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Research Article

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Production of Secondary Metabolites by Immobilized Cells of *Stereocaulon ramulosum* (Sw) Rausch (lichen) in Different Design of Bioreactors

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ABSTRACT

Lichen cells from the thalli of Stereocaulon ramulosum were maintained with kaolinite and sodium acetate as precursor of phenolic biosynthetic pathway in three different systems: continuous flow, fixed and continuous agitation. Fractions were collected and monitored by TLC and spectrophotometry, showing increases in the concentration of metabolites produced by the cells, being the most effective method continuous flow. The cells showed vitality after submission in liquid medium, and capable of producing. This methodology shows the advantage of being economical and effective for the production of substances of pharmacological interest from lichens without causing environmental damage or collection of large amounts of material for obtaining significant amounts of substances.

Keywords: Bioproduction; Chilean lichens; *Stereocaulon ramulosum*

INTRODUCTION

Lichens comprise about 17000 species are symbiotic organisms composed of a fungus that is associated a photosynthesizing partner that can be either an algae or a cyanobacterium or both. In all lichens, the fungus forms a thallus or lichenized stroma that may contain characteristic secondary metabolites, mainly of them with any kind of bioactivity. Several lichen extracts have been used for various remedies in folk medicine, and a variety of biological activities of lichen metabolites, including antibiotic [1,2], antimycobacterial [3], antiviral [4], anti-inflammatory [5,6], allelophatic [7] and antipyretic properties [8], have been indicated by screening processes. Thus, there are considerable interest in lichen metabolites as potential sources of pharmacological agents. The chemistry of about one third of all lichen species has been studied up to now, and about 350secondary metabolites are known from lichens. The chemical structures of approximately 200 of them have been established. They are extracellular products of relatively low molecular weight crystallized on the hyphae cell walls. They are usually insoluble in water and can be extracted into organic solvents. The amount of these compounds is between 0.1 and 10% of thallus dry weight, and sometimes up to 30% [9]. The lichen-forming Ascomycete genus *Stereocaulon* (Stereocaulaceae, Lecanorales) traditionally includes species that have a dimorphic thallus consisting of a crustose primary thallus and a fruticose secondary thallus. The primary thallus consists of basal granules or squamules (phyllocladia) that are tightly attached to the substrate. In most of the species the primary thallus disappears early in the development. The fruticose secondary thallus, or pseudopodetium, arises from the primary thallus by the elongation of thalline tissue into often richly branched stalks. Pseudopodetia support persistent phyllocladia or phyllocladoid branchlets, apothecia, and inmost species cephalodia [10]. *Stereocaulon* has been reported by producinga series of metabolites with biological activity. For example, compounds with structure type depsidones produced by *S. alpinum* showed activity against inhibition of platelet-type 12(*S*)-lipoxygenase with IC_{50} of 28.5 µM [11], pseudodepsidones 1 and 2 showed inhibition of protein tyrosine phosphatase 1B (PTP1B) with MIC of 6.86 and 2.48 µM, respectively [12], and depsides with MIC values against *Mycobacterium aurum* superior to 125 µg/mL [13]. *Stereocaulon azoreum* and *S. sasakii* produce compound type lobaric acid with activity over different therapeutic blank, as inhibition of arachinodate 5-lipoxygenase with IC_{50} of 7.3 µM, inhibition of cyclooxygenase with IC_{50} of 29.2 µM, inhibition

of DNA synthesis in T-47D, ZR-75-1 from breast carcinomas and K-562, inhibition of proliferation of mitogen-stimulated lymphocytes, significant inhibition of contractile activity of Guinea pig *Taeniacoli* at 5.8 µM and of cysteinyl-leukotrienes formation at 5.5 µM, non-competitive inhibition of protein tyrosine phosphatase 1B(PTP1B), inhibition of the polymerization of tubulin and inhibition of growth on fourteen cancer cell lines [14]. *Stereocaulon ramulosum* produced structures type atranorin with moderate inhibition of protein tyrosine phosphatase 1B and methyl haematommate with antifungal activity [15].

