Effect of various culture conditions on growth and production of salazinic acid in *Bulbothrix setschwanensis* (lichenized ascomycetes) *in vitro*

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Results of the studies on the effect of various culture conditions on the growth of *Bulbothrix setschwanensis in vitro*, with special reference to the production of salazinic acid have been presented.

APART from the use of lichens as food, medicine and in dyes and perfumes[1], they have been used as crude drugs all over the world. A variety of products isolated from lichens show a wide range of potentially useful biological activities. Norsoloronic acid shows inhibition of monoamino oxidase, lichesterinic acid and usnic acid inhibit some bacteria, while lichen polysachharides have antitumour and antivirus activities. Usnic and diffractaic acids have anti-inflammatory effects and other depsides inhibit the growth of insects. Phenolic compounds isolated from the cultured tissues and natural thalli of lichens showed inhibitory activities of Epstein–Barr virus (EBV) early antigen activation, melanin biosynthesis, superoxide dismutase-like (SOD-like) activity, etc.[2].

There are several reports on culture of lichen tissue producing many phenolics. In general, lichen tissue cultures grow much faster than natural thalli, but more slowly than many other organisms. If lichen tissue cultures are to have industrial uses for their wide range of potentially useful biological activities, we must improve the growth rate and productivity of the metabolites[3].

In our previous studies[4] cultured lichen tissues composed of mycobiont- and photobiont-producing atranorin (a depside) and salazinic acid (a depsidone) were obtained from the natural thallus of *Bulbothrix setschwanensis* (Zahlbr.) Hale.

In the present paper, the results of our investigations have been presented on the influence of pH, carbon and nitrogen sources on the growth of cultured tissue of *B. setschwanensis* (Zahlbr.) Hale, *in vitro*, with special reference to the production of salazinic acid (Figure 1) which is the source of a rich brown dye for dyeing protein fibres, particularly wool.

Growing tissue used in this study was obtained from the natural thallus *B. setschwanensis* following the technique described by Yamamoto *et al.*[5]. Three to four-month-old cultured tissues composed of both mycobiont and photobiont (Figure 2 *a*) were further subcultured in the following culture media: (1) Malt–yeast extract (MY) containing malt extract 10 g, yeast extract 4 g, agar 15 g in 1 l of water; (2) Bold's basal medium[6] (BBM); and (3) modified BBM[4]. Cultures were maintained at 18°C with alternating photoperiod of 8 h light (400 lux)/16 h dark and 50–80% relative humidity in culture room in media MY(Figure 2 *b*), BBM (Figure 2 *c*), and modified BBM (Figure 2 *d*).

In the first set of experiments, the media were adjusted to pH in the range of 5–9. The cultured tissues were taken out from the petri plates at the interval of 10 days up to 30 days and then finally dried at 40°C for 72 h after 60 days. The dried cultured tissues were measured and used for determination of salazinic acid content.

In the second set of experiments, carbon sources (fructose, glucose, sucrose or mannitol) in concentrations of 2, 4, 8, 16, or 32% (w/v), amino acids (L- or D-asparagine, glutamine, alanine, glycine) in concentrations of 0.2, 0.4, 0.8 or 1.6% (w/v), and vitamins (thiamin (B_1), riboflavin (B_2), ascorbic acid (C) or biotin (H)) in concentrations of 1, 10, 100 or 1000 ppb were individually added in the modified BBM[4]. The cultures were grown for 60 days in the conditions described above. Six replicates of each concentration were used.

In the third set of experiments, 8% sucrose was added in the medium and then further made into three subsets by adding 4% mannitol (w/v), 0.8% L-glutamin (w/v) or 10 ppb ascorbic acid. After 60 days, cultured tissues were taken out from the petri plates and dried at 40°C for 72 h.

The chemical data have been obtained by the standardized method of TLC[7] using standard solvent systems BDA (benzene : dioxane : acetic acid, 180 : 45 : 5; 230 ml) and HEF (hexane : ethylether : formic acid, 130 : 80 : 20; 230 ml). Identification of lichen substances was made by comparison with standard lichen substances and samples of several species containing atranorin, norstictic and salazinic acids and corresponding natural thalli.

Quantitative estimation of salazinic acid was done by the spectrophotometric method[8]. Two mg tissue of natural thallus and its derived cultured tissue were separately extracted twice with 5 ml absolute acetone for 24 h at room temperature with frequent shaking. Then acetone was evaporated under reduced pressure and the extract was dried at room temperature for 12 h. After washing with chloroform, the residue was taken up in 1.0 ml of a KOH–EtOH solution (KOH : H_2O : EtOH = 2.8 g :

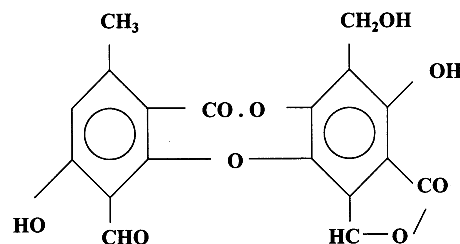

Figure 1. Salazinic acid (depsidone) (after Hamada[8]).

