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Production of Lichen Metabolites by Immobilized Cells of *Cladonia clathrata* Ahti & Xavier Filho

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With 7 Figures

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Summary

Pereira E. C., Da Silva N. H., Andrade L. C., Vicente C. & Legaz M. E. 1999. Production of lichen metabolites by immobilized cells of *Cladonia clathrata* Ahti & Xavier Filho. – Phyton (Horn, Austria) 39 (1): 79–89, 7 figures. – English with German summary.

The production of lichen metabolites by immobilized cells of *Cladonia clathrata* Ahti & Xavier Filho, collected from sandy soils of the tableland of Alhandra, Paraíba, NE of Brazil is reported. Cells were entrapped in kaolinite, and 0.1 mM, 1.0 mM and 10 mM sodium acetate was added to immobilized cells as a precursor of lichen phenolics. Entrapped cells produced the highest amount of some substances that have not been reported in the literature, although they were also detected from organic extracts of the thallus in natura. The concentration of the precursor seemed to enhance the production of a specific compound, protocetraric acid, by cells main-

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tained at 0,1mM; a non identified compound at 1.0 mM and the Cph-2 substance at 10mM. The data lead us to conclude that the immobilized cells have a better performance for producing metabolites. However, the major phenolic of this lichen species, fumarprotocetraric acid, was produced at very low amounts by cells loaded with 1.0 mM acetate.

Zusammenfassung

PEREIRA E. C., DA SILVA N. H., ANDRADE L. C., VICENTE C. & LEGAZ M. E. 1999. Die Produktion von Flechtenstoffwechselprodukten in Zellen von *Cladonia clathrata* AHTI & XAVIER FILHO. – Phyton (Horn, Austria) 39 (1): 79–89, 7 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Es wird über die Produktion von Flechtenmetaboliten durch Zellen von *Cladonia clathrata* AHTI & XAVIER FILHO, welche auf sandigen Böden des Tafellandes von Alhandra, Paraiba, NO-Brasilien gesammelt wurden, berichtet. Die Zellen wurden in Kaolin eingeschlossen und 0,1 mM, 1,0 mM und 10 mM Kaliumazetat als Precursor von Flechtenphenolen der Zellen hinzugefügt. Die verankerten Zellen erzeugten so hohe Substanzmengen, wie in der Literatur noch nie berichtet wurde, obwohl sie sonst auch in organischen Extrakten der Thalli in natura bestimmt werden konnten. Der Precursor schien die Produktion einer spezifischen Verbindung, der Protocetrar-Säure zu erhöhen, wenn die Zellen einer Konzentration von 0,1 mM ausgesetzt waren, aber auch einer noch nicht identifizierten Verbindung bei 1,0 mM und die CpH-2-Substanz bei 10 mM. Die Ergebnisse führen zum Schluß, daß verankerte Zellen eine bessere Leistungsfähigkeit aufweisen, Metabolite zu erzeugen. Die Hauptphenolkomponente dieser Flechtenart, die Fumarprotocetrar-Säure wurde jedoch nur in sehr kleinen Mengen in den Zellen gebildet, denen 1,0 mM Azetat angeboten wurde.

Introduction

Lichen compounds have been refereed through the centuries as useful for many purposes, since cosmetics to medicinal employment (LLANO 1951, SEU-SALERNO & BLAKEWAY 1987, HALE 1983, NASH 1996).

Brazilian lichens showed these qualities, with emphasis on antimicrobial, antineoplasic, and pharmacological actions (PEREIRA et al. 1997, SANTOS et al. 1997). Thus, for extracting, purifying and using these lichen substances, a very high amount of biomass with very difficult turnover is irreversibly used and, thus, destroyed (VICENTE et al. 1995). To solve this problem, the immobilization of lichen enzymes (MOSBACH & MOSBACH 1966, GARCIA-JUNCEDA & VICENTE 1986) or lichen cells (VICENTE & MOLINA 1993), is an alternative to biomass destruction. *Cladoniaceae* from Brazilian North-eastern have been used to cell immobilization in order to produce ribitol (PEREIRA et al. 1995a) or usnic acid (PEREIRA et al. 1995b), using alginate and kaolinite as matrix for entrapping, respectively.

