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Optimization of Culture Conditions for Lichen *Usnea ghattensis* G. Awasthi to Increase Biomass and Antioxidant Metabolite Production

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Summary

The aim of this study is to optimize the culture conditions for lichen *Usnea ghattensis* G. Awasthi in order to increase biomass and antioxidant metabolite production. The cultured lichen consisted of usnic acid produced by mycobionts and photobionts after 2 to 3 months of inoculation. Cultures grown in the media with excess carbon sources showed significant increment in the biomass growth, usnic acid production and total polyphenol mass fraction after six months of inoculation. The methanol extract of six-month-old cultures grown in the malt-yeast extract (MYE) medium containing 0.01 mol/L of sucrose and 0.01 mol/L of polyethylene glycol showed a significantly high inhibition of lipid peroxidation activity up to 89 %. A significant correlation (R^2=0.89) of $p<0.01$ was also found between total polyphenol mass fraction and the inhibition of lipid peroxidation in this lichen species.

Key words: lichen, usnic acid, lipid peroxidation

Introduction

Lichens, symbiotic organisms of fungi (mycobiont) and algae (phycobiont), synthesize characteristic secondary compounds, the 'lichen substances'. These secondary metabolites are unique with respect to those of higher plants. Usnic acid is uniquely found in lichens, and is especially abundant in genera such as *Alectoria, Cladonia, Usnea, Lecanora, Ramalina* and *Evernia*. Many lichens and extracts containing usnic acid have been utilized for medicinal, perfumery, cosmetic as well as ecological applications. Usnic acid as a pure substance has been formulated in creams, toothpaste, mouthwash, deodorants and sunscreen products (*1*). Besides, lichen metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects (*2*). Even though these manifold activities of lichen metabolites have now been recognized, because of difficulties in collection of substantial amounts of lichen biomass, their therapeutic potential has not been fully explored yet and thus remains pharmaceutically unexploited (*3*). Many natural lichens and their derived cultures in the laboratory have been screened for their potential biological activities (*4–10*). However, in general, lichen cultures grow much faster than the natural thalli, but more slowly than many other microorganisms. Hence the large scale industrial production of the lichen metabolites has never been accomplished. Therefore, growth rates of the cultured lichen must be improved to enlarge the access to lichen-derived substances which possess possible applications in pharmaceutical research. With this background, the aim of the present study is to optimize the culture conditions for the improvement of their growth, and to determine the possible relation between culture conditions and the antioxidant activity of lichen secondary metabolites.

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Materials and Methods

Lichen material

The lichen *Usnea ghattensis* G. Awasthi, which produces usnic acid (lichen substance) under natural conditions, was collected from silver oak trees in Mahabaleswar (Satara District, Maharashtra State, India) in July 2003 and determined. Part of the material was preserved as herbarium specimen (U. V. Makhija, 03.391 Ajrekar Mycological Herbarium, Lingmala, Maharashtra, India).

Lichen culture

Lichen cultures were started within 7 days after the collection using methods described earlier (*8*). The natural lichen thalli (5 mg) were cut into 1-cm square pieces, washed with tap water overnight and homogenized with 5 mL of distilled water under sterile conditions. The suspensions were passed through a sterilized 500-μm mesh. Further, the filtrates were passed through a nylon filter with a 150-μm mesh. Small segments from the second filtration were picked up with sterilized stainless steel loop and inoculated onto slant medium in Petri dishes 9 cm in diameter. The inocula were grown at 18 °C, with alternating photoperiod of 8 h light (400 lux)/16 h dark and 50–80 % relative humidity in plant tissue culture room for a period of 3 months. The culture medium used was malt-yeast extract (MYE) containing malt extract 20 g/L and yeast extract 2 g/L solidified with Sabouraud dextrose agar (20 g/L).

After three months, the cultured mycobiont and photobiont and the natural thallus were extracted with acetone (Figs. 1 and 2). The chemical data were obtained by the standardized method of thin layer chromatography (TLC) using standard solvents benzene/dioxane/acetic acid (volume ratio of 180:45:5) and hexane/ethyl ether/formic acid (volume ratio of 130:80:20) (*11*). Lichen substances were identified by comparison with standard lichen substances and samples of several species containing atranorin, norstictic and usnic acids and the corresponding natural thalli.

