

Oxidation reactions are required to produce atranorin from acetate by alginate-immobilized cells of *Cladonia verticillaris*.

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Summary. Atranorin, a *p*-depside of the β -orcinol series, is produced by several *Cladonia* species. Immobilized cells of *Cladonia verticillaris* in calcium alginate are able to produce atranorin when they are supplied with 1.0 mM acetate as a precursor. Production of the depside is enhanced by adding an oxidant agent (NAD⁺ or FMN) to the incubation media and its secretion to these media is facilitated by permeabilizing the immobilized cells with 2 % *iso*-propanol.

Abbreviations: FMN = flavin mononucleotide; HPLC = high performance liquid chromatography; NAD⁺ = Nicotinamine adenine dinucleotide,

Introduction

Lichens produce depsides (phenolics composed by two or three monocyclic units linked by an ester bond) derived from orsellinic acid. Two main series of depsides are recognized (Culberson, 1969):

1. the orcinol series, which comprises orsellinic acid derivatives without any additional substituent at the C3 position in the ring, such as lecanoric acid, and

2. the β -orcinol series, which comprises orsellinic acid derivatives with a C-1 unit at C3 position in the ring, such as atranorin (Fig. 1).

Orsellinic acid is synthesized by an aromatic synthase (Gaucher and Shepherd, 1968) which produces a 8C lineal polyketide intermediate before cyclisation. However, labeling experiments have indicated that an additional C-1 unit must be added to the

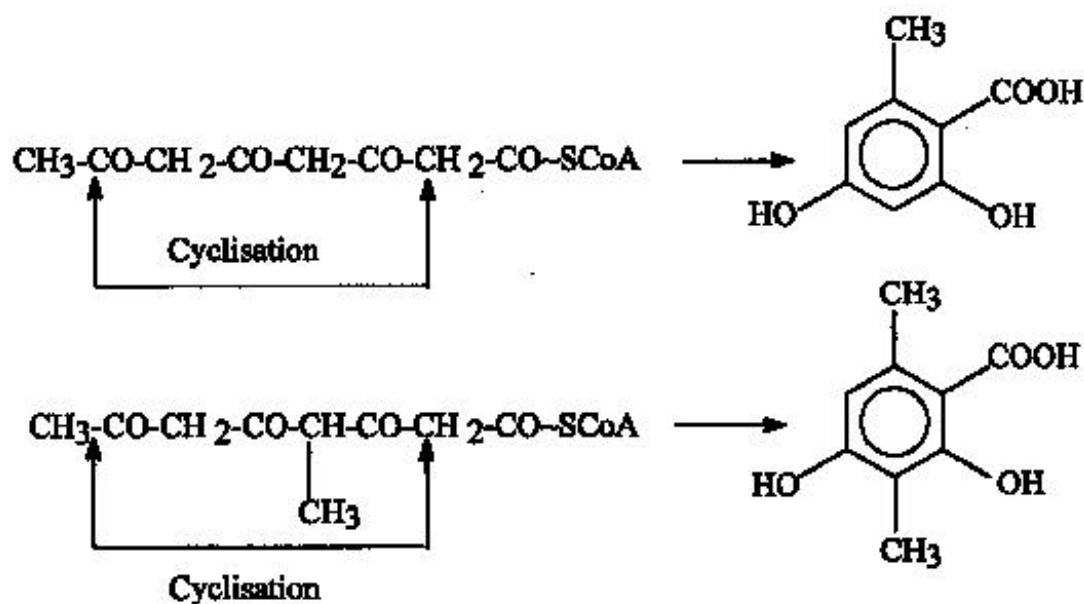


Fig. 1. Mechanism for the production of orsellinic acid (top) and methyl-3-orsellinate (bottom).

polyketide chain prior to cyclisation to produce methyl-3-orsellinate, the basic unit of the β -orcinol series (Yamazaki et al., 1965; Yamazaki and Shibata, 1966). The ester linkage between two monocyclic phenolics is catalyzed by several orsellinate depside hydrolases (Schultz and Mosbach, 1981; Vicente, 1991) thus acting as esterases.