Since the technique for continuous phenolic and poliol production is efficient, and maintains the cell vitality for weeks (GONZALEZ et al. 1984), this paper shows the phenolic production by immobilized cells of *Cladonia*

clathrata Ahti & Xavier-Filho, using kaolinite as an innert matrix, and sodium acetate as precursor.

Material and Methods

Plant material

Cladonia clathrata Ahti & Xavier Filho, collected from sandy soils of tableland (cerrado), in Alhandra County (Paraiba, NE of Brazil), was used throughout this work. Thalli were dried at room temperature (25 ± 3 °C) until cell obtention and phenolic purification.

Cell immobilisation

Three thallus samples (2.0 g in air-dry weight each one) were gently macerated in a mortar with 20 ml of double-distilled water. The material was filtered through four-layered cheesecloth and then, centrifuged for 10 min at 3500 g. Each pellet was mixed with 50 g of kaolinite, previously hydrated with double-distilled water (100 ml) and loaded onto a glass column (30 cm × 7 cm i.d.). Solutions (50 ml) of 0.1 mM, 1.0 mM and 10 mM sodium acetate were supplied to the respective column.

The bioreactor was maintained at room temperature (25 ± 3 °C) for 18 days, under continuous white light (125 $\mu mol.m^{-2}.s^{-1}$).

Fraction obtention and analysis

Aliquots of washing solutions were collected at 3, 6, 12 and 18 days of the experiment, and then added the same volume (25 ml) of fresh acetate solutions onto the columns. The eluates were extracted with 25 ml of diethyl ether/ethyl acetate (65:35 v/v), followed by a second extraction with chloroform/acetonitrile (60:40, v/v). Both organic extracts were mixed and their absorbance measured in an UV spectrophotometer at 254 nm and 366 nm.

The extracts were dried in air flow and used for thin layer chromatography (TLC) assays. The chromatography was carried out on silica gel F plates and developed on B solvent system: hexane/diethyl ether/formic acid, 130:80:20, v/v (Culberson 1972). The spots were visualized under UV short and long waves. The chromatograms were later sprayed with H_2SO_4 (10%), and heated at 100 °C for colour reaction of the spots, and measured the Rf values.

The organic extracts were joined for each acetate concentration (0,1 mM; 1.0 mM; 10 mM), and analysed by high performance liquid chromatography (HPLC), in a Hitachi liquid chromatograph, coupled to an UV detector at 254 nm. Separation was performed by using a reverse phase C18 column and methanol/water/acetic acid (80:19,5:0,5 v/v) as mobile phase, at a flow rate of 1.0 ml min^{-1} (Legaz & Vicente 1983). Substances were identified by their retention time (RT) value, and atranorin, and usnic, fumarprotocetraric and protocetraric acids from Sarsyntex (France) were used as external standards.

Results and Discussion

Cladonia clathrata was described as new lichen species by Ahti et al. (1993). According to these authors, *C. clathrata* produced fumarprotoce-

traric and protocetraric acids, the substance Cph-2, and sometimes also Cph-1. Some reports about *C. verticillata* seem to be referred to *C. clathrata*, such as those of BARROS & XAVIER FILHO 1972 and BEZERRA et al. 1973. However, the material described by CARRAZONI & WANDERLEY 1974 lead us do not agree with their results, since authors found evernic acid in this species, and this compound, quoting AHTI et al. 1993, is not known in the *Cladonia* genus.

