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Non-invasive Methods for Determination of Cellular Growth in *Podophyllum hexandrum* Suspension Cultures

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Abstract Culture conductivity and on-line NADH fluorescence were used to measure cellular growth in plant cell suspension cultures of *Podophyllum hexandrum*. An inverse correlation between dry cell weight and medium conductivity was observed during shake flask cultivation. A linear relationship between dry cell weight and culture NADH fluorescence was obtained during the exponential phase of batch cultivation in a bioreactor under the pH stat (pH 6) conditions. It was observed that conductivity measurement were suitable for biomass characterisation under highly dynamic uncontrolled shake flask cultivation conditions. However, if the acid/alkali feeding is done for pH control the conductivity measurement could not be applied. On the other hand the NADH fluorescence measurement allowed online-in situ biomass monitoring of rather heterogenous plant cell suspension cultures in bioreactor even under the most desirable pH stat conditions.

Keywords: *Podophyllum hexandrum,* NADH measurement, conductivity measurement, on-line biomass measurement

INTRODUCTION

Reliable and rapid methods for measurement of cell growth and assessment of growth related bioprocess kinetics are essential for the bioreactor design in plant cell cultivations. Accurate measurement of cell growth in plant cell based suspension cultures has been rather difficult due to non-homogeneous nature of plant cells, hairy roots or embryo cultures [1,2]. The growth parameters commonly estimated for the *ex situ* measurement of growth of plant cell lines are fresh weight, dry cell weight (DCW), packed cell volume and cell counting. But the estimation of all these parameters is time consuming, requires relatively larger sample volumes and is not very reliable. Further, it may disturb plant cell metabolism due to gradual change in culture volume by regular sampling. Other methods, which have been employed with varying degrees of success, include turbidity [3], cell volume after sedimentation [4], and neural network approach [5]. Amongst non-invasive methods, medium conductivity has been successfully used as an indirect method for measurement of plant cell growth in suspension cultures [6]. An attempt has recently been made by Suresh *et al.* [7] to indirectly estimate the biomass of hairy root cultures using medium conductivity and osmolarity but under uncontrolled conditions. A lot of biological components whose measurement would be of great value for an optimal process control have fluorophore properties (*e.g.* NAD(P)H, chlorophyll, some antibiotics, lignin derivatives *etc.*). NADH may be the most important among these substances because of its central position in metabolic pathways. NADH absorbs light peaking at 366 nm and fluoresces with a maximum at 460 nm. Fluorometric determination of NADH is a well known technique. The first application of this technique was demonstrated by Duysens and Amesz [8] when NADH fluorescence was measured in the whole cells. Later, this technique was applied to continuous cultures of microorganisms [9]. Cellular NADH level has become a popular *in situ* indicator of cellular mass and/or metabolic state of cells. This compound has been conveniently measured through on-line fluorescence for various microbial systems for biomass estimation [10] and establishment of physiological state [11-13]. NAD(P)H fluorescence technique has also been applied to plant cell cultures of *Catharanthus roseus* to study a variety of different metabolic perturbations, including transition from aerobic to anaerobic conditions [14]. However, online monitoring of biomass in plant cell suspension cultures using NADH fluorescence techniques has not been reported so far, in spite of its significant importance to establish the mass activity and status of the cell particularly in pH stat bioreactor operations where conductivity methods can not be applied. *Podophyllum hexandrum* suspension cultures investigated in the present study contained clumps of different sizes [15]. Application of indirect methods would, therefore, be desirable for the estimation of cellular growth in suspension cultures.

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The major advantages of using conductometry and fluorometry for measurement of cell growth are that: (i) these are rapid, economical, efficient and reproducible, (ii) these are non-invasive and non-destructive, which do not adversely affect the growing plant cells, (iii) these require small volume of (or no) samples, and (iv) these are independent of homogeneity and morphology of the culture. The present study highlights the applicability of these two indirect methods for measurement of cell growth in suspension cultures of *P. hexandrum*, which produces podophyllotoxin, the precursor of anticancer drugs, etoposide (VP-16-213) and teniposide (VM-26) [16].