Lichen phenolics have been suggested to form a protective screen on the lichen cortex that filters radiation and, thereby, protects photosynthetic pigments in the photobiont cells (Fernández et al., 1996; Hidalgo et al., 1992). We have observed that specimens of *Cladonia verticillaris* show strong colour differences depending on whether they grow under full sunlight, or under a canopy of several shrubs that filters about half of the incident light intensity, as well as the age, size and the situation of different tissues. Fumarprotocetraric acid and atranorin accumulate in response to high irradiation values (Legaz et al., 1986) and this implies that light positively regulates depside

production and deposition (Mateos et al., 1993).

Many depsides are used for industrial or pharmacological purposes. For instance, atranorin and its derivatives are common constituents of the "oak moss absolute", characteristic of many perfumes (Richardson, 1988; Schultz and Albroscheit, 1989). Since this depside behaves as a powerful inhibitor of elastase (Proksa et al., 1994), it appears to be of great interest for the cosmetic industry. However, provided the slow growth rate of lichens, the quantities of lichen biomass required to supply significant amounts of these metabolites to the industry can be considered as prohibitive. Lichen tissue cultures have been attempted for many lichen species in order to improve phenol production (Yamamoto et al., 1998) but the growth rate of calli is very slow. We herein provide evidence of a new method of atranorin production based on immobilized cells of *C. verticillaris* that can be employed to circumvent the need of high amounts of lichen biomass.

Material and methods

Cell immobilization

Cladonia verticillaris (Raddi) Fr., collected from sandy soils of tableland in Alhandra (Paraiba, NE of Brazil) was used throughout. The voucher specimen is in the Herbarium of the Faculty of Biology of the Complutense University, MACB 46637. The thallus of this lichen species accumulates the depsidone fumarprotocetraric acid and the depside atranorin (Legaz *et al.*, 1986). Thalli were dried under air flow conditions and stored at room temperature in the dark until required.

Samples of 2.0 g of dry thalli were completely soaked in distilled water and gently macerated in a mortar to obtain an homogenate. Then, 20 ml of sodium alginate, medium viscosity, in aqueous solution (4 per cent, w/v) were added and mixed with the homogenate, and the mixture was extruded drop-wise with a syringe into 50 ml of 0.2 M CaCl₂ (Kierstan and Coughlan, 1985) to produce beads of gellified calcium alginate. These gellified drops were supplemented with 50 ml 1.0 mM sodium acetate, defined as bath solution, and maintained at 25°C for 90 days. Where indicated, 40 µM FMN or 14 µM NAD⁺, the most common coenzymes for oxidation-reduction reactions (Dixon and Webb, 1979), were added to the acetate solution. A second series of immobilized cells was prepared and maintained under the same conditions but the bath solution contained 2 per cent *iso*-propanol (v/v) in order to permeabilize lichen cells. *Iso*-propanol, as well as other alcohols, organic solvents and enzyme systems have currently been used to permeabilize different plant cells and yeasts (Bindu *et al.*, 1998; Tandon *et al.*, 1999). At the times indicated, an aliquot of 5 ml of the bath solution was collected to extract lichen phenolics and replaced with the same volume of the corresponding fresh medium.

Phenolics extraction and analysis

Phenolics were extracted by mixing the aliquot of the bath solution with 5 ml of diethyl ether : ethyl acetate (65:35 v/v). The mixture was vigorously stirred and the organic phase was separated with a micropipette (Pedrosa and Legaz, 1991). The extraction was repeated three times. Organic

extracts were mixed and dried in air flow. The residue was redissolved in 2.0 ml methanol and analyzed by HPLC, according to Legaz and Vicente (1983), using a Varian 5060 liquid chromatograph equipped with a Vista CDS 415 computer. Chromatographic conditions were as follows: column, reverse phase MCH-10; mobile phase: methanol :water: acetic acid (80:19.6:0.4 v/v/v); injection: 10 µl; flow rate: 0.7 ml min⁻¹ in isocratic mode; pressure: 76 atm; detector: UV set at 238 nm. Atranorin and fumarprotocetraric acid from Sarsyntex (France) were used as external standards.

