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RESEARCH ARTICLE

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Production of phenolics by immobilized cells of the lichen *Pseudevernia furfuracea*: the role of epiphytic bacteria

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Abstract Immobilized lichen cells from the thalli of the lichen *Pseudevernia furfuracea*, supplied with acetate as the only source of carbon, continuously produced phenolic substances, atranorin and physodic acid, over 23 days. Epiphytic bacteria associated with the lichen thallus grew actively, probably using both acetate and reduced compounds supplied by lichen cells, since their active growth was avoided by including 10 μM 3,3′-dichlorophenyl-1,1′dimethylurea in the bath solution. Penicillin largely impeded the growth of epiphytic bacteria and decreased phenolic production, which was recovered only at the end of the experimental period, just when the bacteria started a slow, but active growth. We suggest the cooperation of epiphytic bacteria in the biosynthesis of both atranotrin and physodic acid.

Keywords *Pseudevernia furfuracea* · Cell immobilization · Phenolics · Lichens · Epiphytic bacteria

Introduction

Lichen phenolics are unique compounds in the Plantae and Fungi kingdoms. They are composed of two monocyclic phenols joined either by an ester bond (depsides), both ester and ether bonds (depsidones) or a furane heterocycle (dibenzofurans, usnic acids). These substances have been widely used in folk medicine, cosmetics and perfume industry for centuries. Moreover, lichen phenolics have long been used as powerful topic antibiotics against Gram-positive bacteria [1,21]. Their high cytotoxic activity is now investigated for their use as carcinostatic agents [10]. Some lichen despidones, such as virensic acid and granulatin, are active against the human immunodeficiency virus (HIV) by inhibiting viral integrase [12]. In addition, vulpinic acid is anti-inflammatory [6], whereas usnic and diffractaic acids are analgesic and antipyretic [3, 14].

Therapeutic cosmetics use several depsides to prevent skin aging by inhibiting the enzymes involved in the process; and these are largely inhibited by atranorin [18]. The depsidones pannarin and 1′-cloropannarin [5] and the depside atranorin largely inhibit elastase and trypsin. However, the use of atranorin as a photoprotector must be carefully evaluated before utilization, since this depside produces severe allergic dermatitis, without any light requirement, in sensitive patients [2, 19].

Despite the importance of such compounds, the main pathways of their biosynthetic processes are hardly known [4, 8, 22]. Bioproduction of lichen phenolics can be achieved, at a semi-industrial scale, by means of immobilization techniques of cells and enzymes, as an alternative to the use of large amounts of lichen biomass to isolate lichen compounds or to study biosynthetic phenolic enzymes. These techniques have great advantages, such as the very small biomass required for immobilization, its inexpensive maintenance and the very fast rate of metabolite production.

For example, several *Cladonia* species produce atranorin. Immobilized cells of *C. verticillaris* in 4% calcium alginate can produce atranorin when they are supplied with 1.0 mM acetate as a precursor. The addition of an oxidant agent (NAD^+ or FMN) to the incubation media enhances the production of the depside; and its secretion into these media is facilitated by permeabilizing immobilized cells with 2% *iso*-propanol [20]. Cells of the lichen *C. substellata*, when immobilized in kaolinite and supplied with acetate, produce large amounts of usnic acid at room temperature, which can be recovered from the washing solution. The production of usnic acid starts on day 4 after immobilization and depends on the concentration of the precursor supplied, being maximum for 10 mM sodium acetate [16].

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However, production of usnic acid by cells of *C. corallifera* saturates from 1.0 mM acetate, probably because this phenol feed-back regulates several of the enzymes involved in the overall process [17].

As yet, no studies on the role of epiphytic bacteria on the activity of lichen immobilisates have been performed, although they have been found in several immobilized systems. No effect of these epiphytic bacteria on the stability of immobilisates has been reported. However, when glucose is supplied instead of acetate to immobilized cells of *C. substellata*, catabolite repression prevents usnic acid production. In addition, proteobacteria rapidly develop and disaggregate the immobilisates. This indicates that these antagonist microorganisms are probably foreign contaminants rather than epiphytic bacteria.