Both mycobionts and symbionts that derived from the natural fragment of lichen thalli of *U. ghattensis* were examined and it was found that symbionts could only produce the usnic acid. They were further subcultured under various culture conditions in order to optimize the conditions and enhance the growth rate and production of usnic acid. They were further subcultured in the Petri dishes containing solid or semi-solid water agar and MYE media with the addition of different amounts of carbon and nitrogen sources, and were kept under 18 °C with alternating photoperiod of 8 h light (400 lux)/16 h dark and 50–80 % relative humidity in plant tissue culture room for 6 months. In the first set of experiments, carbon sources (glucose, sucrose or polyethylene glycol (PEG)), nitrogen sources, amino acids (L- or D-asparagine, glutamine, alanine, glycine) in the concentrations of 0.005, 0.01, 0.02, 0.05 or 0.1 mol/L, and vitamins thiamin (B1), riboflavin (B2), ascorbic acid (C) or biotin (H) in the concentrations of 0.01, 0.1, 1.0 or 10 μg/mL were individually added to the MYE medium. In the second set of experiments, symbionts were subcultured in the MYE medium prepared with the additional 0.01 mol/L

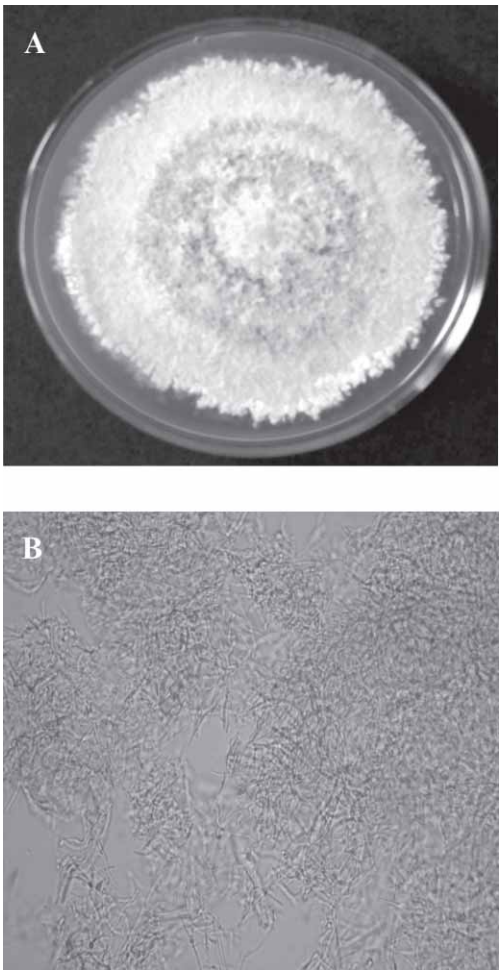

Fig. 1. A) Cell aggregates obtained from the fragment of natural lichen thallus of *Usnea ghattensis* grown in MYE solidified with Sabouraud dextrose agar, supplemented with 0.01 mol/L sucrose and 0.01 mol/L polyethylene glycol. B) Symbionts composed of colourless fungal hyphae and green algal cells

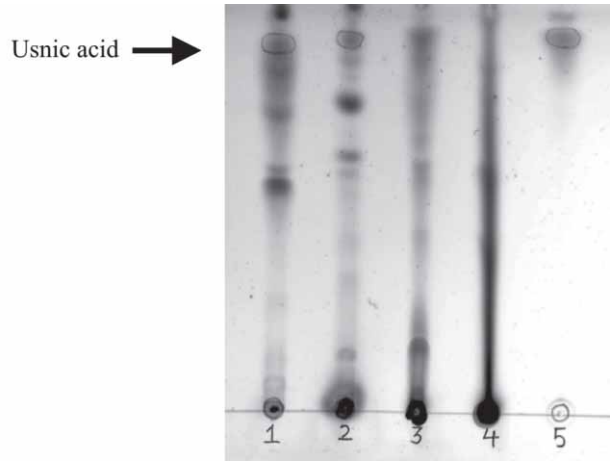

Fig. 2. TLC chromatogram of lichen substances in natural thallus of *Usnea ghattensis* G. Awasthi and cultured tissue in solvent system benzene/dioxane/acetic acid (volume ratio of 180:45:5). Lane 1: natural thallus of *Usnea ghattensis*, lane 2: cultured tissue, lanes 3 and 4: lichen species containing atranorin and norstictic acid and lane 5: usnic acid purchased from Sigma-Aldrich (USA)

of sucrose, 0.01 mol/L of PEG and 0.005 mol/L of glycine. In all experiments, the pH of the medium was adjusted to 5.8–6.2. After every two months and finally after 6 months, cultured tissues were taken out from the Petri dishes and dried at 40 °C for 72 h. The biomass of the cultured mycobionts and photobionts obtained under various conditions mentioned above was weighed. The cultured lichen biomass was extracted in a Soxhlet apparatus by using 20 mL of 10 % (by volume) solutions of acetone and methanol as solvents at room temperature. The extracts were then filtered, concentrated 4-fold *in vacuo* and freeze dried. Acetone extract was used for the estimation of the usnic acid mass fraction, and methanol extract was used in lipid peroxidation assay.