Electron microscopy

At specific incubation times, beads were washed and fixated in 1% osmium tetroxide solution buffered at pH 7.4. The material was dehydrated in ethanol and embedded in Epon/Araldite. Thin sections were cut with a LKB Ultratome I and examined in an electron microscope Carl Zeiss (Germany) EM 902 at 80 kV.

Results and Discussion

Production of atranorin by cells supplemented with FMN

Fig. 2 shows a chromatographic profile of the phenolics extracted from a sample of *C. verticillaris* thallus. Atranorin is separated as a well-defined peak with a high degree of selectivity and a resolution higher than that shown in previous reports (Legaz *et al.*, 1986). Thus, the separation method used in this work is sufficiently accurate to quantify this lichen phenolic.

Our measurements showed that a small amount of atranorin was dissolved from immobilized cells of *C. verticillaris* in calcium alginate to media supplemented with acetate or acetate plus FMN (Fig. 3). This initial release was observed from non-permeabilized cells as well as from those previously permeabilized with *iso*-propanol. This fact indicates that the first release concerns the original amount of atranorin contained by the living thallus before immobilization. An additional indication supporting this evidence was that atranorin secretion at day 8 of the incubation of the beads significantly decreased in all the cases. From this time, atranorin production and release was

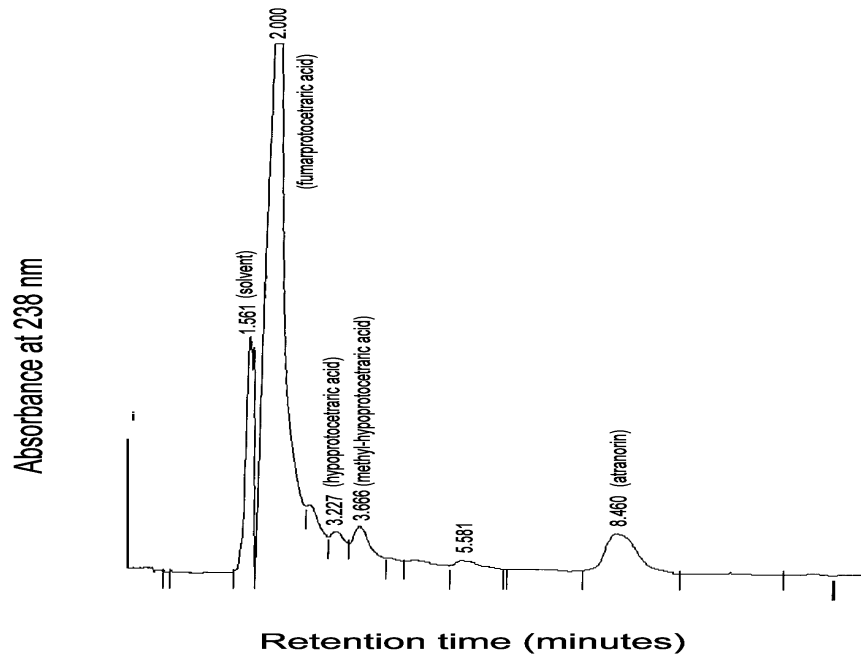
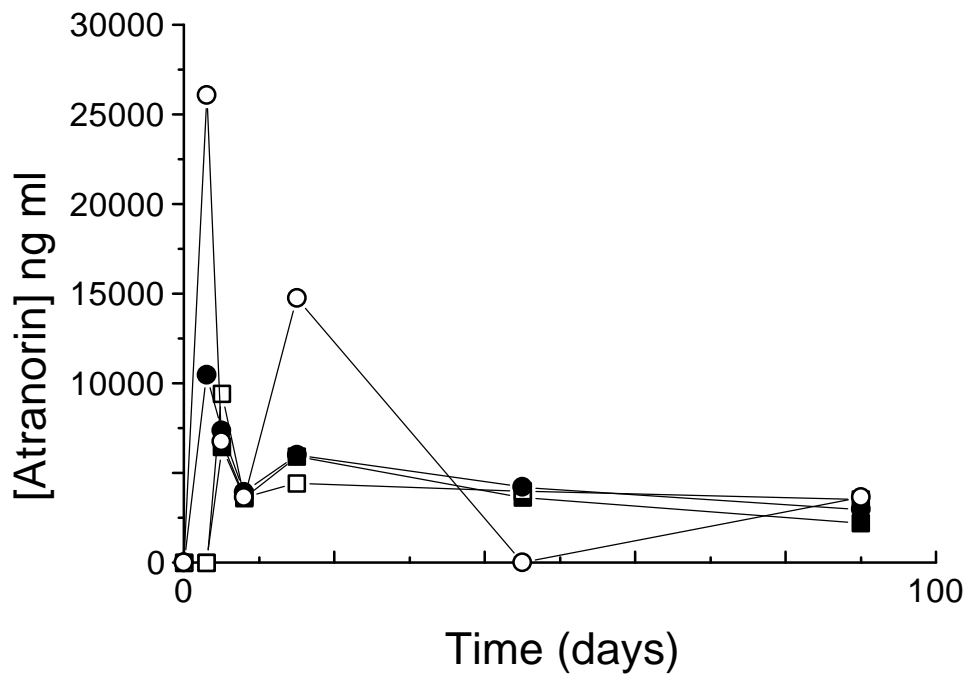