The chemical composition of crude extracts obtained from *C. clathrata* was analysed by TLC. The tests revealed that the thallus in natura synthesized both fumarprotocetraric and protocetraric acid, besides Cph-2 substance. The Rf values of the acids (32 and 25, respectively) coincided with those of the standards, and the spot with a Rf value of 13, probably could be identified as Cph-2 substance. However, three additional, unknown compounds were registered in the chromatogram (Fig. 1).

The organic extracts of the wash solutions from the immobilized cells were spotted beside the crude extract of the thallus in a TLC plate. Chromatograms revealed that immobilized cells produced unidentified substances, the Rf values of which were higher than those assigned to fumarprotocetraric and protocetraric acids used as standards. Cells maintained on 0.1mM acetate produced four substances, while the chromatograms

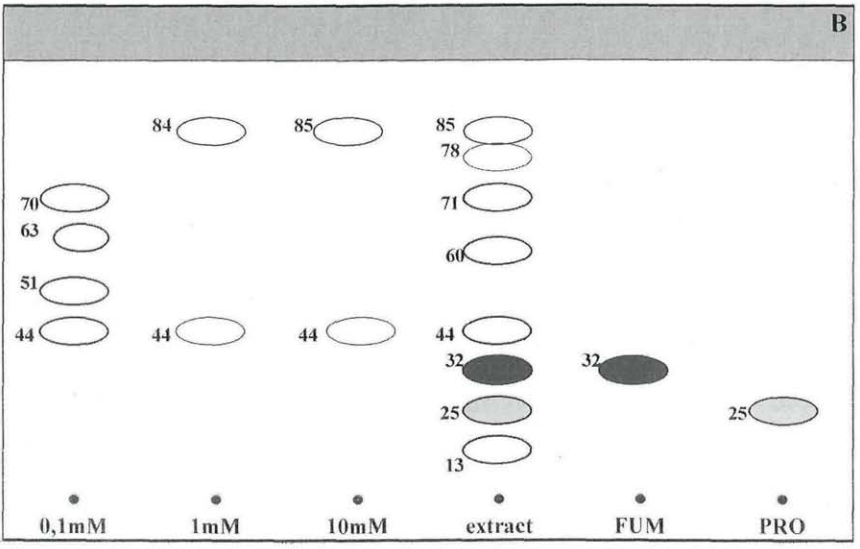

Fig. 1. Diagram of a thin layer chromatogram using organic extracts of washing solutions obtained from immobilized cells of *Cladonia clathrata* loaded with sodium acetate at different concentrations (0.1 mM, 1.0 mM and 10 mM), organic extract of *C. clathrata* thallus (extract), and fumarprotocetraric acid (FUM) and protocetraric acid (PRO) as standards.

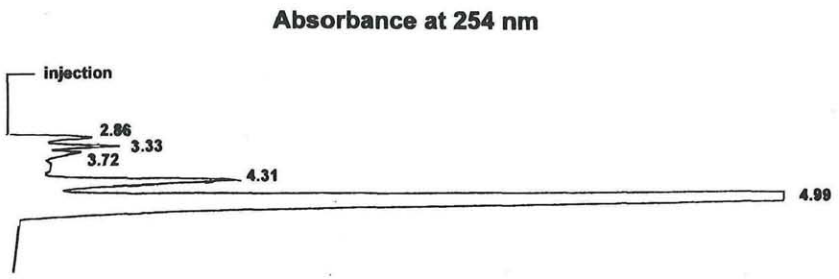

Fig. 2. HPLC traces of organic extracts obtained from *C. clathrata* thallus using concentration of dry crude extract of 5.0 mg per ml of the solvent. Numbers near the peaks indicate retention time values in min.

corresponding to eluates from columns loaded with 1.0 mM and 10 mM acetate showed only two compounds. However, three of them have Rf values similar to those of the spots corresponding to the in natura thallus (Fig. 1).