Despite of several reports of biological activity for lichens, including for *Stereocaulon* species, the extraction and purification of natural products require the collection of large amounts of biological material. The problem is greater when no large amounts for testing are available. To minimize this problem, biotechnological methods are being developed, mainly involving tissue culture and cell immobilization. These techniques have great advantages, such as the very small biomass required for immobilization, its inexpensive maintenance, longevity of cells under immobilized systems, and the very fast rate of metabolite production [16].

Lichens has been reported as important source of secondary metabolites with biological activity and very interesting for biotechnology processes, for example, the production of compounds as atranorin for immobilized culture can be an important application of these area. Biotechnological processes consisting of the use of bioreactors with immobilized lichen cells is today the most accurate techniques for depsidone production. It has been used mainly to investigate the enzymatic pathways of depsidone biosynthesis and, in addition, it has been revealed as a very efficient method that produces high yields in product preparation. Moreover, bioreactor can be used to modify the chemical structure of particular phenolic molecules in order to increase a particular biological activity and a decrease of undesirable cytotoxic effect [17]. The use of immobilized cells of lichens has been studied previously, for example immobilized cells of *Cladonia verticillaris* in 4% calcium alginate can produce atranorin when they are supplied with 1.0 mM acetate as precursor [18]. Cells of *C. substellata* lichen, 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 [19]. Immobilized lichen cells from the thalli of the *Pseudevernia furfuracea*, supplied with acetate as source of carbon, continuously produced phenolic substances, atranorin and physodic acid, over 23 days [20].

Since the problem of destroying is lichen biota be emphasized, since the collection of large amounts of the thallus for industrial purposes, implies in a difficult or irreversible replacement of this organism to the environment [16]. This way, in this paper the production of secondary metabolites by immobilized cells of *Stereocaulon ramulosum*, using an acetate precursor and different apparatus immobilization system is described. The success of the technique can allow further studies for production of bioactive compounds of lichen in scale-up.

MATERIALS AND METHODS

Biological Material

Stereocaulon ramulosum collected in July 2011from El Colorado (38°38”53´S; 71°14”54´W: 444 msnm), Maule Region, Chile was used throughout this work. Vouchers (600, 601) of collected samples were deposited in the Lichen Herbarium of the Talca University, and included geographical coordinate as well as substrate type (soil, rocks, in slopes between mosses and others lichens). Lichen thallus was cut into small pieces, dried in air and stored at room temperature (25ºC) in the dark until required.

Cell Immobilization

The experiment was performed in three different systems (Figure 1), depending on the conditions of the precursors:

System 1: Bioproduction under continuous flow system: 58.3 g to sterilized kaolinite previously hydrated with sterile deionizeted water (66.6 mL) was mixed with 2 g of lichen thallus fragments, and this mixture was placed in a separation funnel in which 50 mL of 10 mM sodium acetate was added and used as precursor of phenolic biosynthetic pathway. This system maintained in a flow of precursor entry and exit of substance continuously. The experiment was kept at ambient luminosity and room temperature (25 ± 3°C) and analyzed for seven weeks.

System 2: Bioproduction by fixed system: 58.3 g to sterilized kaolinite previously hydrated with sterile deionizated water (66.6 mL) was mixed with 2 g of lichen thallus fragments. This mixture was placed in an Erlenmeyer in which 50 mL of 10 mM sodium acetate was added and used as a precursor of phenolic biosynthesis. This system remained still no flow of precursor.

System 3: Bioproduction in continuous agitation system: 58.3 g to sterilized kaolinite previously hydrated with deionizeted water (66.6 mL) was mixed with 2 g of lichen thallus fragments. This mixture was placed in an Erlenmeyer in which 50 mL of 10 mM sodium acetate was added and used as a precursor of phenolic biosynthesis. This system was placed on a rotator shaker at 30 rpm, 18°C for all time of experiments.