Fig. 2. Chromatographic profile in HPLC showing the separation of the major phenolic compounds of *C. verticillaris* and the characteristic retention time values for each one of these compounds.

Fig. 3 (below). Quantification of atranorin secreted to the incubation media by beads of immobilized cells supplemented with acetate (squares) or acetate plus FMN (circles). Cells were (empty symbols) or not (filled symbols) permeabilized with *iso*-propanol during incubation. Values are the mean of three replicates. Standard error was never higher than the symbols.



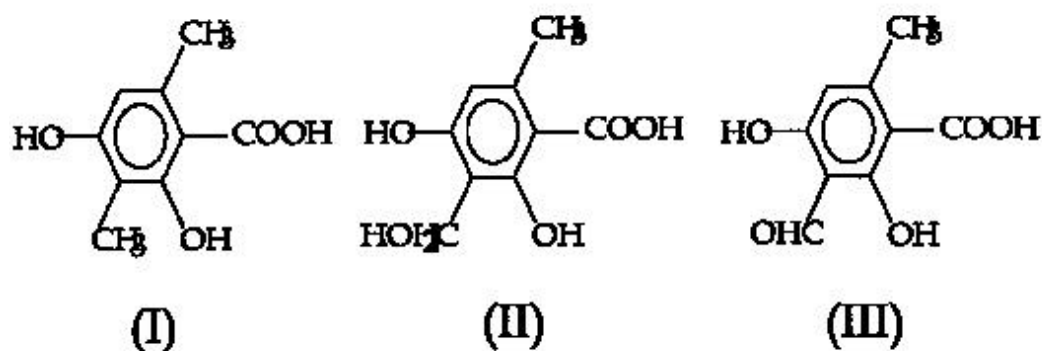


Fig. 4. The successive oxydation stages of methyl-3-orsellinate to produce the A ring of atranorin. In the B ring, the C1 substituent at 3' position remains as methyl group.

stabilized until the end of the experiment (90 days) in non-permeabilized cells. However, a net peak of atranorin production and secretion was observed at day 15 of incubation for permeabilized cells and followed by a decrease. This is consistent with cell permeabilization facilitating the uptake of both acetate and FMN and, consequently, the release of the newly synthesized atranorin to the medium. The very long period needed to use the exogenously supplied FMN by immobilized cells suggest that the enzyme which oxidized the methyl substituent of methyl-3-orsellinate, the first precursor of atranorin (I in Fig. 4), to an aldehyde function (III in Fig. 4) could be a NAD(P⁺)-dependent oxido-reductase rather than a flavoprotein with oxido-reductase activity and, thus, its affinity to flavine coenzymes could be lower than that shown for its adequate coenzyme.