The crude extract of the thallus was also analyzed by HPLC. When concentrated, (5.0 mg ml^{-1}) the chromatogram showed six peaks, being the last two peaks identical to those of standards, protocetraric and fumarprotocetraric acids. When diluted (data are not shown), the chromatogram of the crude extract showed only the peaks corresponding to these two acids, probably by dilution of the other compounds below the lowest concentration which produced a signal higher than the noise. The retention time of fumarprotocetraric and protocetraric acids used as standards were 5.10 min and 4.35 min, respectively.

Cells loaded with 0.1 mM acetate mainly produced a compound with a retention time value of 3.34 min., probably the Cph-2 substance (AHTI et al. 1993) and another substance with a retention time value of 7.68 min (Fig. 3A). Other minor peaks were registered, but corresponding to a very low concentration of compound. The major peak in the chromatogram from washing solutions of the cells loaded with 1.0 mM acetate showed a retention time value of 4.47 min, identified as protocetraric acid. The chromatogram also revealed peaks at 3.38 min (Cph-2 substance), 6.25 min, and 7.71 min, this last probably identical to that produced by immobilized cells loaded with 0.1 mM acetate, without any correspondence with phenolics characteristic of this lichen species (Fig. 3B). Other metabolites, biogenesically related to both protocetraric and fumarprotocetraric acids, have been described in the literature. SUNDHOLM & HUNECK 1981 were able to identify hypoprotocetraric acid from *Ramalina hypoprotocetrarica* and *R. tumidula*, whereas ELIX & VENABLES 1993 characterized 4-O-methyllividic acid from *Hypotrachyna livida*. According to the chromatogram B in Fig. 3, peaks with retention time values of 6.25 min and 7.71 min could be tentatively identified as hypoprotocetraric and methylhypo-protocetraric

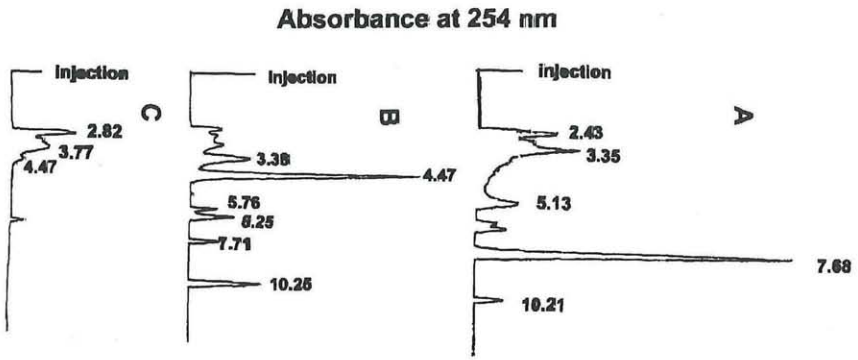

Fig. 3. HPLC traces of organic extracts obtained from the washing solution of *C. clathrata* cells immobilized in kaolinite and loaded with (A) 0.1 mM sodium acetate; (B) 1.0 mM sodium acetate and (C) 10 mM sodium acetate. Numbers near the peaks indicate retention time values in min.

acids, respectively. By comparison with protocetraric acid (retention time 4.47 min in Fig. 3B) , relative retention time values, calculated as 6.25/4.47 = 1.39 and 7.71/4.47 = 1.72, were very similar to those described by HUNECK & YOSHIMURA 1996 for hypoprotocetraric and methylhypoprotocetraric acids, respectively.