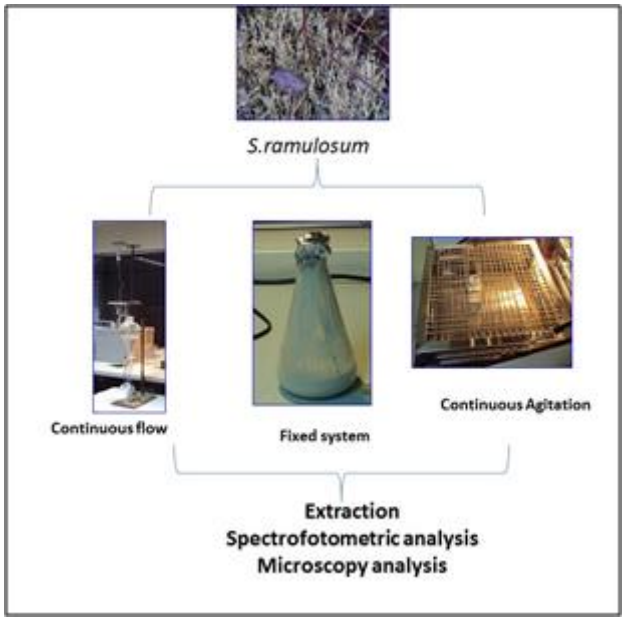

Figure 1: Schema of work for immobilized lichen

Collection and Fraction Processing

The collection of fraction (20 mL) containing the substances synthesized and released to acetate solution was carried out each 7 days for 5 weeks. After that, the collections were performed weekly resulting in 15 fractions, corresponding to five for each immobilization system. The same volume of the fresh precursor was replaced after each collection, except to continuous flow, which the precursor passed continuously throughout the system. The substances present in the fractions were extracted in a separation funnel, through strong stir (5 min) and rest (2 min), with mixture (20 mL) of diethyl ether/ethyl acetate (65:35, v/v)and then with chloroform/acetonitrile (60:40, v/v) [21]. The upper(diethyl ether/ethyl acetate) and the lower (chloroform/acetonotrile) organic phases were collected from the aqueous solution, and then submitted to spectrophotometer at 266 nm and 310 nm for detecting the presence of the substances synthesized during the immobilization process. All the extracts were assembled at room temperature (25ºC) and then dried until constant weight.

Analysis of the Extracts

The dried material was resuspended in the same solvents utilized in extraction and submitted to ascending thin layer chromatography on silica gel 60 $F_{254+366}$ (Merck) plate using the A system solvent (toluene/ethyl acetate/formic acid, 8:1:1, by volume), according to Culberson (1972) [22]. The chromatoplate was analyzed under ultraviolet light at 254 nm and 366 nm, and then sprayed with H_2SO_4 and heated at 100^0C for color reactions. For extracting the lichen thallus (2 g) the same solvents utilized for the extraction of the immobilized lichen were used, and applied as a reference. The chromatographic spots obtained were compared using the Rf values and color reaction.

Cell Vitality

After time course of experiment, the immobilized lichen material was submitted to an optical microscopy analysis for evaluating the percentage of dead and alive cells of the algal layer utilizing Supravital staining with Neutral Red 0.1% utilized previously in others works with the same objectives [23,24].

RESULTS AND DISCUSSION

Lichen substances are formed by phenolic units that have origin from a polyketonecarboxyl derived of the acetic acid that the enzymatic lichen systems use to from acetate [25]. By this reason, sodium acetate (NaAOc) is used as precursor for the synthesis of phenolic compounds of interest. By other hand, the light is the source of energy for the photobiont. The light energy is converted on carbohydrates through photosynthesis, and these compounds synthetize by the algae or cyanobacteria are transfer to fungus [26-28]. From of the graphics building with the spectrophotometric data is possible to notice the productivity of the fragments of thallus of *S. ramulosum* during the five weeks of experiment. The cells produced their metabolites continuously (Figure 2). It can be observed an increasing of productivity after of the second week, with emphasis to the immobilized thallus in the continuous flow system.