Preservation of immobilized cell vitality

Observation of immobilized cells during the two first weeks of the incubation period showed adequate preservation of both fungal (Fig. 5A) and algal (Fig. 5B) ultrastructure. In fact, Molina *et al* (1994) found that, at this stage, both algal and fungal cells are able to divide inside the beads of alginate. After 90 days of incubation, observation by transmission electron microscopy showed a high degree of disorganization of the phycobiont cells, indicating an effective loss of vitality. The

ultrastructural deterioration seemed to be similar to that found after a parasitic action of the mycobiont on its algal partner, described in some unbalanced symbiotic states (Molina & Vicente 1996). The chloroplast completely disappeared and the cytoplasm was thoroughly occupied by large vacuoles, an unusual situation in full-functioning phycobionts (Fig. 5D). Some epiphytic bacteria were co-immobilized with both algal and fungal cells (Fig. 5C). According to Zook (1983), such bacteria may contribute to the maintenance of the symbiotic state.

Atranorin production by cells supplemented with NAD⁺

Despite this loss of vitality, the beads were washed with abundant sterile, distilled water and supplemented with acetate or acetate supplemented with NAD⁺ instead of FMN. Atranorin secretion was stabilized at very low values in non-permeabilized cells but those supplemented with NAD⁺ strongly secreted atranorin after 3 days of incubation, although this secretion decreased thereafter (Fig. 6). This clearly indicates a significant atranorin production under the influence of NAD⁺. Apparently, the atranorin production requires some oxidation reactions. Acetate supplied to immobilized cells must be used by a fungal aromatic synthetase to produce methyl-3-orsellinate (Fig. 4I), the precursor of atranorin. In natural conditions, acetate, or better acetyl-