It is particularly interesting that fumarprotocetraric acid, the major depsidone of *C. clathrata*, cannot be produced by immobilized cells of this lichen species. Traces of this compound found after incubation of cells in 0.1 mM acetate (Fig. 3A) could be considered as a remaining amount of this phenol, dissolved in the acetate solution from the untreated, disrupted thallus samples, but immobilized cells seemed to be unable to synthesize the depsidone. Both protocetraric and fumarprotocetraric acids could be considered as atranorin derivatives. Protocetraric acid could be produced by atranorin oxidation, a process which normally required NAD^+ as a cofactor (GARCÍA-JUNCEDA et al. 1991, VICENTE & XAVIER FILHO 1993). However, the production of fumarprotocetraric implied the use of succinyl-CoA as an additive on the C2' position of protocetraric acid in a coupling reaction which involved a FAD-dependent dehydrogenation reaction (Fig. 4). Apparently, the mechanical disruption of the lichen thallus did not affect the production of NAD^+ by the mycobiont cells in a sufficent amount to assure the continuous production of protocetraric acid. However, the supply of succinyl-CoA, FAD or both, failed after thallus disruption and, then, fumarprotocetraric acid cannot be produced. The excess of protocetraric acid could be used to produce the unusual hypoprotocetraric and methylhypoprotocetraric acids (Fig. 4).

The cells loaded with 10 mM acetate produced small amounts of protocetraric acid (retention time 4.457 min).

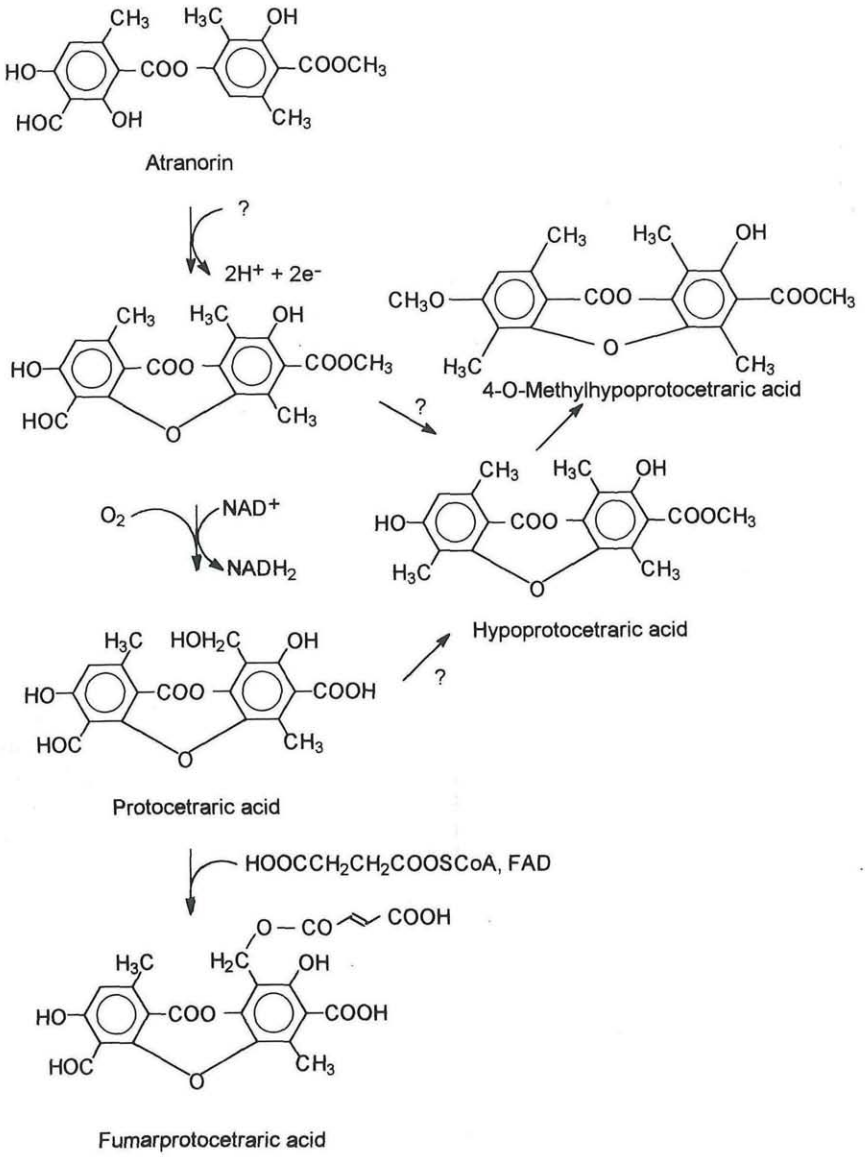

Fig. 4. Proposed pathway for the biosynthesis of fumarprotocetraric acid and derivatives.