Estimation of usnic acid mass fraction

The estimation of usnic acid mass fraction in the cultured lichen biomass composed of mycobionts and photobionts was carried out following the procedure published by Jayasankar and Towers (*12*) and details of the procedure with slight modification had been reported earlier (*13*). The mass fraction of usnic acid in the cultured mycobionts and photobionts was calculated using a standard curve obtained with the usnic acid, purchased from Sigma-Aldrich (USA).

Analysis of antioxidant component in the extract

Antioxidant component in the methanol extract of *Usnea ghattensis* was determined following the procedure published by Espin *et al.* (*14*). TLC plate, coated with silica gel G (Fluka Chemie, Switzerland) to 0.25-mm thickness, was spotted with 20 μL of the extracts. The plate was then developed in a solvent system of ethyl acetate/methanol/water (volume ratio of 10:2:1). After drying, the developed plate was first observed under UV light at a wavelength of 365 nm and sprayed with 0.4 mM DPPH radical in methanol. Furthermore, the developed TLC plate was sprayed separately with spray solution 1 (1 % solution of iron(III) chloride in water mixed immediately before use with an equal volume of a 1 % solution of potassium hexacyanoferrate(III) in water (Barton's reagent)), which gave a blue colour in the presence of phenolic compounds, and spray solution 2 (2 % iron(III) chloride in ethanol), which, when heated to 105 °C for 5–10 min, gave either a blue colour, indicating the presence of phenolics with trihydroxy groups, a green colour, indicating phenols with dihydroxy groups, or a red/brown colour, indicating the presence of other phenolics.

Inhibition of lipid peroxidation assay

The lipid peroxidation inhibition in the methanol extract of *Usnea ghattensis* was measured following the procedure published by Liegeois *et al.* (*15*). An aqueous solution of linoleic acid and AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) solution was prepared as described (*15*). A volume of 30 μL of an aliquot of 16 mM linoleic acid solution was added to a UV cuvette containing 2.81 mL of 0.05 M phosphate buffer, pH=7.4. The oxidation reaction was initiated at 37 °C by adding 150 μL of 40 mM AAPH solution in the presence of aliquots of 20 μL of 0.2 mg of TLC-eluted usnic acid per mL of methanol. For the control experiment, 20 μL of 0.2 mg of standard antioxidants Trolox (a vitamin E analogue, purchased from Sigma-Aldrich (USA)) or ascorbic acid with 20 μL of 10 % methanol (by volume) were used as positive control. A volume of 20 μL of methanol with reagent mixture (without standard antioxidant and extract) was used as negative control to check if methanol has an effect on the lipid peroxidation inhibition. The rate of peroxidation at 37 °C was monitored by recording the increase in absorbance at 234 nm caused by the formation of conjugated diene hydroperoxides. The inhibition of lipid peroxidation was calculated by the following equation:

$$\text{Inhibition}=([A_0 - A_1]/A_0)\times 100 \qquad /1/$$

where A_0 is the absorbance of the control reaction (without antioxidants and extract) and A_1 is the absorbance of the reaction in the presence of the extract.

In all cases, three independent experiments, each with five measurements, were performed. The results are the means of these measurements.

Determination of total phenolic compounds

Total soluble phenolics in the *Usnea ghattensis* extracts were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (*16*) using pyrocatechol as a standard. The concentration of total phenolic compounds in the extract was determined as microgram of pyrocatechol equivalent by using an equation that was obtained from standard pyrocatechol graph given below:

$$A=0.001\times m(\text{pyrocatechol})+0.0033 \qquad /2/$$

where A is the absorbance of the sample and m is the mass of pyrocatechol in μg.

Statistical analysis

Effect of carbon or nitrogen concentrations in the media on the growth of lichen composed of mycobionts and photobionts, and usnic acid production were analyzed statistically by one-way analysis of variance (ANOVA). Student's *t*-test was used for determining statistical significance of usnic acid and the total polyphenolic content in the inhibition of lipid peroxidation. A value of $p<0.05$ was considered as statistically significant. Sigma Stat v. 3.0 software was used.