*For correspondence.

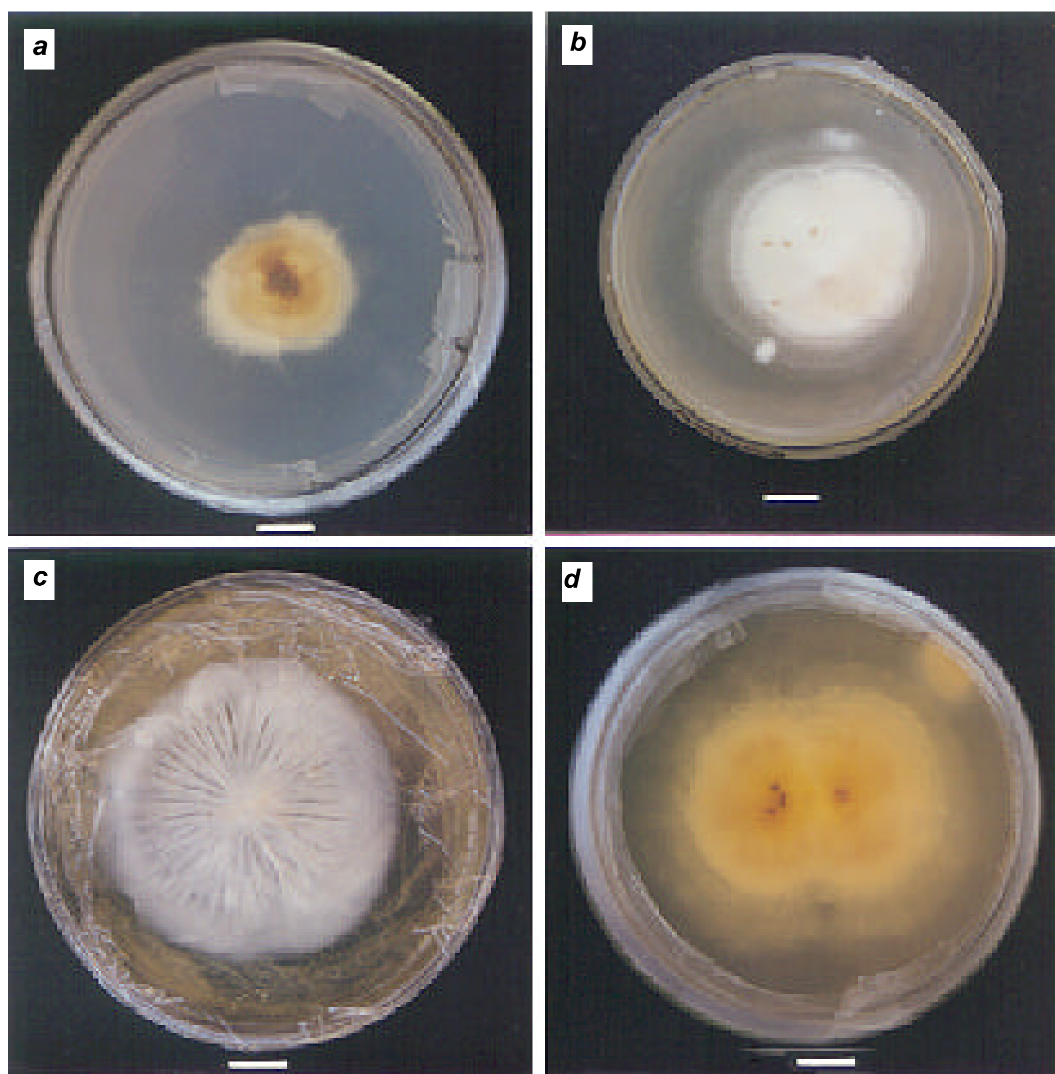

Figure 2. Cultured tissue of *Bulbothrix setschwanensis* over a period of two months in various media; ***a***, 3–4-month-old cultured tissues composed of both mycobiont and photobiont grown in modified BBM; ***b***, Cultured tissues (mycobiont only) grown in malt–yeast extract containing 8% sucrose; ***c***, Cultured tissues (mycobiont only) grown in BBM containing 8% sucrose; ***d***, Cultured tissues (both mycobiont and photobiont) grown in modified BBM containing 8% sucrose; Bar = 1 cm

Table 1. Effect of culture conditions on growth rate and salazinic acid content over a period of 8 weeks