The production of metabolites by immobilized cells of *C. clathrata* was measured at 3, 6, 12 and 18 days (Figs. 5, 6 and 7). The cells supplied with 0.1mM sodium acetate showed their best production around of the sixty

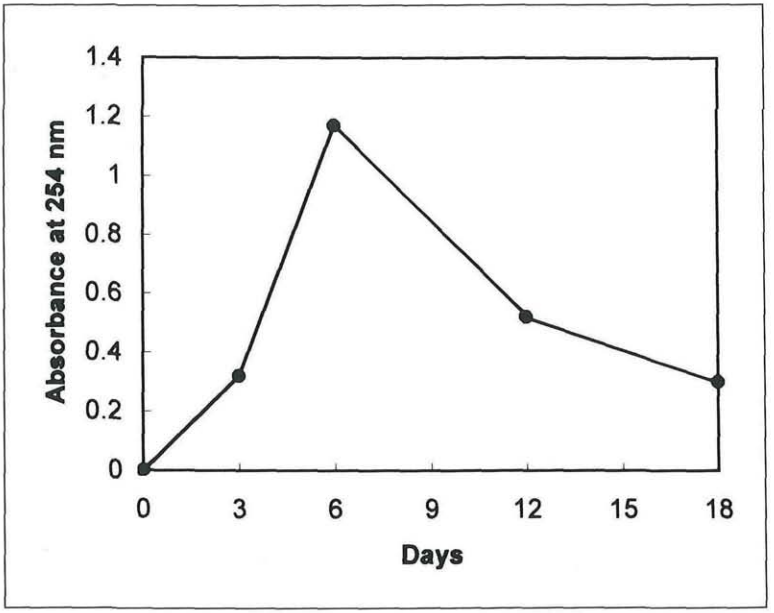

Fig. 5. Production of metabolites by cells of *C. clathrata* immobilized in kaolinite and loaded with 0.1 mM sodium acetate.

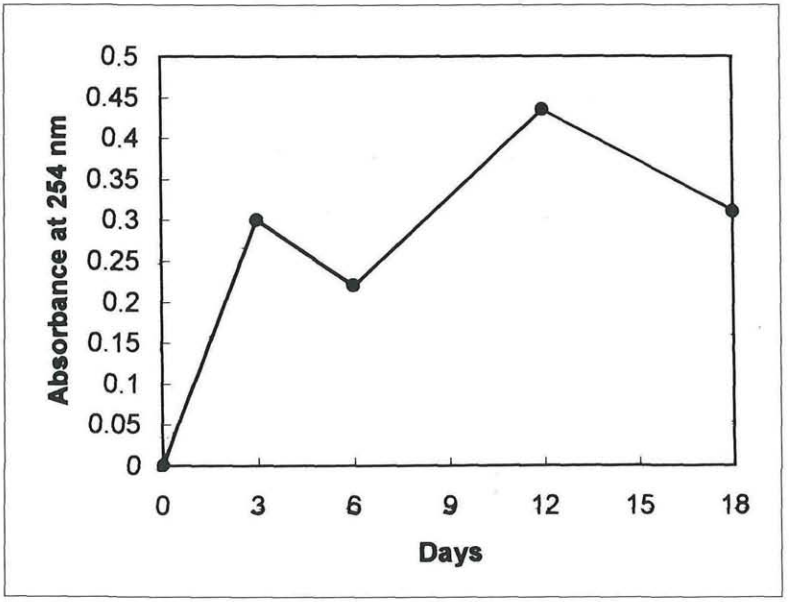

Fig. 6. Production of metabolites by cells of *C. clathrata* immobilized in kaolinite and loaded with 1.0 mM sodium acetate.