MATERIALS AND METHODS

Cultivation of *P. hexandrum* in Shake Flask

P. hexandrum cells were cultivated in 50 mL of Murashige and Skoog (MS) medium [17] contained in 250 mL Erlenmeyer flask, supplemented with glucose (72 g/L), IAA (1.25 mg/L) and polyvinylpyrrolidone (10 g/L) (initial pH = 6) [18]. A three-week, dark-grown suspension culture was used as inoculum (8 g/L) for the cell cultivation. The cultures were incubated at 20°C on a gyratory shaker at 125 rpm in the dark. Samples (15 mL) were collected every 4 days to measure DCW, medium conductivity, podophyllotoxin and residual glucose. The conductivity (expressed in millie Siemens; mS) of the culture broth was measured by digital conductivity meter (EC-CON 10, Eutech Instruments, Singapore).

Cultivation of *P. hexandrum* in 3-L Bioreactor

P. hexandrum cells were cultivated in a 3-L custom-made stainless steel bioreactor (height: 23 cm and diameter: 13.5 cm) with a working volume of 1.5 L in the same MS medium and at culture conditions mentioned above. A low-shear impeller (Setric impeller) was used at 100 rpm to minimize the cell damage. The physical parameters such as temperature and pH were controlled at 20°C and 6.0 respectively by bio-controller (ADI 1030, Applikon Dependable Instruments, The Netherlands). An NADH fluorescence probe (Ingold, Switzerland) was inserted in the bioreactor through a lateral port to measure the fluorescence emitted by the plant cells at 460 nm when light of 366 nm was passed through the culture broth. The probe displayed the online NADH fluorescence levels of the plant cell suspension culture continuously. Samples (15 mL) were collected in duplicate after every two days and analysed for DCW, podophyllotoxin and residual glucose.

Measurement of Cell Growth, Podophyllotoxin and Residual Glucose

The samples were centrifuged at 3,000 rpm for 15 min. The cells were then washed with distilled water, centrifuged again at 3,000 rpm for 15 min and weighed to get the fresh weight. The cells were then dried at 60°C for 16 h to determine dry cell weight (DCW). Podophyllotoxin and residual glucose were estimated as described elsewhere [15].

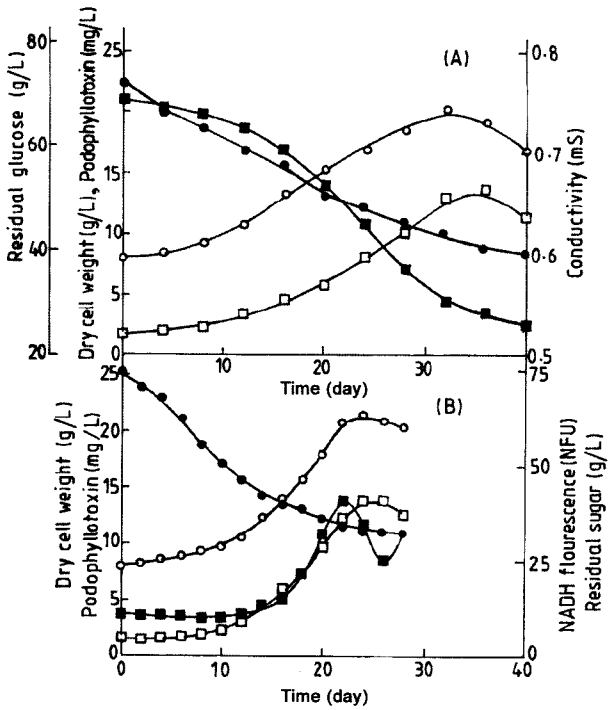

Fig. 1. A. Batch profiles of cell growth, medium conductivity, podophyllotoxin and residual glucose during cultivation of *P. hexandrum* in shake flask [O dry cell weight, ■ medium conductivity, □ podophyllotoxin, ● residual glucose]. B. Batch profiles of cell growth, NADH fluorescence, podophyllotoxin and residual glucose during cultivation of *P. hexandrum* in bioreactor [O dry cell weight, ■ NADH fluorescence, □ podophyllotoxin, ● residual glucose].