Results and Discussion

Effect of culture conditions on growth and usnic acid mass fraction

The influence of various culture conditions on the growth of mycobionts and photobionts and the usnic acid production (a lichen substance) of a lichen species *Usnea ghattensis* under laboratory conditions have been studied. The growth of bionts and usnic acid production were found to vary quantitatively in the water agar and MYE media (Table 1). In the water agar medium, the growth of bionts and usnic acid production were found to be lower than in the cultures grown in MYE medium. Although the lower mass fraction (5 %) of agar in the

Table 1. Comparison of usnic acid production, total polyphenol mass fraction and total antioxidant activity of a lichen *Usnea ghattensis* G. Awasthi cultured for 6 months in the laboratory under various culture conditions

	m(dry biomass)/g	*w*(usnic acid)/ (μg/g dry mass)	*w*(total polyphenol)/ (mg/g dry mass)	Inhibition of lipid peroxidation/%
Culture conditions				
Agar 5 %	0.7	0.0	2.2	13
Agar 10 %	1.2	0.0	5.9	14
Agar 20 %	1.8	0.3	5.8	19
Agar 20 %+sucrose 0.01 mol/L	1.4*	0.3**	12.0**	33*
Agar 20 %+PEG 0.01 mol/L	1.6	0.2	11.4	41**
Agar 20 %+glycine 0.005 mol/L	2.2	0.2	4.2	14
Agar 20 %+sucrose 0.01 mol/L+ PEG 0.01 mol/L	3.0*	0.4***	7.4***	21*
Malt-yeast extract medium (MYE)	1.3	0.1	35.0	27
MYE+agar 10 %	1.3	0.3*	7.4	29
MYE+agar 20 %	2.5	0.7*	16.5	44**
MYE+sucrose 0.01 mol/L	4.9*	1.3	38.0***	89***
MYE+PEG 0.01 mol/L	3.6	1.0	23.5**	59***
MYE+glycine 0.005 mol/L	2.8	0.3	18.6	31
MYE+sucrose 0.01 mol/L+PEG 0.01 mol/L	7.7**	3.8**	46.3**	76***
MYE+sucrose 0.01 mol/L+PEG 0.01 mol/L+glycine 0.005 mol/L	5.7*	1.6*	13.6	38*
Natural thallus of *U. ghattensis*	1.0	13.4	62.4	88
Usnic acid (Sigma-Aldrich)				13
Standard water soluble antioxidants				
Trolox (vitamin E analogue, Sigma-Aldrich)				65
Ascorbic acid				51

*p<0.05, **p<0.01, ***p<0.001
Values are expressed as the average of five measurements
A volume of 20 μL of 0.2 mg of TLC-eluted usnic acid per mL of methanol was used for inhibition of lipid peroxidation assay; usnic acid (Sigma-Aldrich), Trolox or ascorbic acid were used as positive control
One-way analysis of variance test (ANOVA) was performed for determining the influence of carbon and nitrogen concentrations on growth rate, usnic acid production and total polyphenol mass fraction, and Student's *t*-test was used for determining usnic acid mass fraction and inhibition of lipid peroxidation

medium initially promoted the growth of mycobiont, it did not produce usnic acid even after 6 months of inoculation. However, a significant increase in the growth of bionts and the production of usnic acid were observed when the agar mass fraction was increased from 10 to 20 % in water agar medium. A 20 % of agar in MYE medium yielded 2.5 g of dry biomass of the bionts and 0.7 μg of usnic acid per g of dry biomass after 6 months of inoculation. In the same culture period, 20 % of agar in water yielded 1.8 g of biont dry biomass and 0.3 μg of usnic acid per dry biomass.

Many lichen species were also grown in MYE and other media supplemented with excess carbon or nitrogen for the enhancement of the growth of mycobionts and photobionts and the production of lichen substances (*17–19*). We have also carried out experiments by adding excess carbon and nitrogen sources in the water agar and MYE media to improve the growth rate of the symbionts (Table 1 and Fig. 3). Carbon sources like sucrose or PEG in concentrations ranging from 0.005 to 1.0 mol/L

