



Antimicrobial activity of extracts of the lichen *Parmelinella simplicior* and its isolated mycobiont.

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ABSTRACT

Lichens are well known for their unusual secondary metabolites. Lichen sample was collected from Bhimashankar and Varasgaon–Panshet regions. Fungal partner was successfully isolated, cultured and maintained in the laboratory for further analysis. Detailed thallus structure and the behavior of its isolated mycobiont was studied with the help of Scanning Electron Microscopy. The common depside Atranorin and depsidone Salazinic acid were found in natural lichen as well as in isolated mycobiont. Their presence was confirmed by High Performance Liquid Chromatography. Antimicrobial activity of the lichen extract and the extract of its isolated mycobiont was assayed against various micro-organisms. These results confirmed that the isolated mycobiont has the capacity to produce lichen substances that are the natural products of the whole lichen even in absence of algal partner. This study reveals that mass production of these unique secondary metabolites from isolated mycobiont is possible any time during the year and could be used as an easily accessible source of natural secondary metabolites for the antimicrobial properties in the pharmaceutical industry.

KEY WORDS: Lichen, Mycobiont, SEM, Secondary metabolites, Antimicrobial activity

INTRODUCTION

Lichens are organisms formed by symbiotic association and integration of an alga and a fungus. Lichen symbiosis is very successful one and thallus shows no resemblance to either partner.

Lichens are well known for their unusual secondary metabolites. However the lichenflora is not sufficiently voluminous for its economic exploitation. Also they are slow growers and very sensitive to pollution. Due to uncontrolled pollution these lichens may become extinct or endangered. The lichens are long known to produce large number of secondary metabolites which are unique to them and having usage in herbal medicine. Various lichen substances like depsides, depsidones, dibenzofurans, usnic acid, xanthenes, anthroquinones produced by mycobionts were summarized by Ahmadjian [1] Nobuo Hamada [2-4] in his series of experiments have shown that the lichen substances are produced by the mycobionts. It is well known fact that the fungal partner of the symbiosis is responsible for the production of lichen substances which are not found in higher plants or even in free living fungi.

Standardized methodology and further refinement of routine analytical TLC procedures for detecting and comparing lichen metabolites have been reported by Culberson et.al. [5] Other advanced methods like HPLC [6].

HPTLC [7] have been used for the identification of different lichen products. Behera et.al [8] reported antibacterial activity of methanol extracts of some lichen cultures. Mason Hale [9] had present a summary of lichen structures as viewed with the SEM, based on the results obtained from about 50 genera.

These are the reasons that isolation and extraction of mycobiont for secondary metabolites is necessary. The main objectives of this study were to standardize the methodology for isolation of mycobiont, study the behaviour of this fungus *in vivo* and *in vitro*, and to screen their antimicrobial potential.

MATERIALS AND METHOD

Collection of Material

The natural thallus was collected when fresh in September 2008 from Bhimashankar and Panshet- Varasgaon

regions in Pune, Maharashtra state, India. The dried specimen is preserved in the Department of Botany, Sinhgad College of Science, Pune, India. It was identified as *Parmelinella simplicior* on the basis of morphological and chemical criteria. It produces a common depside Atranorin and depsidone Salazinic acid predominantly.

Isolation of mycobiont

The separation, isolation and culture of mycobiont offers a fascinating opportunity to study the components morphologically and biochemically.

For isolation of mycobiont different inocula and also different media like PDA, 2% malt agar, Malt Yeast Extract Agar, MGYP (Malt extract-10 gm, Yeast Extract-2.5 gm, Peptone -1 gm, Glucose -10 gm, Agar-Agar-10 gm, volume made to 500 ml.) were tried.

To isolate the mycobiont and grow on culture medium apothecia were soaked in water and surface sterilized with 0.01% HgCl₂. After washing with sterile distilled water, small pieces of apothecia were inoculated on the nutrient medium. The inoculated plates were incubated at $\pm 20^{\circ}\text{C}$.

Daily checks for contamination and immediate transfer of non contaminated inocula on to fresh medium were found essential in the isolation process. Depending upon the type of experiment, lichen mycobiont was cultured either on solid or on liquid medium.

Medium composition was varied to promote growth rate of isolated mycobiont by using different sugars and nitrogen compounds.

Scanning Electron Microscopy

The cellular structures which are unique to the lichen symbiosis and which form new morphological entity as lichen thallus from two totally different symbionts was of particular interest.

