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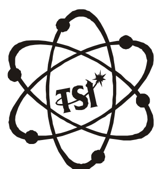

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Production of rhodocladonic acid by immobilized cells of *Cladonia miniata* var. *parvipes* in calcium alginate

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ABSTRACT

Cell aggregates of *Cladonia miniata* var. *parvipes* immobilized in calcium alginate were used to assay their ability to produce rhodocladonic acid. Immobilisates were supplied with sodium acetate or calcium acetate as precursors for phenol biosynthesis, and with ampicillin to prevent bacterial contamination. Rhodocladonic acid was actively produced during incubation of immobilized cells mainly on calcium acetate, although cell vitality progressively decreased since long exposures of alginate to calcium ions increased the rigidity and mechanical resistance of immobilisates and produced cell plasmolysis. Ampicillin slightly improved the production of rhodocladonic acid, probably by impeding the competition of bacterial populations using acetate as a source for growth and the use of rhodocladonic acid by lowly specific redox enzymes.

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KEYWORDS

Alginate;
Ampicillin;
Cell immobilization;
Cladonia miniata var. *parvipes*;
Rhodocladonic acid.

INTRODUCTION

Cladonia miniata var. *parvipes* produces two main lichen phenolics, rhodocladonic and barbatic acids (**Figure** 1). Barbatic acid is a depside from the β-orcinol series composed of two units of orsellinate derivatives that are synthesized by an aromatic synthase[1], containing one subunit with methyl-transfer activity[2]. An 8C lineal polyketide intermediate is produced before cyclization to form methyl-3-orsellinate. Depsides from the orcinol and β-orcinol series are produced by esterification of two orsellinate derivatives[3], catalyzed by several orsellinate depside hydrolases[4], thus acting as esterases.

On the other hand, the most widespread quinones in lichens are antraquinones, such as parietin from *Xanthoria parietina* and emodin from *Nephroma laevigatum*. Synthesis of antraquinones has been studied in this last lichen species which not only produces emodin but also some emodin derivatives. When thalli of *N. laevigatum* are supplied with ^{14}C-2-acetate or ^{13}C-2-acetate, emodin is synthesized through the acetate-malonate pathway from eight molecules of acetyl-CoA[5], whereas its chlorination is specifically achieved on C7 position by an haloperoxydase, characterized from the same lichen species[6]. *In vivo* chlorination of

secondary metabolites is carried out by chloroperoxy dases which are also able to catalyze bromination and iodination of several substrates[7,8]. Before the characterization of the chloroperoxydase from *N. laevigatum* by Cohen and Towers[6], only a bromoperoxydase from *X. parietina* has been described. The enzyme contains vanadium, essential for its catalytic activity and it shows high affinity by bromide but it is inhibited by chloride and fluoride[9]. Rhodocladonic acid was firstly considered as an antraquinone but it is now defined as a furan-naphtoquinone after more precise structural analyses[10]. Lichens synthezise naphtoquinones through the acetate-malonate pathway, coincident in part with some common enzymatic reactions of the biosynthetic pathway of barbatic acid, although higher plants may produce other naphtoquinones by four different ways[11].

Studies on the synthesis of lichen phenols has often been carried out by using immobilized cells and enzymes[12,13]. Cell aggregates of *Lasallia pustulata* were immobilized in 20% polyacrylamide to facilitate the study of a process involving at least two consecutive enzymatic reactions, the hydrolysis of lecanoric acid by an orsellinate depside hydrolase to produce two molecules of orsellinic acid and the subsequent decarboxylation of orsellinic acid to produce orcinol[14]. Although polyacrylamide induces cell death, the enzyme remains active for many days.