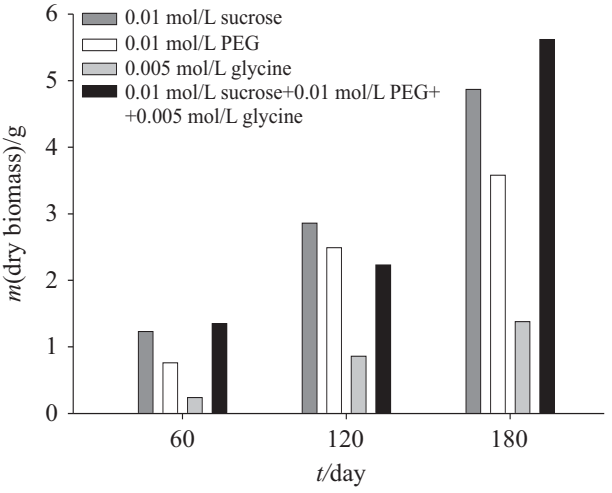

Fig. 3. Growth rate of lichen *Usnea ghattensis* cultured in MYE medium supplemented with various concentrations of sucrose, polyethylene glycol (PEG) or glycine

in MYE medium showed linear increase in usnic acid production by the cultures (Fig. 4). The cultures grown in MYE medium having excess of 0.01 mol/L of sucrose and 0.01 mol/L of PEG showed almost doubled growth rate of the cultures grown in water agar medium with the same concentration of sucrose and PEG. The combination of 0.01 mol/L of sucrose and 0.01 mol/L of polyethylene glycol in MYE medium produced 7.7 g of dry biomass with 3.8 μg of usnic acid per g of dry biomass, whereas MYE medium without carbon source yielded 1.3 g of dry biomass and 0.1 μg of usnic acid per g of dry biomass. In order to know the significance of the carbon sources, *i.e.* sucrose or PEG concentration in the media on the growth and usnic acid production, we performed the one-way analysis of variance test (ANOVA). The results showed that sucrose and PEG in both water agar and in MYE media had significant effect ($p<0.01$) on the biomass growth and usnic acid production in this species.

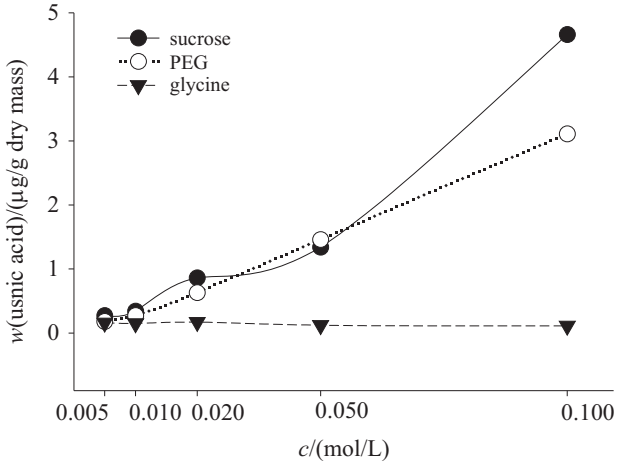

Fig. 4. Effect of sucrose, polyethylene glycol (PEG) or glycine concentrations in the MYE medium on the usnic acid production in a lichen *Usnea ghattensis* cultured *in vitro* for 6 months. Data are the mean of five measurements

As far as nitrogen sources such as amino acids glycine, asparagine, alanine, or vitamins thiamin (B1), riboflavin (B2), ascorbic acid (C) or biotin (H) are concerned, only glycine promoted the growth of bionts, but their rate of growth and usnic acid production were lower than when the carbon sources were added to the MYE medium (Figs. 3 and 5). Vitamins and other amino acids did not promote the growth of cultured bionts and the usnic acid even after 6 months of their inoculation.

These results suggest that the addition of amino acids and vitamins as nitrogen sources is less effective than sucrose and polyethylene glycol as far as the growth of bionts and the production of usnic acid in *Usnea ghattensis in vitro* culture is concerned.

Our results are in agreement with those that reported suppression of usnic acid production by amino acids (*20*). In our previous (*13*) and the present studies, it was found that increased carbon concentration in the medium increased the growth of biomass and usnic acid production in this lichen species. This indicates that the

