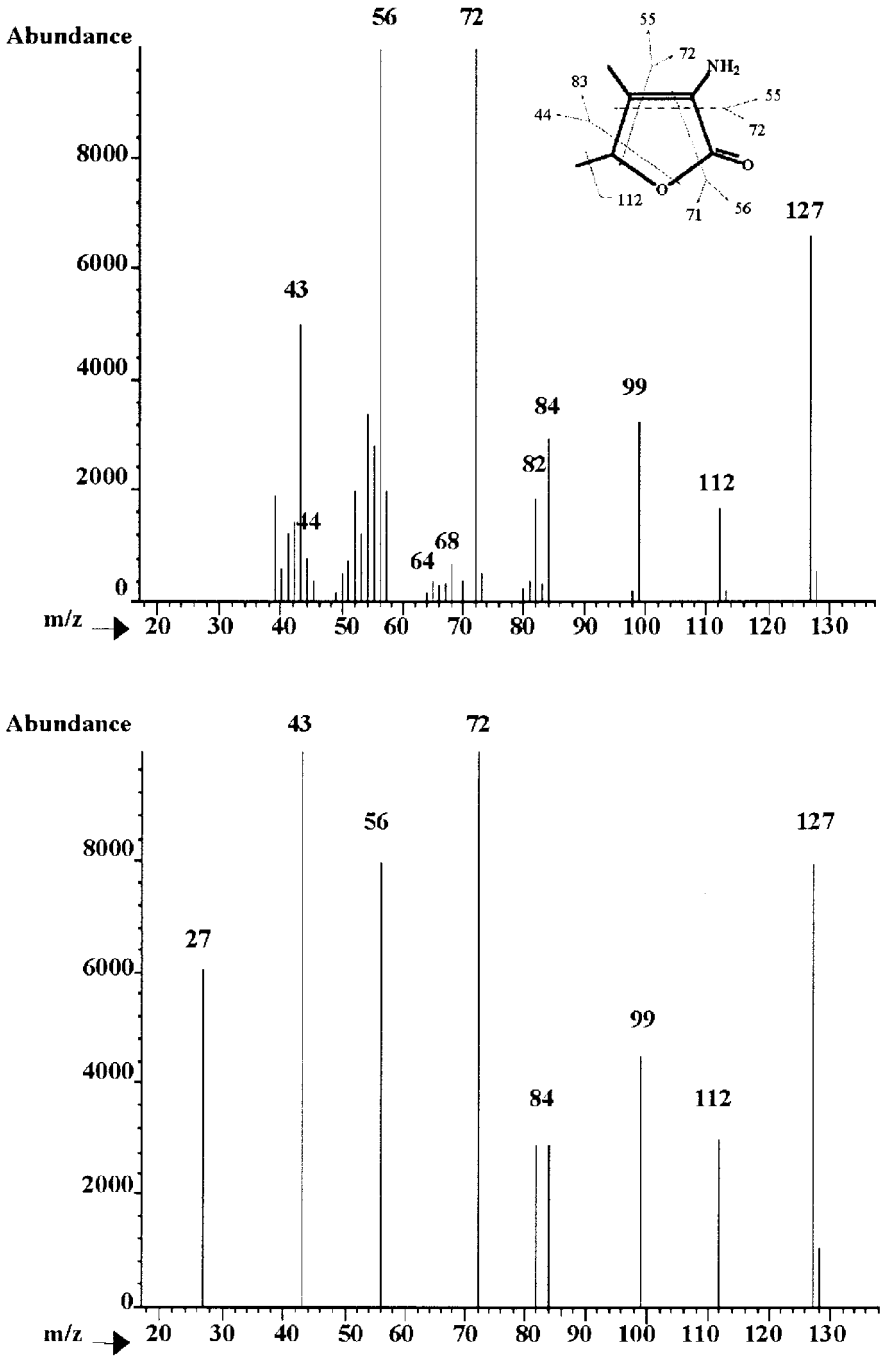

Figure 5. EI mass spectra and fragmentation pattern for the putative sotolone biosynthetic precursor, 3-amino-4,5-dimethyl-2(5*H*)-furanone, present in ethanolic extracts from hairy root cultures of *T. foenum-graecum* (upper), and the authentic compound (lower).

aromatic fraction, which eluted before 25 min in the GC-MS chromatogram (Figure 3). From GC-MS analyses of the oleoresin extracts, 48 compounds were detected (Figure 3), but only 9 were truly identified using authentic compounds as standards (Table 2). The mass spectra analysis of the compounds from those extracts containing the fenugreek aroma clearly revealed the presence of sotolone and the compound 3-amino-4,5-dimethyl-2(5*H*)-furanone (Figures 4 and 5), which had been previously postulated as a compound structurally related to sotolone (*28, 29*). The lack of the aroma in the essential oil agrees with reports in the literature that some kinds of *Trigonella* seeds do not produce the characteristic fenugreek aroma and typically do not contain sotolone (*27*).

From the synthetic pathways postulated for sotolone formation (*12, 26, 56*−*58*), it has been suggested that this compound might originate from HIL. This hydroxylated amino acid is usually present in *T. foenum-graecum* (*59, 60*) and *L. helvus* (*29*), a mushroom from Europe. On the other hand, Blank et al. (*27*) found that sotolone was generated in model systems by thermally induced oxidative deamination of HIL through 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone, using different carbonyl compounds as carbonyl reactant. In the present work we were unable to find the HIL oxidative deamination product (*10*). However, the compound 3-amino-4,5-dimethyl-2(5)-furanone, previously found in *L. helvus* (*29*), with a molecular weight and mass spectrum close to those of sotolone, was clearly present

in the hairy root cultures (Figure 5; Table 2). It is unknown whether these compounds in this system were produced by chemical degradation, due to the action of the air upon the biomass components, or whether an enzymatic mechanism is involved. However, the results suggest that the synthetic pathway in the mushroom *L. helvus* and in the plant *T. foenum-graecum* might share several intermediary compounds, independent of whether a biochemical or chemical process, or a combination of them, is involved. These possibilities might be explored using the hairy root cultures as an experimental model to compare the biochemistry in the fenugreek plant and the mushroom. To our knowledge this is the first study that reports the presence of sotolone and its putative intermediary precursor, the compound 3-amino-4,5-dimethyl-2(5)-furanone, in hairy root cultures of *T. foenum-graecum* and insight into its biotechnological significance in the production of the fenugreek aroma.

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