Cell immobilization in calcium alginate preserves cell vitality. Cells of *Evernia prunastri* immobilized in calcium alginate produce the depside atranorin from sodium acetate. The synthesis of the depside involves molecular oxygen and NADH, required by an oxidase and an alcohol dehydrogenase respectively, to produce haematommic acid, the orsellinate derivative involved in atranorin production[15], a pathway that has been demonstrated for the first time by using lichen cells immobilisates. Alginate immobilisates of *Cladonia verticillaris* were also used to elucidate the origin of the carboxylic C4 chain which esterified the hydroxyl function attached at C3' position in the molecule of fumarprotocetraric acid[16]. However, the supply of sodium acetate as a substrate for depside synthesis produces swelling of immobilisates and loss of cell material to the media, since sodium replaces calcium in the alginate mesh. Alginate immobilisates have also been used to produce not only lichen phenolics[17] but also other metabolites, such as ribitol or mannitol[18].

In the present paper it is demonstrated for the first time the production of a furan-anthraquinone, rhodocladonic acid, using a very simple system to immobilize cells of *Cladonia miniata* var. *parvipes* in calcium alginate. The study has been carried out by supplying sodium and calcium acetate as a substrate in order to improve the efficiency of immobilisates in furan-naphtoquinone production and potential changes of immobilisate structures. The use of calcium instead of sodium acetate is proposed to avoid disaggregation of immobilisates achieved by the displacement of calcium from the matrix due to sodium exogenously supplied. This preliminary study, once optimized the production of rhodocladonic acid, could be applied for studying enzymes involved in the process, as revealed for other lichen phenolics[19], or even to improve the industrial production of this compound, commonly used in the tint of textile fabrics and in perfumery[10].

MATERIAL AND METHODS

Cell immobilization

Cladonia miniata Mey. *var.* parvipes (*Vain.*) Zahlbr. was collected in the Serra de Itabaiana (NE Brazil) on burned wood. Thalli were dried under airflow conditions and stored at room temperature in the dark until required.

Samples of 0.5 g dry thalli were completely rinsed in distilled water and gently macerated in a mortar to obtain an homogenate; 100 mL sodium alginate, medium viscosity, solution (4% w/v) were added and mixed with the homogenate and the mixture was extruded dropwise with a syringe into 50 mL 0.2 M $CaCl_2$[20]. The beads of calcium alginate were supplemented with 50 mL sodium acetate or calcium acetate, with or without 0.2 mg ampicillin mL^{-1}, and maintained, gently stirred, at 22^0C for 20 days in the light of a photon flux rate of 150μmol m^{-2} s^{-1}. An aliquot of 5.0 mL bath solution was collected at different times to extract lichen phenolics and replaced with the same volume of fresh medium.

Phenolics extraction and analysis

Phenolics were extracted by mixing the aliquot of the bath solution with 5 mL diethyl ether:ethyl acetate

(65:35 v/v). The mixture was vigorously stirred and the organic phase separated with a micropipette. Aqueous phase was then extracted with 5 mL chloroform:acetone (50:50 v/v), stirred for 5 min and the organic phase recovered and mixed with the first one[21]. Organic extracts were dried in air-flow. The residue was redissolved in 2-0 mL methanol and analysed by HPLC according to Feige et al[21]. Using a Varian 5060 liquid chromatograph equipped with a Vista CDS 415 computer. A reverse phase MCH-10, 5 μm particle diameter, 250 mm × 4.6 mm column was used. Two solvent systems were employed. System A was MilliQ water containing 1% (v/v) ortho-phosphoric acid and system B was 100% methanol. The run started with 30% B and continued isocratically for 1 min at 0.7 mL min-1. After this, 10 μL was injected and the solvent B was increased to 70% within 14 min, then up to 100% in 30 min, and then isocratically in 100% B for a further 8 min. At the end of the run, system B was decreased to 30% within 1 min and the column flushed with 30% B for 16 min before a new run was started. Detection was carried out by a UV set at 254 nm. Barbatic acid was used as an external standard. The amount of rhodocladonic acid recovered from the medium is referred to the mass of immobilized thallus, 0.5 g of lichen tissue by each bioreactor. The mass of alginate was not included then in the quantification of the amount of the furan-naphtoquinone.