Culture condition	Dry wt (mg)	Salazinic acid content (μg/mg dry wt)
Malt–yeast extract (mycobiont)	3.27 ± 1.12	NP
BBM (mycobiont)	5.38 ± 0.16	NP
Modified BBM (both bionts)	9.21 ± 0.03	1.31 ± 0.001
Modified BBM + 8% sucrose	16.88 ± 0.10	3.22 ± 0.007
Modified BBM + 8% sucrose + 4% mannitol	17.04 ± 0.28	2.42 ± 0.013
Modified BBM + 8% sucrose + 0.8% glutamin	15.07 ± 0.69	1.73 ± 0.07
Modified BBM + 8% sucrose + 10 ppb ascorbic acid	11.77 ± 0.13	0.64 ± 0.29
Natural thallus		11.09

Results are means ± SE of six independent determinations; NP, not present.

150 ml : 100 ml) to produce a wine-red colour, incubated for 30 min and then transferred to an absorption cell of UV–VIS spectrophotometer. Absorbance was read at 470 nm to determine the concentration of salazinic acid in this study. The concentration of salazinic acid corresponding to each absorbance of the reaction mixture was determined by the 2,4-dinitrophenylhydrazone method[9]. The pH of all media used here was adjusted to 6.6 before autoclaving with 1 N NaOH or 1 N HCl. All chemicals used in these experiments were of analytical reagent grade (Himedia Laboratories Pvt Ltd, India).

It is evident from the experiments that the growth of cultured tissues increased 2–3 fold when the medium was adjusted to pH 6.5–6.6 (Figure 3 *b*) and the biomass production was dependent on the pH. The relationship (r^2) between the pH of the medium and the growth of the tissue was $r^2 = 0.9142$ (Figure 3 *a*).

The cultures of *B. setschwanensis* grew remarkably in the medium with 4% sucrose or mannitol. The maximum growth (16.88 mg dry wt.), however, was obtained at 8% concentration of sucrose (Table 1). The growth rate in medium with sucrose was greater than that in medium