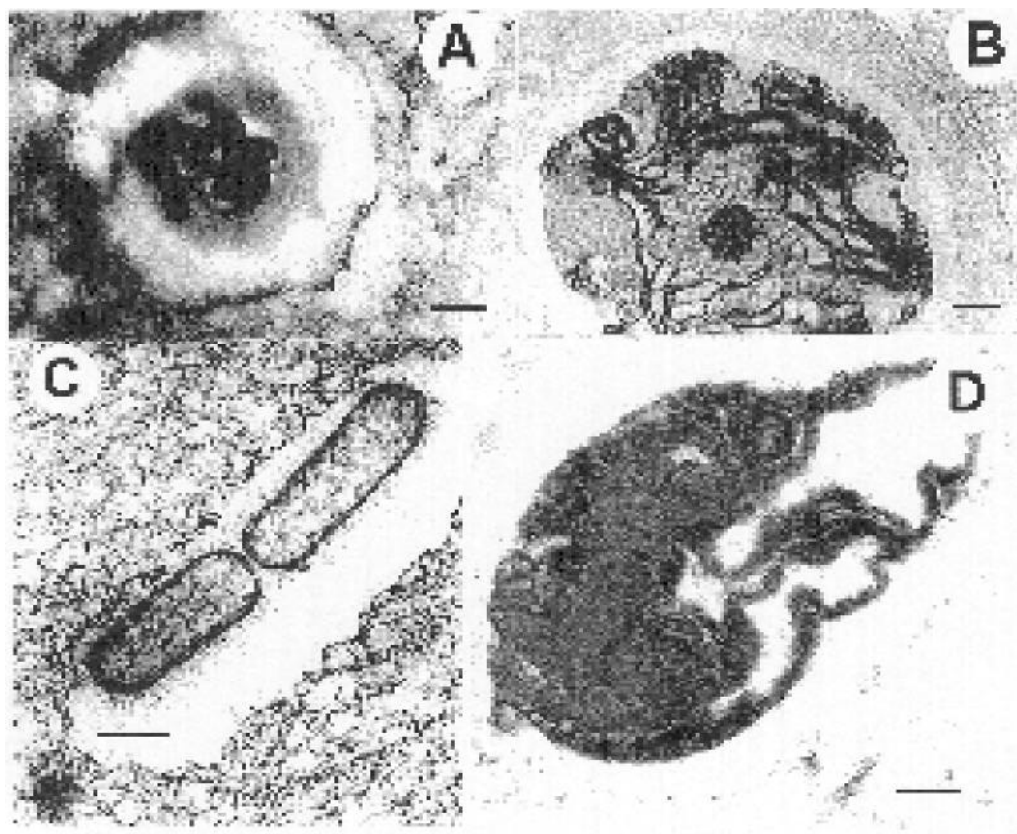


Fig. 5. Ultrastructure of immobilized lichen cells observed by transmission electron microscopy. In A) mycobiont cells and in B) phycobiont cells of recently immobilized thallus homogenate (bar = 0.5 μm). In C) epiphytic bacteria co-immobilized together with lichen cells (bar = 0.06 μm). In D), phycobiont cells of immobilizates after 90 days of incubation at room temperature on acetate plus FMN (bar = 0.3 μm).

CoA, is produced by the mycobiont after glycolytic degradation of part of sugars photosynthesized by the phycobiont and supplied to the fungal partner. Thus, immobilized fungal cells of *C. verticillaris* are independent from the sugars supplied by the lichen algae because they can obtain sufficient acetate to produce atranorin from the external solution. Since the A ring of the depside contains an aldehyde function at the 3-position whereas the first product of an aromatic synthase is methyl-3-orsellinate (Fig. 4.I), the methyl substituent must be oxidized to aldehyde probably through two successive oxidation reactions, first to alcohol (Fig. 4.II) and finally to aldehyde (Fig. 4.III) and probably this last reaction requires NAD^+ (or alternatively

FMN, working then oxido-reductases with low efficiency) as a cofactor.

In conclusion, we have herein reported a new method of atranorin production based on the use of immobilized cells that is of potential interest for the industry. This procedure to obtain atranorin is much more rapid and efficient than that of culture tissues and requires very small amounts of lichen thalli. It concerns a method to obtain a metabolite used on a large scale in perfume and cosmetics industries without large lichen biomass consumption.

Acknowledgements

This work was supported by a grant from the Dirección

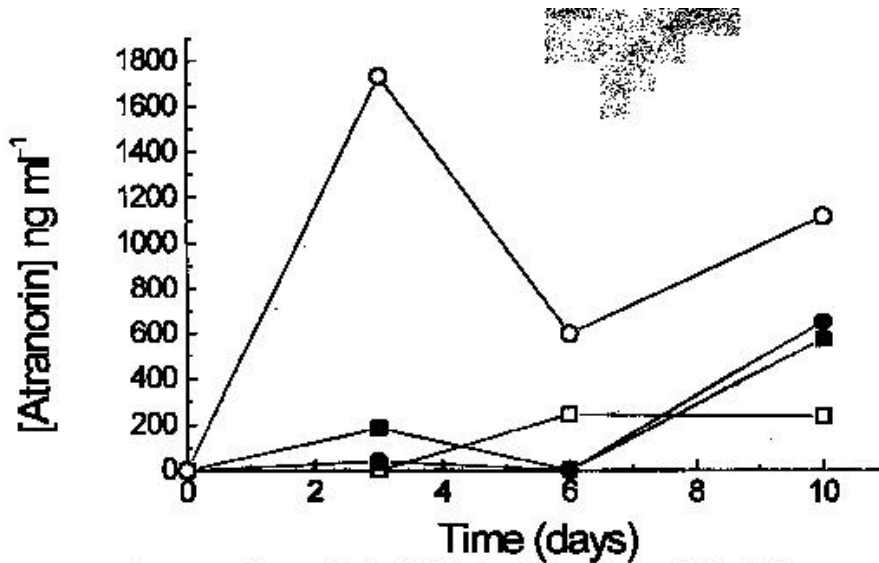


Fig. 6. Atranorin recovery from media in which the beads were incubated after 90 days on acetate (squares) or acetate plus NAD⁺ (circles). For the supplement of acetate plus NAD⁺ were used the corresponding beads previously incubated on acetate plus FMN. Empty symbols indicate permeabilized whereas filled symbols indicate non-permeabilized cells. Values are the mean of three replicates. Standard error was never higher than the symbols.

General de Investigación Científica y Tecnológica (Ministerio de Educación y Cultura, Spain), PB96 0662. We are indebted to M. Vicente-Manzanares for critical reading of the manuscript.

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