Eluted peak tentatively identified as rhodocladonic acid was analyzed by MS. Mass spectra was recorded on a VG micromass 7070F mass spectrometer at 70 eV linked on line to a Finnigan Incos data system. The detector was a quadrupole used in scan mode from m/z of 35 to 650[22].

Light microscopy analysis

Sections of immobilisates of about 8 μm thickness were obtained by using a freezing microtome and observed using a Zeiss invertomicroscope. Photographs were obtained by a Coolpix 5000 digital camera from Nikon.

Assay of cell vitality

Immobilisate were resuspended in 10 mL distilled water containing 13μg 2,6-dichlorophenol indophenol (DCPIP) and irradiated with white light (photo flux density 200 μmol m^{-2} s^{-1}) for 5 min. Decrease of absorbance at 600 nm was measured[23].

Assay of gel viscosity

Viscosity of calcium alginate gels was measured, before and after incubation on both sodium and calcium acetate solutions, using a Schott AVS 450 viscosimeter, according to the instructions of the manufacturer. The liquid core of alginate beads was extracted, whereas it was possible, with a microsyringe and placed into the viscosimeter. Measurements were achieved at 20^0C.

RESULTS AND DISCUSSION

Vitality of immobilisates

Disaggregated, fully rehydrated thalli of *C.miniata* var. *parvipes* were immobilized in 4% (w/v) calcium alginate, supplied with 10 mM sodium or calcium acetate in light and maintained at room temperature (~22^0C) for 20 days. Vitality of immobilisates, estimated as the ability of immobilized algae to photoreduce DCPIP, is shown in figure 2. A loss of about 41.5% of the photosynthetic ability of immobilisates was recorded at 17 days of incubation on sodium acetate, whereas this capacity was reduced to 17% from the initial activity (zero time) when immobilisates were supplied with 10 mg ampicillin which prevented bacterial growth. The

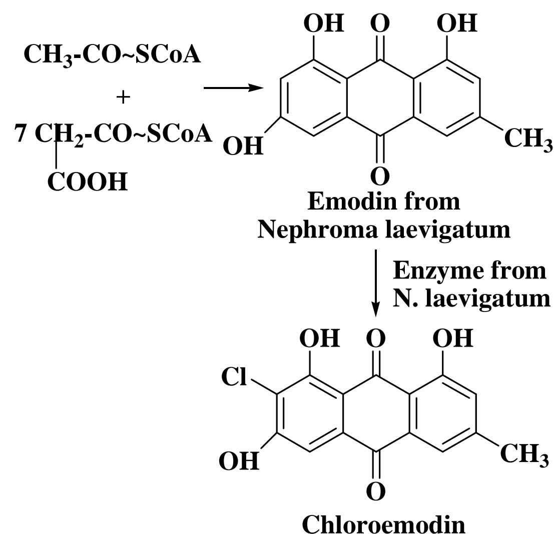

Figure 1: Chemical structures of barbatic and rhodocladonic acids, the main components of the chemosyndrom of *C.miniata* var. *parvipes*

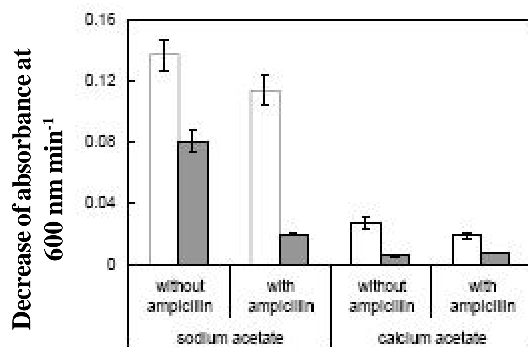

Figure 2: Maintenance of cell vitality of immobilisates of *C.miniata* var. *parvipes* in calcium alginate supplied with sodium or calcium acetate in the light. Vitality has been estimated as the ability of algal cell to photoreduce DCPIP at zero time (white rectangles) and after 17 days (grey rectangles) incubation. Media were supplied with or without ampicillin, as indicated. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols