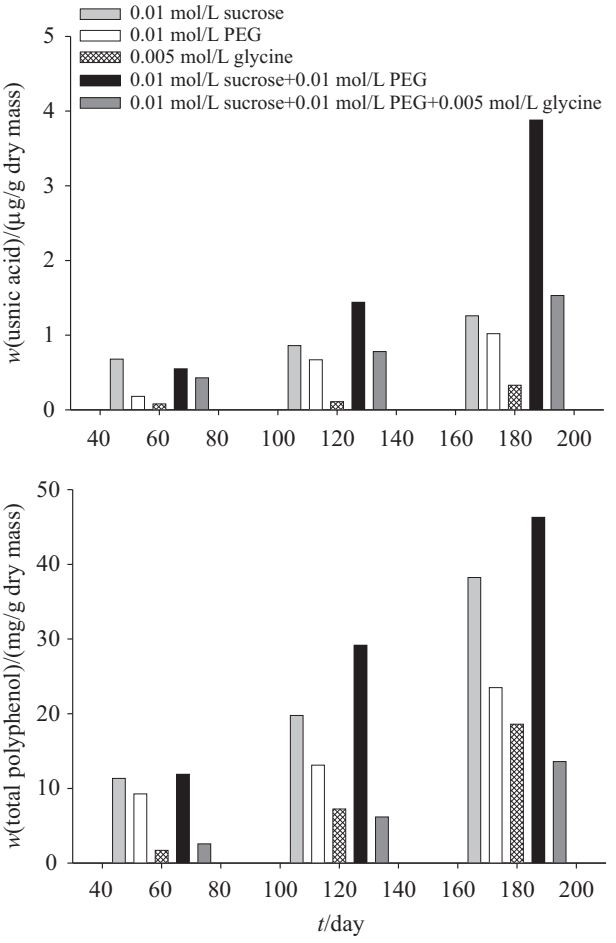

Fig. 5. Usnic acid and total polyphenol mass fractions of a lichen *Usnea ghattensis* cultured in MYE medium with various concentrations of sucrose, polyethylene glycol (PEG) or glycine

excess sucrose or PEG enhance the growth and probably activate the pathways producing lichen substances.

Many researchers have also studied the effects of various carbon and nitrogen sources on the cultures. In a more recent study a small amount of salazinic and usnic acids in axenic cultures of *Usnea orientalis* grown on a malt-yeast medium was detected and the production of both compounds increased when it was grown together with its natural photobiont (*20*).

Effect of culture conditions on the inhibition of lipid peroxidation

The lipid peroxidation inhibition potential of the methanol extract of a lichen *Usnea ghattensis* cultures grown under various conditions is presented in Table 1. The increase of usnic acid and total polyphenol mass fractions in the cultured tissue increased the inhibition of lipid peroxidation. In order to know the significance of the effects of culture conditions on the inhibition of lipid peroxidation, Student's *t*-test was performed to establish the relationship between usnic acid mass fraction and the lipid peroxidation inhibition. Usnic acid mass fraction in the cultured tissue showed no significant effect on the lipid peroxidation inhibition. However, the increase of total polyphenol mass fraction in the cultured

tissue showed highly significant ($p<0.01$) effect on the inhibition of lipid peroxidation activity. Furthermore, the standard antioxidants Trolox and ascorbic acid inhibited >50 % of lipid peroxidation. The methanol extract of the cultures grown in the MYE medium having 0.01 mol/L of excess sucrose showed 89 % inhibition of lipid peroxidation, which is almost equivalent to the 88 % inhibition of lipid peroxidation shown by the natural thallus of the lichen *Usnea ghattensis*. The methanol extract of the cultures grown in media having glycine alone showed lower lipid peroxidation inhibition. In order to explore the relationship between total polyphenol mass fraction and the inhibition of lipid peroxidation activity, total polyphenol mass fraction was correlated with the inhibition of lipid peroxidation and a strong correlation with $R^2=0.89$ ($p<0.01$) was found. The extract was qualitatively analyzed to determine the antioxidant component present in it. The results indicate the presence of phenolics with trihydroxy groups in the extract. Lichen produces secondary metabolites which are mainly phenolic compounds (*21*). Our results are in agreement with those that report the ability of phenolic compounds to scavenge free radicals and active oxygen species (*22*).

The present results show that the inhibition of lipid peroxidation depended on the amount of phenolic compounds produced by the symbionts under the culture conditions.

Conclusion

As the growth of lichens in nature is very slow, the optimization of culture conditions reported here may help in obtaining more abundant biomass production of lichen and considerably enlarge the access to lichen-derived substances for possible applications in pharmaceutical research. Since the total phenolics may be the mixture of many types of phenolic compounds, at present the exact active compound(s) responsible for the inhibition of lipid peroxidation activity in this cultured lichen *Usnea ghattensis* is unknown. Therefore, it remains to isolate and purify antioxidative compounds in this species.

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