Cultivation of *Lobelia inflata* L. Hairy Root Culture in Bioreactor

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Abstract

We have studied the biomass and alkaloid production of genetically transformed hairy root cultures of *Lobelia inflata* L.. The hairy root clone 8009/h7 transformed with *Agrobacterium rhizogenes* strain R1601 was cultivated on B5 liquid media containing 3% sucrose. The bioactive compounds were analysed by HPLC, the total alkaloid content was determined by spectrophotometry in the hairy roots and in the liquid medium. The bioreactor cultures produced an increased biomass and quantity of total alkaloids, but lobeline was not measurable in the tissues and in the medium. 1.7 mg/g total alkaloid content was found in the hairy root tissues. The total alkaloid production in the culture medium (14.3 mg) was higher than in the tissues (10.46 mg). Polyacetylenes were detected both in the tissues and in the liquid medium.

INTRODUCTION

Lobelia inflata L. (Lobeliaceae) contains di-substituted and mono-substituted piperidine alkaloids. The herb contains more than 20 alkaloids, giving a total alkaloid content of about 0.5% (Kaczmarek, 1961). The main alkaloid is lobeline, which has a stimulatory effect on the respiratory centre. The investigation of the growth and alkaloid production in cell suspension-, callus- and organized cultures from *L. inflata* showed that these cultures are able to synthesize the characteristic alkaloids of the intact plant (Krajewska et al., 1987; Szőke et al., 1992, 1998). The biosynthetic activity of genetically transformed hairy root cultures of *L. inflata* has also been studied (Ishimaru et al., 1991, 1992; Yonemitsu et al., 1990; Bálványos et al., 2001, 2002). These results showed, that growth regulators and alkaloid precursor amino acids have significant effect on the growth and alkaloid synthesis of *L. inflata* hairy roots. In order to increase the biomass production of hairy root cultures, we cultivated the hairy roots in bubble column bioreactor.

MATERIALS AND METHODS

Bioreactor Culture

We have studied hairy root cultures of *Lobelia inflata* L. previously infected by microinjection of *Agrobacterium rhizogenes* R 1601. The axenic hairy roots were cultivated on solid, hormone-free culture media consisting of MS (Murashige and Skoog, 1962) or Gamborg B5 salts and vitamins (Gamborg et al., 1968) with 2 % sucrose (Bálványos et al., 1997).

In order to achieve greater biomass formation, we cultivated the *L. inflata* hairy roots in a cylindrical, glass-fronted, 10 litre bubble bioreactor. A cylindrical nylon net was placed in the middle of the reactor to stabilize the cultures. The sterile air was laid on through the bottom of the bioreactor by a Midisart 2000 sterile filter.

We inoculated hairy root culture of *L. inflata* (clone 8009/h2) which was previously cultivated on solid B5 medium, under dark conditions (the fresh weight of the inoculum was 6.98 g). The bioreactor contained liquid B5 medium, with 3% sucrose. We cultivated the hairy roots under dark, at 23 ± 2 °C.

During the fermentation period the culture medium was charged in three phases:

- In the first phase, 1 litre of B5 liquid medium was applied for 16 days of cultivation.
- In the second phase, the starting medium was replaced with 2 litres of fresh B5

medium. At this stage we cultured the hairy roots for 19 days.

- In the last phase, 3 litres of fresh B5 medium were added into the bioreactor vessel, and the cultivation lasted for another 12 days.

On the 47 th day the tissues were collected. The fresh and dry weight was measured (after lyophilisation).

Chemical Analysis of Biologically Active Compounds

The biologically active compounds (alkaloids and polyacetylenes) of the lyophilised bioreactor culture and the liquid culture medium were analysed by HPLC. The lyophilised and powdered hairy root sample (5 g) was extracted with 1 × 70 mL, and 2 × 50 mL of 0.1 N HCl: methanol (1:1, v/v) for 3 × 20 minutes using a Labsonic U ultrasound device. After centrifugation (6000 rpm for 10 min, 2500 g) and filtration the methanol was evaporated and the remaining aqueous phase was made up to a solution (25 mL) with 0.1 N HCl. This solution and the samples of the culture medium (1 L was evaporated to 50 mL) were purified by solid-phase extraction (SPE). Aliquots (10 mL) were passed drop wise through a pre-activated (2 × 2.5 mL methanol then 2 × 2.5 mL water) SPE cartridge (Supelclean LC-8, 3 mL), which was then washed with 1 × 2.5 mL water. After air-drying of the cartridge, the alkaloid-containing fraction was eluted from the tube with 2 × 2.5 mL methanol. After the evaporation of the methanol the samples were soluted with 1.5 mL methanol for high-performance liquid chromatography (HPLC). The HPLC system consisted of a Spectra Physics P4000 quaternary gradient pump, a FOCUS scanning UV-VIS detector in combination with a Rheodyne 7125 injector. Separation was achieved on an Eurospher 100-C8 reverse-phase Vertex column (250 × 3 mm i.d.), with a pre-column (5 × 3 mm i.d., Knauer) using 33.2: 66.8 (v/v) acetonitrile: 0.1% trifluoracetic acid at a flow-rate of 1 mL/min.