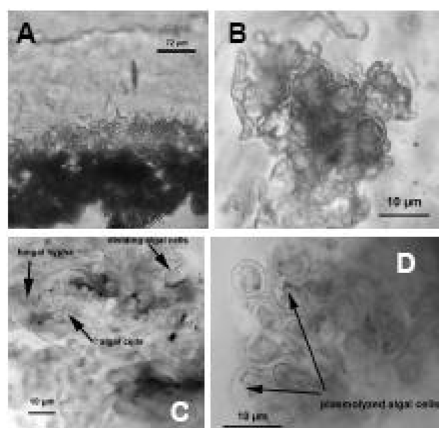

Figure 3: Cross sections of thallus of *C.miniata* var. *parvipes* recently collected (A), of cell aggregated immobilized in calcium alginate and maintained for 15 days in sodium acetate (B) or in calcium alginate (C and D)

loss of the ability to photoreduce DCPIP varied from 22% to 40% from the initial activity when immobilization of lichen cells was achieved by using calcium acetate as a nutritional supply (Figure 2).

Structural stability of immobilisates

C.miniata var *parvipes* possesses a cylindrical thallus showing a dense cortex (Figure 3A) below which a thin layer of algal cells can be observed. Fungal medulla limited a central, air-filled space inside the structure. When disaggregated cells were immobilized in calcium alginate and supplied with sodium acetate, many

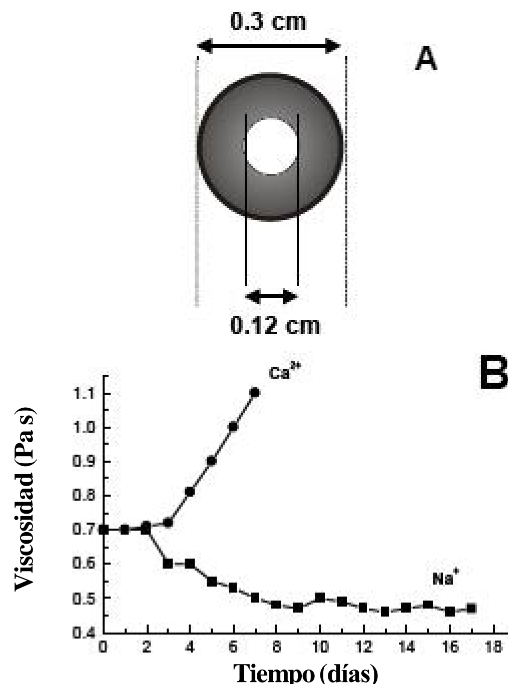

Figure 4: (A) Diagrammatic representation of one alginate bead, of approximately 0.3 cm in diameter, with a liquid core of about 0.12 cm in diameter. Alginate from this core was extracted with a microsyringe and assayed for viscosity changes. (B) Time-course of viscosity of alginate-bead core during incubation of immobilisates on 10 mM sodium acetate or calcium acetate

algal cells maintained physical contact with fungal hyphae (Figure 3B) and the cytoplasm of the phycobiont occupied the complete volume of algae although an incipient plasmolysis was sometimes observed. The physical contact between myco- and phycobiont was maintained, in some extent, in immobilizates supplied with calcium acetate (Figure 3C), although many algal cells appeared strongly plasmolyzed (Figure 3D). Probably, the continuous supply of calcium cations increased the rigidity and mechanical resistance of the matrix changing the liquid core into a gelatinous state[24] (Ertesvåg et al. 1996). This has been confirmed by analysing changes of viscosity of alginate beads after incubation of sodium or calcium acetate. As it is shown in figure 4, continuous incubation of beads on sodium acetated slightly diminished the viscosity since Ca^{2+} was replaced by Na^{+} in some extent[24]. However, continuous supply of Ca^{2+} for 17 days of incubation rapidly increased the viscosity of alginate beads, since this cation slowly diffused into the beads to reach the liquid core of immobilisates. Viscosity of the immobilisates core maintained in cal-