Materials and methods

Lichens growing on *Pinus sylvestris* L. were collected in Valsain (Segovia, central Spain). About 2.0 g dry weight of lichen thalli were gently macerated in a mortar and mixed with sufficient amount of polyhydroxyurethane spheres (≤ 3 mm diameter), packed in a 20×3 cm cylindrical column supplied with 50 ml of 1.0 mM sodium acetate. Alternatively, 10 μM 3,3′-dichlorophenyl-1,1′-dimethylurea (DCMU), a herbicide that uncouples the photosynthetic non-cyclic electron transport, was added to the nutrient solution on day 11. When indicated, 10-ml samples were removed from the column and replaced by 10 ml of fresh acetate solution. In parallel, a second bioreactor was used, including 3,000 units of sodium penicillin in the wash medium. Quantitation of viable bacteria was performed from 1.0 ml of this sample. Phenolics were extracted according to Pedrosa and Legaz [15], by mixing with the bath sample 9.0 ml of diethyl ether:ethyl acetate (65:35, v/v) and strongly shaking for 5 min. The organic phase was recovered and the medium was newly mixed with 9.0 ml of chloroform:acetonitrile (50:50, v/v). After shaking for 5 min, both organic phases were mixed and dried in an air-flow. Solid residue was redissolved in 2.0 ml of methanol for chromatography. High-performance liquid chromatographic separation was carried out by using a Varian 5000 liquid chromatograph equipped with a Vista CDS 401 computer according to Legaz and Vicente [11]. 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 in isocratic mode; pressure: 76 atm; temperature, 25 °C; detector: UV set at 238 nm. Atranorin from Sarsyntex (France) was used as an external standard.

Results and Discussion

Bioreactors of *Pseudevernia furfuracea* cells immobilized by adsorption in polyhydroxyurethane have been used to verify the hypothetical action of epiphytic bacteria on the biosynthesis of lichen phenolics. As shown in Fig. 1, the number of viable bacterial cells strongly decreased when penicillin was added to the bath medium, but it increased linearly in the absence of the antibiotic. However, a lag phase was established for approximately 4 days before the accelerated growth phase. The acceleration of growth rate coincided with the maximal accumulation of phenolics. This can be interpreted as a positive co-operation between lichen cell metabolism and bacterial growth. In fact, adding 10 μM DCMU to the medium in the bioreactor on day 11 resulted in a clear decrease in the bacterial growth rate (Fig. 1). It is possible that, rather than using acetate supplied from the medium, epiphytic bacteria used reduced (or reducing) metabolites obtained from the lichen cells, such as sugars or glutathione, largely produced by *P. furfuracea* [9]. This would agree with the observation about the blackening of monumental stones caused by sulfur bacteria associated with crustose lichens, which used the reducing power derived from the photosynthetic activity of the algal partner to reduce sulfate to sulfide [13].

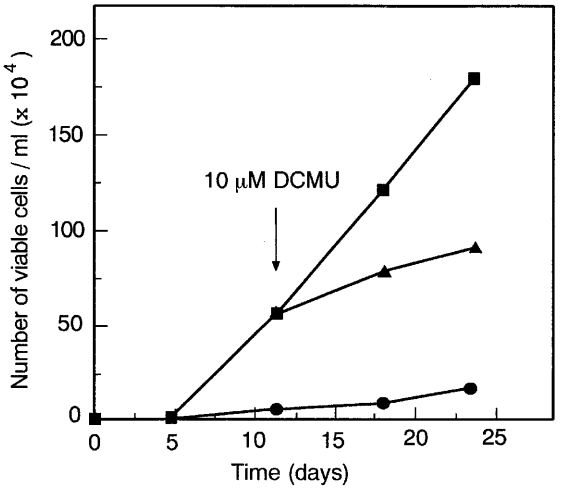

Fig. 1 Growth of bacterial cells in immobilisates of *Pseudevernia furfuracea* in polyhydroxyurethane supplied with 1.0 mM sodium acetate (■) or 1.0 mM sodium acetate containing 3,000 units of penicillin/ml (●). Arrow indicates the addition of 10 μM 3,3′-dichlorophenyl-1,1′dimethylurea (*DCMU*) to the acetate-containing medium (▲). Initial number of cells is 0.77×10^4