A lichen thallus is a heterogeneous structure and fungal hyphae are arranged in layers of different densities and contain intercellular material which may be fibrillar, gelatinous or crystalline. SEM gives good impression of the three dimensional inner organization of the thallus.

Cross sections of air dried lichen thallus were prepared by hand with a sharp blade and then mounted directly on the metal stubs. Thin glass pieces covered with isolated mycobiont were processed for the SEM photography. Platinum coating was done with vacuum coater to form ion emitting layer.

Identification of lichen substances by High Performance Liquid Chromatography

Lichen thallus and isolated mycobiont were extracted in acetone.

HPLC analysis was carried out on Shimadzu Prominence System, C 8 Luna column

(C 8 Luna, 4.6 x 250 mm, 5 μm) and UV detector at 25°C with solvent system – Methanol- Water- Phosphoric Acid (80:20:09 v/v/v). The detection wavelength was 254 nm and injection volume was 10 μl . Flow rate was 1 mL/min. [6] Lichen substances were identified by their peak symmetry and their retention time by comparison with authentic substances.

Antimicrobial activity

Screening of the extracts of the lichen thallus and isolated mycobiont against microorganisms was carried out by agar well diffusion method.

Microorganisms used for screening of antimicrobial activities were *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus megatarium*, *Escherichia coli*, *Candida albicans*, *Streptococcus faecalis*.

Nutrient agar was inoculated with 100 μL of bacterial suspension. The wells were filled with extracts (40 μL). The inoculated plates were incubated for 48 hrs. at $\pm 37^{\circ}\text{C}$.

The antimicrobial activity was evaluated by measuring the inhibition zone. All tested microorganisms were procured from National centre for Industrial Microorganisms, National Chemical Laboratory, Pune, India.

RESULTS

Isolation of mycobiont

From different media tried for isolation of mycobiont, MGYP medium best supports the growth of fungal partner.

In the pure culture of isolated mycobiont, colony texture was white smooth and with wavy margin. Radial extension rate of the culture was 0.8 cm/day (Fig.1). The growth of isolated mycobiont was found to be rapid as compared to natural thallus. After prolonged incubation, as nutrients became scarce, the mycelium aggregated at various points to form compact aerial aggregates (Fig. 2).

Fig 1 Isolated Mycobiont of *Parmelinella simplicior*

Fig 2 Compact aerial aggregates of mycelium

Separation of mycobiont from solid medium was impractical. Besides that mass production was found best in liquid cultures. So for analysis of secondary metabolites Liquid cultures were found to be more convenient. Also addition of maltose and ammonium nitrate favoured the higher growth of mycobiont.

Production of secondary metabolites

Lichen substances were identified by TLC method. In *Parmelinella simplicior*, Atranorin and Salazinic acid were found prominently.

Thin Layer Chromatography studies revealed that isolated mycobiont also produced same lichen substances as produced in natural thallus. Presence of these lichen acids in natural thallus and isolated mycobiont were confirmed by HPLC. These results support the fact that the fungus alone is responsible for the production of these characteristic unique compounds in lichens.

Scanning Electron Microscopy

The SEM gave an excellent view of the three dimensional inner organization of the thallus and hyphal distribution. Transverse sections of *Parmelinella simplicior* showed upper cortex which consisted of randomly oriented closely packed cells. Beneath the upper cortex there is algal layer within which contact between the partners of symbiosis is established. Here globular photobiont cells covered with fungal hyphae are noticeable. Hyphal orientation in medulla was clearly evident. Numerous small interwoven hyphae can be seen.

The shape of the outermost cells of the cortex has an important influence on the habit of the lichen. The surface is often covered with a thin homogenous cuticle, but in number of lichens the outermost cells become necrotic and give the thallus scurfy appearance. These tiny granules are called pruina. They may also be an accumulation of Carbonates and oxalates. The margins of this foliose thallus are covered with whitish dust supports this observations (Fig.3).

The thickness of the algal layer varies in different lichen genera and the algae are situated in the part of the thallus where the hyphae are sufficiently loosely interwoven to leave enough space for the alga and where they have optimum light intensity. If by chance, the position of the thallus is changed in nature, i.e. lobe of a foliose thallus is reversed, the algal layer which usually lies near the upper surface of the thallus, migrates to the new upper surface and establishes itself inside a tissue which was originally part of the medulla in the lower part of the thallus before reversal (Fig.3).