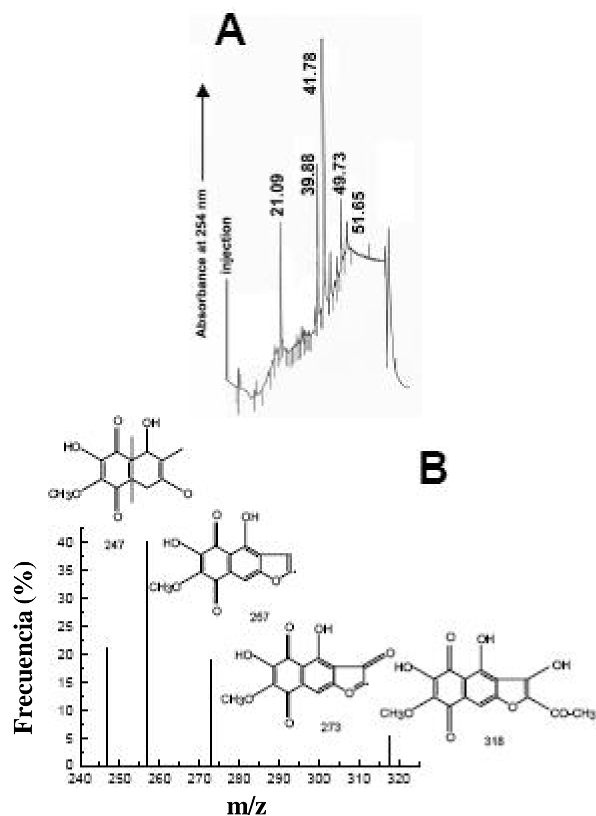

Figure 5: (A) Chromatographic trace of total phenols produced extracted with acetone from *C.miniata* var *parvipes* thalli. Numbers near the peaks indicated retention time value in min. B) Mass spectrum of the substance eluted at 49.73 min in HPLC

cium acetate cannot be measured because of this core must be too viscous to be removed with a microsyringe.

Separation and identification of rhodocladonic acid isolated from immobilisates bath

Organic-soluble metabolites produced by immobilisates and secreted to the medium were extracted with organic solvents, as described above, and analyzed by HPLC. Standard barbatic acid produced an efficient peak with a retention time value of 41.78 min. This peak represented more than 52% of the total area counts recorded in chromatograms obtained after analysis of the phenolics extracted from recently collected thalli (Figure 5A). Minor peaks at 21.09 min, 39.88 min and 51.65 min were also obtained, but they represented less than 7% of the total area counts. The peak eluted at 49.73 min has been identified as rhodocladonic acid, since its mass spectrum produced fragments with mass (m/z) values of 318, 273, 257 and 247, identical to those described by Huneck and Yoshimura[25] for this furan-naphtoquinone. The mass spectrum as well as the structure of these fragments is shown in figure 5B.

Efficiency of the production of rhodocladonic acid by lichen immobilisates

The amount of rhodocladonic acid extracted from the incubation media containing sodium acetated rapidly decreased from 7 days of incubation of immobilisates with independence on the presence or absence of ampicillin. However, the production of the furan-naphtoquinone was slightly recovered after 10 days of incubation, mainly by immobilisates maintained on ampicillin (Figure 6A) and this increase was continuously achieved to 23 days, whereas that found in the absence of ampicillin newly deseapeared from 17 days incubation. The production of rhodocladonic acid by cell immobilisates supplied with calcium acetate oscillated but a clear maximum at 15 days of incubation was found. This production was not significantly affected by the addition of ampicillin to the incubation media (Figure 6B).