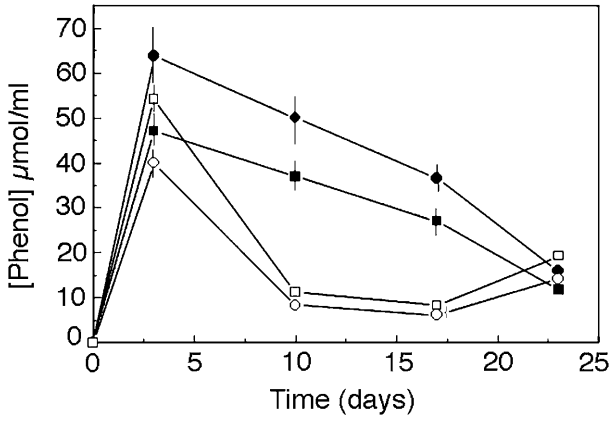

Fig. 2 Atranorin (*circles*) and physodic acid (*squares*) secretion to the bath media by immobilized cells of *P. furfuracea* in polyhydroxyurethane supplied with 1.0 mM sodium acetate (*filled symbols*) or 1.0 mM sodium acetate containing 3,000 units of penicillin/ml (*empty symbols*). The addition of DCMU does not significantly modify the production of the phenolics. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols