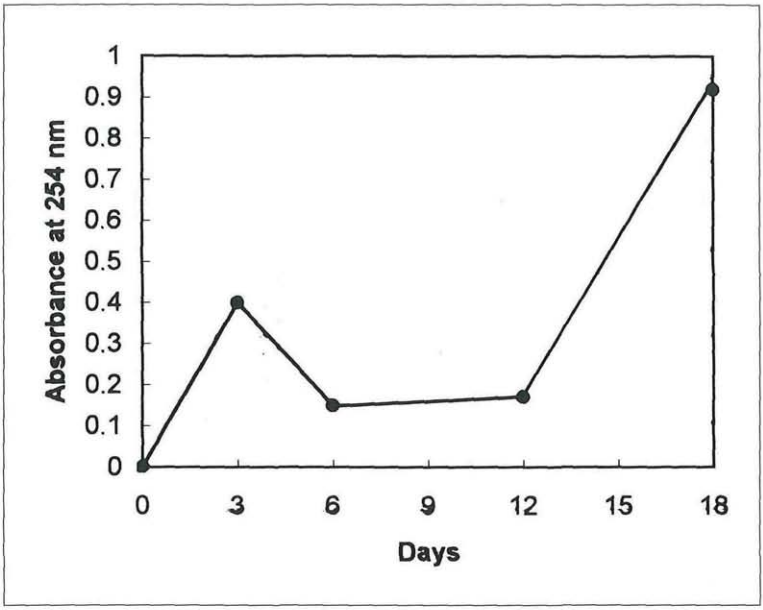

Fig. 7. Production of metabolites by cells of *C. clathrata* immobilized in kaolinite and loaded with 10 mM sodium acetate.

day, while the material supplied with 1.0 mM acetate, produced more phenolics at 12 days of experiment. However, 10mM sodium acetate induced phenolic production only after 12 days.

Conclusions

Since these data, it was possible to conclude that immobilized cells of *C. clathrata*, when incubated with 1.0 mM sodium acetate show a more efficient production of lichen metabolites, in relation to the other concentrations of the precursor used here.

The cells product highest amounts of substances not refereed in the literature, however detected in the organic extracts of the thallus in natura. The immobilised cells had a satisfactory performance for producing metabolites, but the principal compound of the species, fumarprotocetraric acid, was detected through HPLC analysis, only in the samples from the cells maintained at 0.1 mM of the precursor.

Acknowledgements

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Recensiones

HAUSEN [Björn M.] & VIELUF [I. K.] 1997 Allergiepflanzen – Pflanzenallergene. Handbuch und Atlas der allergieinduzierenden Wild- und Kulturpflanzen. Kontaktallergene. Allergische Frühreaktionen. 2. Auflage. – Gr. 8°, XII + 567 Seiten, zahlreiche Abbildungen, farbig und schwarzweiß; geb. – Ecomed Verlagsgesellschaft, D-86899 Landsberg. – DM 168,– ISBN 3-609-64082-0.

Teil I dieses Bandes trägt den Titel „Allergische Spätreaktionen (Kontaktallergie) und entspricht der ersten Auflage des Buches aus 1988, die in Phyton 31(1): 95–96 besprochen worden ist. Dort ist alles wesentliche über diesen Teil gesagt, das vollinhaltlich auch auf die Neuauflage zutrifft, weshalb auf diese Rezension verwiesen sei. Die neue Auflage ist erfreulicherweise überarbeitet und ergänzt, so ist z.B. der inzwischen in Mode gekommende Australische Teebaum (*Melaleuca alternifolia*) ausführlich behandelt. Der Umfang ist von 311 Seiten der 1. Aufl. auf 324