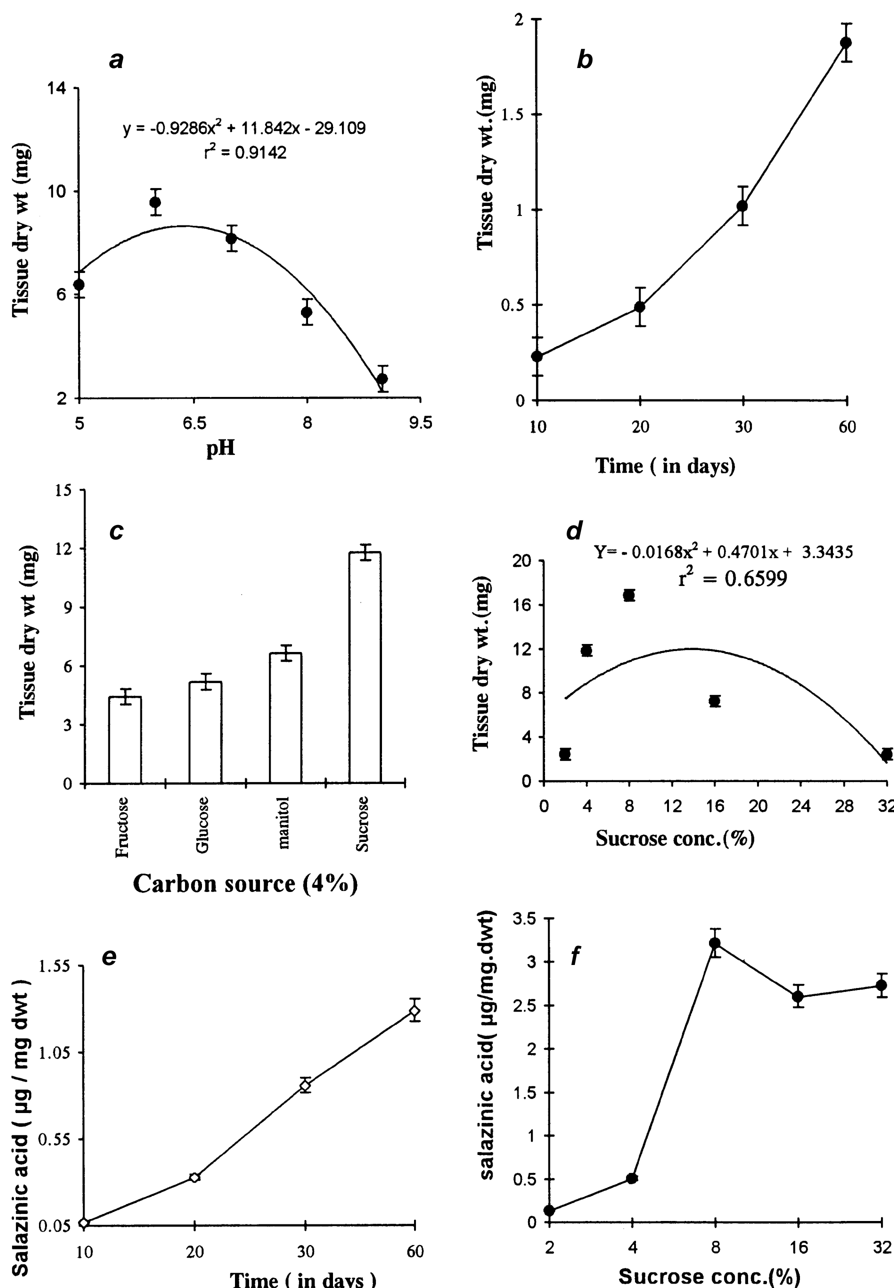

Figure 3. Effect of various culture conditions on (*a–d*) growth and (*e–f*) production of salazinic acid in *B. setschwanensis in vitro*.

with mannitol (Figure 3 *c*). When the concentration of sucrose was increased from 8 to 16% in the medium, the growth was found to decrease, resulting in dry tissue weight of 7.51 mg (Figure 3 *d*). The relationship between the sucrose concentration of the medium and the growth rate of cultures was $r^2 = 0.6599$. The dry weight of cultured tissue was increased only by 0.16 mg when mannitol was used in combination with sucrose when compared to the medium containing sucrose alone (Table 1).

We have also studied the effect of various amino acids and vitamins as well as common salts containing nitrogen, e.g. ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3), ammonium sulphate [$(NH_4)_2SO_4$] or ammonium tartarate ($C_4H_{12}N_2O_6$) in concentrations ranging from 2 to 32% and phytohormones, e.g. indole acetic acid, gibberlic acid, zeatin or kinetin, in concentrations ranging from 1 to 1000 ppb in the media. However, phytohormones and the salts never favoured the growth.

The results have shown that the growth of the cell aggregates varied with various amino acids. Even a small concentration (0.2% w/v) of amino acid (except glutamin) strongly inhibited the growth.

It was observed that the quantity of salazinic acid produced increased with the growth period (Figure 3 *e*). However, the content of salazinic acid per mg air dry weight varied considerably with the addition of different carbon and nitrogen sources, vitamins and amino acids (Table 1). The maximum production of salazinic acid (3.22 μg/mg dry wt) was favoured by the addition of 8% sucrose in the modified BBM (Figure 3 *f*). The addition of mannitol, glutamin or ascorbic acid in combination with sucrose in the medium adversely affected the production of salazinic acid (Table 1).

Out of the many culture media tested, the cell aggregates composed of both symbionts having fresh fungal hyphae and the algal cells were obtained only on modified BBM. Salazinic acid was detected only in cultured tissues which were composed of both bionts. It is believed that the production of salazinic acid in cultured *B. setschwanensis* tissue is based on a relationship between mycobiont and photobiont and the photobiont plays an important role in the production of salazinic acid. This is concurrent with our previous studies[4].

The addition of extra sucrose was reported to promote rapid growth of mycobionts and the production of lichen substances[10–12]. The present study showed that the highest production of salazinic acid was favoured by the addition of sucrose in modified BBM possibly activating the pathways of salazinic acid production.

Mosbach[13] reported that the biosynthesis of lichen secondary compounds could be the result of an excess of carbohydrates caused by photobiont photosynthesis in combination with a lack of nitrogen. Our results are in agreement with those of Mosbach[13] in this respect, that the addition of excess quantity of sucrose with low amount of nitrogen in the medium increased the production of salazinic acid in the cultures grown for 60 days. There was also a strong relation between them, but when the cultures were grown with the addition of glucose in the same medium, production of salazinic acid was detected only as trace on TLC plates after 3 to 4 months.

The ideal culture conditions for the production of various substances appear to depend upon the nature of the lichen substances, with the production of depsidones being more dependent upon the osmotic pressure than that of depsides[11]. It seems probable that sucrose added in excessive quantities acts as a nutrient as well as an osmoticum. The growth of cell aggregates and the production of salazinic acid seem to be nutritional because the growing medium contains so many compounds – sugars, metals, amino acids, etc.

Our results are based on the examination of cultured tissue over a period of 8 weeks in comparison with the fully developed natural thallus. We have not analysed content of salazinic acid in the initial stages of development of *B. setschwanensis* in nature. However, as the growth of lichenized thallus in nature is slower compared to the cultured material[3], it can be concluded from the above results that the cultured tissue was more productive than natural thallus of this lichen with respect to growth and salazinic acid content studied here.

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