The strong decrease of the rhodocladonic acid production observed in immobilisates supplied with sodium acetate (Figure 6A) cannot be explained by a loss of cell vitality since this loss was enhanced by a supply of calcium acetate (Figure 2) and the production of rhodocladonic acid was nevertheless recovered after 10 days of incubation. In addition, plasmolysis did not affect rhodocladonic acid production (Figure 3D) although it could be related to the lost of vitality (Figure 2). Since plasmolysis of phycobionts implies water stress, which causes decrease of the photosynthetic rate[26], it can be concluded that the photosynthetic supply of metabolites from algae to the fungal partner is not required for the production of rhodocladonic acid when the uptake of exogenous acetate by immobilisates is achieved, although immobilisates were maintained in continuous light. Several epiphytic and saxicolous lichens coexist with epiphytic bacteria, some of those sulfobacteria from the *Desulfotomaculum* genus and related[27]. These bacteria, in addition to their ability to produce sulfide from sulfates, also produce hydrogenases that actively reduce many anthraquinones[28]. Morevover, bacterial and fungal peroxidases can oxidize these antraquinones and after this, quinone nuclei

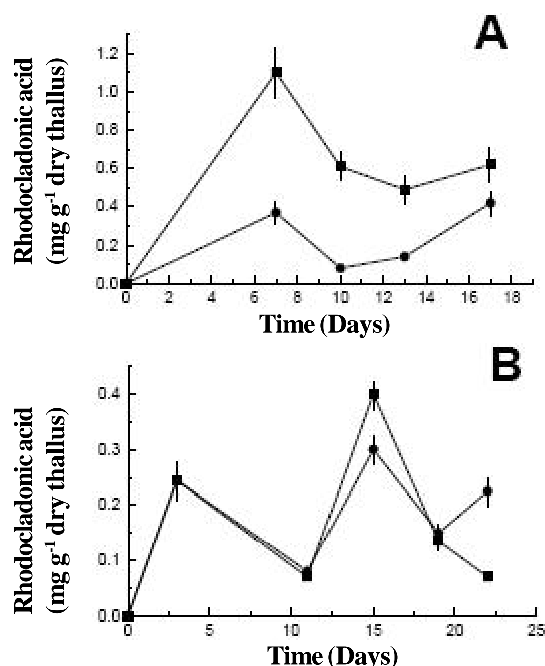

Figure 6: Time-course of rhodocladonic acid production by immobilisates of *C.miniata* var. *parvipes* in calcium alginate supplied with sodium or calcium acetate in the light. Media were supplied with or without ampicillin. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols

could be broken[29]. The loss of rhodocladonic acid from the media where immobilisates are supplied with sodium acetate in absence of ampicillin could be correlated to the slight loss of vitality of the immobilized lichen cells. However, this loss highly increases in the presence of the antibiotic without a sensible change in the recovery of the furan-naphtoquinone (Figures 2 and 6). Thus, it can be hypothesized that epiphytic bacteria, immobilized together lichen cells, collaborate in the maintenance of an amount of rhodocladonic acid that do not induce cell death. This hypothesis is based on the well-known phycocide activity of many lichen phenolics[30] and could be supported by a better permeation of the antibiotic through the external layer of calcium alginate towards the liquid core of the beads, whereas the increase of the rigidity of beads supplied with calcium acetate (Figure 4) could decrease the entry of ampicillin to the gellified core of the immobilisates[24,31], (minimizing the antibiotic action (Figure 6B).

As a conclusion, a supply of sodium acetate to immobilized cells of *C.miniata* var. *parvipes* in calcium alginate assures the production of rhodocladonic acid. A supply of calcium instead of sodium acetate stabilizes immobilisates although decreases the furan-naphtoquinone production by reducing the flow of acetate inside the immobilisates. This technique of immobilization could be used to the study of the enzyme involved in the production of rhodocladonic acid.

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