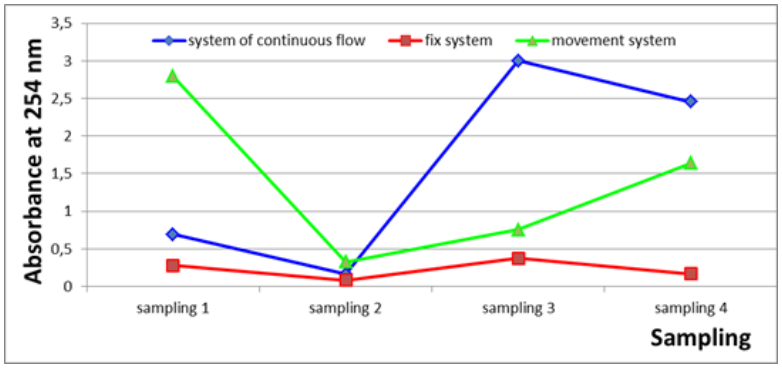

Figure 2: Comparison of production of secondary metabolites during the time of duration of the assay according to the different type of systems

According to the absorbance registers for each one of obtained fractions in different systems of immobilization, it shows an increase in the concentration of the secondary metabolites, being the fix system and the continuous flow the most efficient. Analysis of extracts permits to establish a major production of secondary metabolites in the system of continuous flow (Figure 2).

The extracts came from of thallus fragments submitted to the different systems of production present others bands that probably, it trait of intermediate compounds produced by the bioreactors. Undoubtedly, that the conditions to which were submitted the fragments of *S. ramulosum* in the systems of production of metabolites are different to the found in their natural habitat, which induce to the production of different compounds. Recent studies through Maldi and Electrospray ionization Mass spectrometry showed the presence of Atranorin as major secondary metabolite in the species [29]. Atranorin (ATR) is an important member of the depside group and is found in a variety of lichen species [30]. Studies on bioactive properties of extracts containing ATR have revealed antimicrobial activity [31], antinociceptive and antiinflammatory properties [32] and photoprotective capacity [33]. Isolated ATR showed antinociceptive effects [34] and to inhibit leukotriene B4 synthesis in leukocytes, which might affect inflammatory processes [35]. ATR showed anti-proliferative action against malignant cell lines [30]. In a study of the mitochondrial uncoupling activity of lichen metabolites, ATR was the only compound which did not exhibited toxic effects, indicating it could substitute other related lichen metabolite, usnic acid, which also presents potential medicinal applications, in the formulation of novel therapeutic compounds [36]. ATR has a relevant redox-active action, acting as a pro-oxidant or antioxidant agent depending on the radical [37]. In previous studies with entrapment of cells in different matrixes, the problem mentioned by different authors is the lack of contact between symbionts, during the process of disruption of the thallus for cells obtainment. This procedure difficult transference of enzymes and co-factors from one symbiont to another. This way, species produced the protocetraric and hypoprotocetraric acids, and atranorin instead of fumarprotocetraric acid (FUM), accumulating these intermediate products of FUM biosynthesis [21], what was ratified by adding FNM and succinil-CoA [18] in bioreactors, where lichen fragments produced FUM. The use of fragments increased the productivity, but intermediary products still appeared in the cell washes. These products can be resulted from a reuse of the lichen cells of produced compounds as carbon source, and/or intermediary products released from these lichen cells [2]. This way, to find compounds in cell washes different from those ones synthesized by the lichen thallus, as demonstrated in our study, can result from these mentioned processes. Aspect of lichen from its natural source and immobilized fragments were compared morphologically showing similar structures what it confirm the growth and development of the species under the given condition as shown in Figure 3.

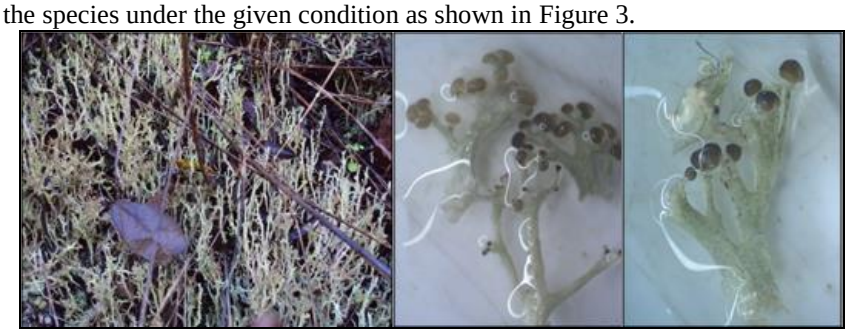

Figure 3: Comparison of tallus *in situ*(A) with tallus immobilized (B) of *S. ramulosum*

Microscopic analysis of tissue confirmed the cell viability of *S. ramulosum* showing crystals of lichen sustances, after of the coloration with Supravital staining with Neutral Red 0.1% (400X). The Figure 4, illustrate the

vitality of the cells of the algal layer at final of the experiment, it can observe that near of the 90% of the cells of the algal layer maintain their vitality.