The total alkaloid content in the lyophilised bioreactor culture, and in the liquid culture medium was determined photometrically. The quantity of total alkaloids was given in terms of dry tissue weight (mg/g). On taking up the concentration series for the determination of the total alkaloid content we used solutions of the lobeline base. The linear regression equation was $y = 0.0013 + 0.00057\ x$, where *y* is the absorbance and *x* is the concentration of lobeline (μg/100 ml). The correlation coefficient was 0.999.

RESULTS AND DISCUSSION

The hairy root clone 8009/h2 was inoculated into the reactor vessel, because of its appropriate growing features. Agitation was achieved entirely by the air bubbles rising through the medium, and a nylon net was applied to fix the cultures. The culture medium was charged in 3 stages to supply the rising requirements of the hairy roots. At the end of the cultivation period, the culture was collected and the fresh weight (62 g) was measured (Fig. 1). After lyophilisation the quantity of dry weight was determined (6.152 g).

The HPLC analysis presented, that measurable amount of lobeline was not found in the tissues or in the medium. However, the hairy roots synthesized large quantities of a so far unknown component which had analogue UV spectrum with lobeline and can be considered as a lobeline derivative. High amount of this component released to the liquid medium.

1.7 mg/g total alkaloid content was measured in the hairy root tissues. The total alkaloid content was similar to other experiments with *Lobelia inflata* hairy roots cultivated on solid medium or in shake flasks (Fig. 2). The total alkaloid production in the culture medium (14.3 mg) was higher than in the tissues (10.46 mg).

Ishimaru et al. (1991, 1992) isolated polyacetylenes (lobetyol, lobetyolin and lobetyolinin) from *L. inflata* hairy roots. We detected polyacetylenes by HPLC in the tissues and in the medium from bioreactor culture of *L. inflata* hairy roots as well.

CONCLUSION

Szőke et al. (1994) have reported the successful establishment of *L. inflata* suspension cultures in bioreactor. The bioreactor cultivation enables greater biomass and

secondary metabolite production. The hairy root cultures are vulnerable; therefore it is necessary to design a suitable bioreactor configuration that can provide adequate mixing while minimizing the hydrodynamic pressure and the intensity of shear stress. We applied nylon net to fix the cultures. It is concluded that due to the greater biomass formation of the bioreactor cultures, the alkaloid production was increased. We detected polyacetylenes in the bioreactor culture of *L. inflata* hairy roots.

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Figures

Fig. 1. *L. inflata* hairy roots cultivated in bioreactor

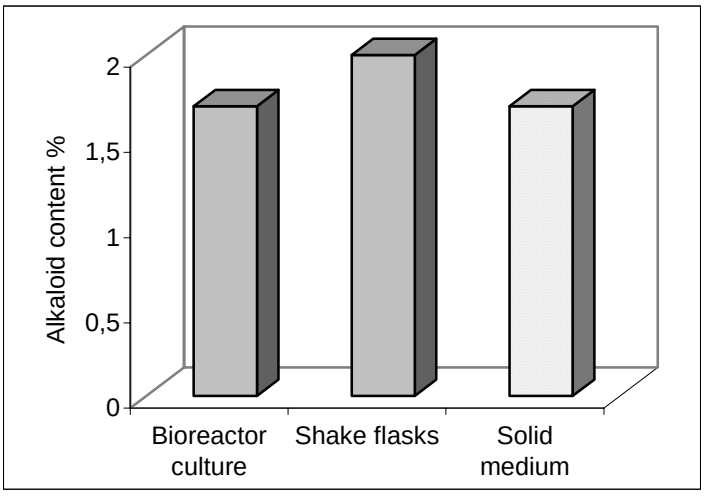

Fig. 2. Alkaloid content of *L. inflata* hairy roots cultivated in bioreactor, shake flasks and solid medium