In SEM photographs of isolated mycobiont growing hyphal tips, anastomosing hyphae, projections in the hyphae are clearly evident. Besides that one more characteristic feature of isolated mycobiont, their self infection is clearly seen. This fact may explain the relatively slow growth rates of lichens. It also represents a form of autophagy where by under poor nutrient conditions these fungi can cannibalize themselves as means of persisting through difficult conditions (Fig.4).

Antimicrobial Activity

When acetone extracts of lichen thallus and its isolated mycobiont were tested against different microorganisms, they were found to be effective against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus megaterium*, *Escherichia coli* and *Candida albicans* (Fig.5 & 6).

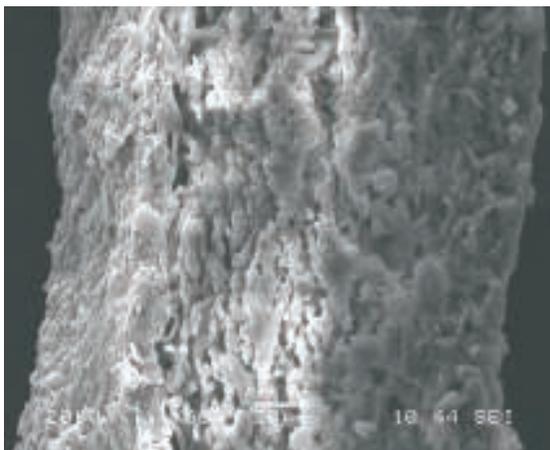


Fig 3 Cross section of *Parmelinella simplicior* Showing anastomosing hyphae



Fig 4 Isolated mycobiont of *Parmelinella simplicior* showing upper surface, medulla, algal cells



Fig 5 Zone of inhibition of *P.simplicior* thallus against *S.aureus*

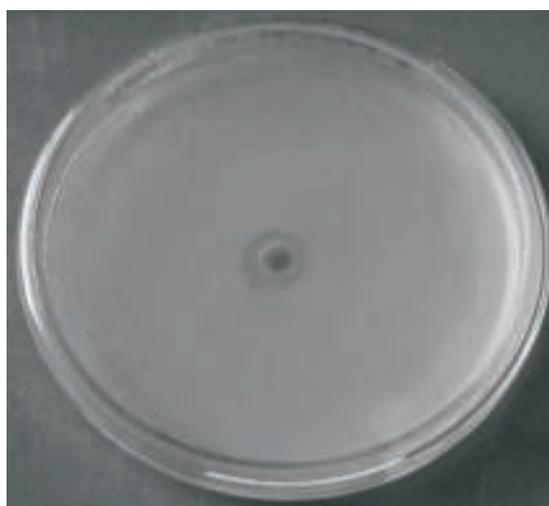


Fig 6 Zone of inhibition of *P.simplicior* mycobiont against *S.aureus*

	<i>Staphylococcus aureus</i>	<i>Streptococcus faecalis</i>	<i>Escherichia coli</i>	<i>Candida albicans</i>	<i>Pseudomonas aeruginosa</i>	<i>Bacillus megaterium</i>	<i>Bacillus subtilis</i>
<i>Parmelinella simplicior</i> (thallus)	+ve	-ve	+ve	+ve	+ve	+ve	+ve
<i>Parmelinella simplicior</i> (mycobiont)	+ve	-ve	+ve	+ve	+ve	+ve	+ve

DISCUSSION

Lichen secondary metabolites are produced primarily by mycobiont and are secreted on to the surface of lichen hyphae either in amorphous forms or as crystals.

Plant product drugs and herbal remedies have been employed since prehistoric times to treat human and animal diseases. Lichens synthesize numerous metabolites –'lichen substances' which comprise of amino acid derivatives, aromatic compounds, dibenzofurans, depsides, depsidones etc.

Lichens and their metabolites have manifold biological activity: - antiviral, antibiotic, enzyme inhibitory, allergenic. Behera [10] reported that the acetone, methanol and light petroleum extracts of lichen *Usnea ghatensis* were effective against *Bacillus licheniformis*, *B.megatarium* and *S. aureus*.

Ali karagoz et.al. [11] reported antibacterial activity of aqueous and ethanolic extracts lichens like *Lecanora muralis*, *Peltigera polydactyla*, *Ramalina farinacea*, *Xanthria elegans*.

On the basis of the results obtained it can be suggested that the extracts of isolated mycobiont could be used as an easily accessible source of natural secondary metabolites for the antimicrobial properties in the pharmaceutical industry.

The growth rate of the cultured mycobiont in the laboratory could be improved to harvest large quantities of these novel secondary metabolites.

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