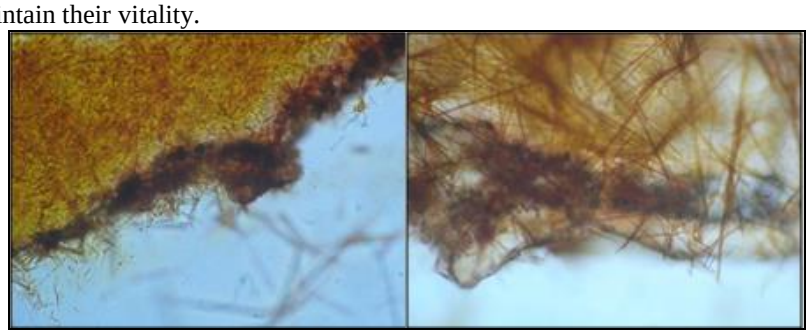

Figure 4: Optical microscopy differentiatingalivealgae cells of the lichen *S. ramulosum*.Vitality of the cells of the algal layer at start of experiment (left) and at the end of the experiment (right)

The cell vitality is an important parameter for considering an immobilization system as satisfactory. The kind of used matrix for entrapment of cells can be a problem, since some of them show any limitation, as polyacrilamide, that plasmolize immobilized cells after some few days [16,38]. The use of spheres made with alginate of medium viscosity, with a surface hardened by calcium chloride, and cells maintained inside them, can preserve the integrity of cells [39], allowing their division, as demonstrated by Molina and Vicente (1993) [40] with *Xanthoria parietina*. By other hand, this matrix does not endure high concentrations of precursors, as demonstrated by Pereira et al. in 1995 [41], when tried to produce ribitol from immobilized cells of *Cladonia verticillaris*. Increase of concentrations of sodium bicarbonate induces the cation exange between the Na of the precursor with the Ca that hardened the sphere that melted at 10 mM. Judging from these data, a kaolinite matrix can be considered more adequate, since it is an inactive clay, and the first production of usnic acid using this matrix was described by Pereira et al. in 1995 [19], what is in accordance with our results, since cells after long immersion in a water solution of sodium acetate was alive and ready for bioproduction.

The concentration of the precursor is another important condition for a more efficient bioproduction for some species. For example, crescent concentrations of sodium acetate (NaOAc) increased the production of usnic acid by entrapped cells of *C. substellata* [42] , while concentrations between 0.1 and 10 mM of this precursor, or up 0.01 to 20 mM, did not cause any difference in phenolic production of, respectively, *C. corallifera* [43] and *Parmotrema praesorediosum* [2]. This way, in our study we choose to use 10.0 mM of sodium acetate due to its high productivity in the most cases of immobilization. By other hand, it would be advisable future studies with another concentration of NaOAc. The different design of the bioreactor also can influence on cells productivity. The first report of different apparatus was made by Lima in 2004 [43], and described by Pereira et al. in 2012 [44].

CONCLUSION

The improvement of productivity depends on the species, but continuous flow seems to be more efficient, since the contact between entrapped cells and precursor is shorter, in comparison to the other systems. In our study, the three systems used showed to be effective for the bioproduction of metabolites. Since these findings, it is suggested a further study to evaluate others concentrations with the purpose of increase the bioproduction of metabolites of interest. In addition, bioproduction of lichens can be achieved, at a semi-industrial scale, by means of immobilization techniques of cells or enzymes, as an alternative to the use of large amounts of lichen biomass to isolate lichen compounds or to study biosynthetic enzymes. These techniques have great advantages, such as the very small biomass required, its inexpensive maintenance and the very fast rate of metabolite production.

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