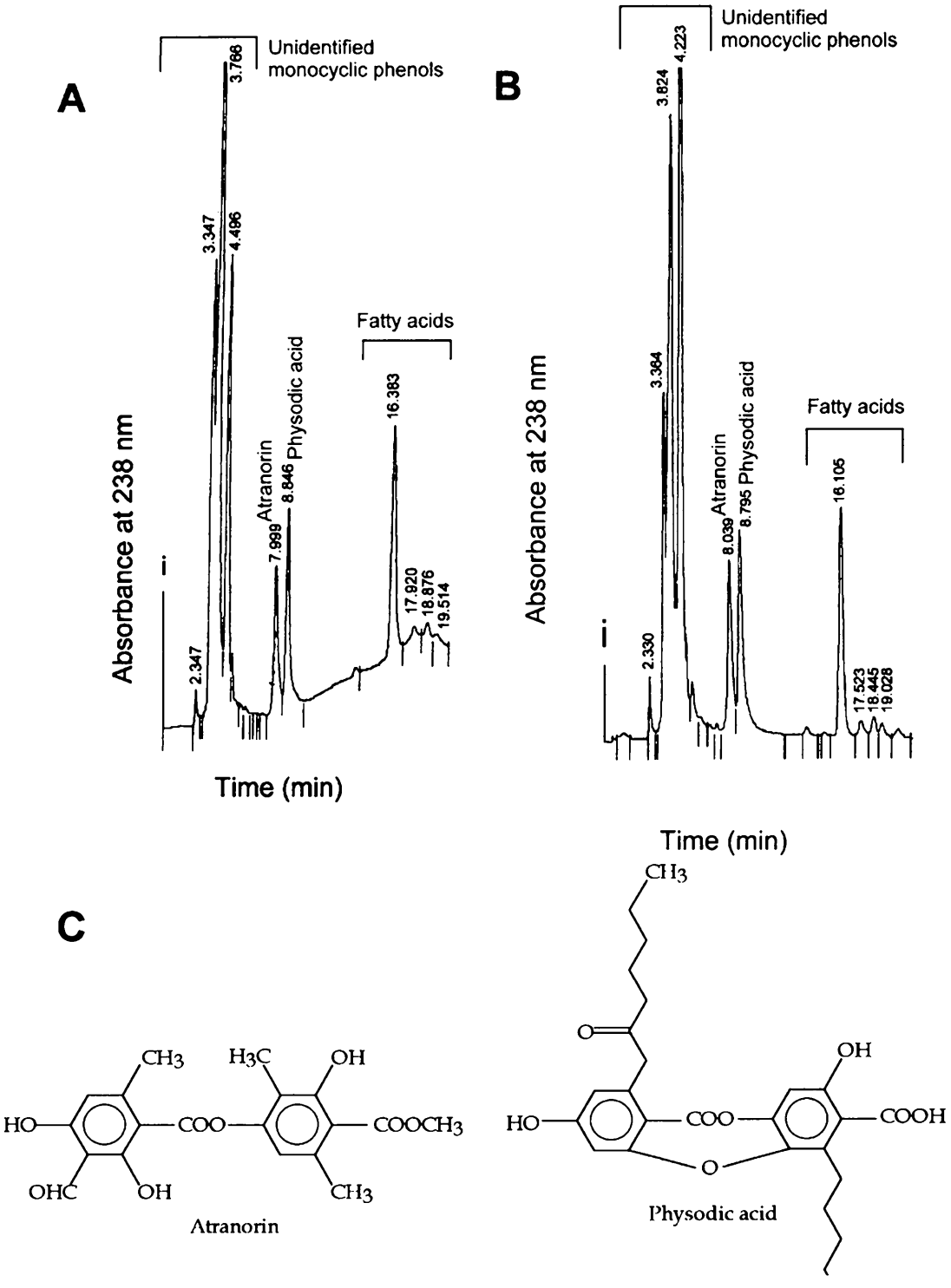

Fig. 3A–C Phenolics produced by *P. furfuracea*. **A** Chromatographic profile of phenolics produced and secreted to the bath medium by *P. furfuracea* cells immobilized in polyhydroxyurethane and supplied for 4 days with 1.0 mM sodium acetate. *i* Injection. **B** Chromatographic profile of phenolics produced and secreted to the bath medium by *P. furfuracea* cells immobilized in polyhydroxyurethane and supplied for 4 days with 1.0 mM sodium acetate containing 3,000 units of penicillin/ml. *Each number* near a peak indicates the retention time (min). **C** Structural formulae of atranorin and physodic acid

Hence, the lag phase of bacterial growth in *Pseudevernia* bioreactors shown in Fig. 1 must be interpreted as the time required to secrete sufficient amount of these reduced compounds to support bacterial growth. The addition of DCMU does not significantly modify the production of both phenolics. However, the pattern of phenol biosynthesis is almost identical to that observed in absence of the inhibitor (DCMU; Fig. 2). This implies

that the fungal component has a sufficient amount of accumulated sugar to support the production of phenols during the complete period of experimentation.

The main phenolics produced by *P. furfuracea*, atranorin and physodic acid, were recovered from bath media with a clear maximum on day 4 after immobilization, decreasing linearly later, in the absence of penicillin. When the antibiotic was included in the medium, concentration of both phenolics also decreased from day 4, but increased slowly from day 17 after immobilization (Fig. 2). This could be interpreted as a degradation of lichen phenolics by the action of contaminating, epiphytic bacteria. However, the qualitative analysis of phenolics secreted from the immobilized lichen cells to the media, shown in Fig. 3A and B, show that there are no significant differences between the phenolics secreted, regardless of the absence or presence of penicillin. Peaks in chromatographic profiles with retention time values of 2.35–4.50 min are interpreted as different monocyclic phenols, precursors or catabolites of the main depside and depsidone and, in this way, the absence of penicillin does not promote the appearance of new peaks, which would reveal the accumulation of new degradation products. Thus, the apparent inhibition of phenolic production by immobilisates with increasing bacterial populations may be explained as a competence between lichen cells and epiphytic bacteria for a unique and limiting pool of exogenous acetate as the only carbon source for growth.

The production of phenolics by immobilized lichen cells loaded with penicillin decreased more than in non-treated bioreactors. One explanation for this could be that lichen cells used some cofactors provided from bacterial metabolism to synthesize both atranorin and physodic acid. The hematommic acid moiety of atranorin is characterized by an aldehyde function as a substituent at C3 (Fig. 3C), which implies an oxidation of the original methyl group of the first product of an aromatic synthase, methyl-3-orsellinate, to alcohol and then its reduction to aldehyde through two consecutive reactions involving NAD^+/NADH [22]. Physodic acid incorporates CH_3-(CH_2)-CO– at the C6 position (Fig. 3C), probably from the corresponding acyl-CoA [7]. A critical bacterial biomass could be required to supply some of these cofactors, as the final increase in phenolics production suggests, but this is a hypothesis to be experimentally confirmed.

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