RESULTS AND DISCUSSION

Conductivity Measurement during Batch Cultivation of *P. hexandrum* in Shake Flask

Fig. 1A shows the growth, medium conductivity, podophyllotoxin and residual glucose profiles during batch cultivation of *P. hexandrum* in shake flasks. Maximum biomass and podophyllotoxin achieved were 20.2 g/L and 13.6 mg/L, respectively. The conductivity of the culture exhibited a decreasing trend during the active growth phase of *P. hexandrum* cells (8–32 days). The reduction in medium conductivity was mainly due to consumption of inorganic salts by *P. hexandrum* cells. An inverse relationship was observed between biomass and medium conductivity during the active growth phase (8–32 days) of the culture (Fig. 2).

The results suggest that the rate of consumption of

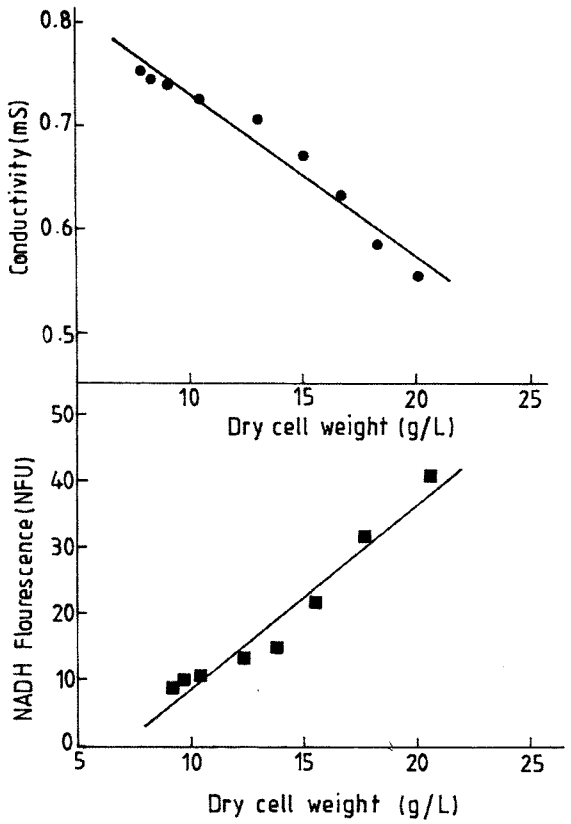

Fig. 2. Linear correlation between cell growth and conductivity and NADH fluorescence during batch cultivation of *P. hexandrum* in shake flask and bioreactor [● medium conductivity, ■ NADH fluorescence].

ionic constituents can be correlated with the cell growth rate. The cell growth coefficient is defined as the increase in cell mass per unit volume per unit conductivity equivalent of ionic nutrients consumed by the cells. A relatively constant value of cell growth coefficient [74 g L^{-1} mS^{-1}] was obtained during the active growth phase (8–32 days) of *P. hexandrum*. The cell growth coefficient varied widely when the culture entered in its stationary phase after 32 days, where the uptake of ionic nutrients (and therefore medium conductivity) was not proportional to cell growth.

Linear relationship between DCW and conductivity change has been reported for suspension cultures of *Coffea arabica*, *Withania somnifera*, *Vinca rosea* and *Nicotiana tabacum* and it was suggested that conductometry could be generally used for biomass determination in plant tissue cultures [19]. Medium conductivity has recently been reported to follow an inverse relationship with biomass during the cultivation of *Tagetes patula* L hairy roots [7].

Fluorescence Measurement during the Batch Cultivation of *P. hexandrum* in 3-L Bioreactor

The main difficulty with the measurement of conductivity as a means of determining the cell mass was that the cultivation had to be carried out without the addition of more ions *e.g.* acid or alkali; in other words, the shake flask studies had to be conducted at uncontrolled pH conditions. This problem was addressed by using NADH fluorescence probe under pH stat conditions. Thus NADH fluorescence was a superior bio-sensing method as it allowed the cultivation at controlled pH conditions.

On-line fluorescence measurement served as an indirect and *in situ* indicator of DCW during batch cultivation of *P. hexandrum* in 3-L bioreactor. The cell growth, NADH fluorescence, podophyllotoxin and residual glucose profiles during the batch cultivation of *P. hexandrum* are presented in Fig. 1B. Maximum biomass and podophyllotoxin produced were 21.4 g/L and 13.8 mg/L, respectively. The different phases of growth were distinctly represented by the profile of fluorescence. The fluorescence during the exponential phase of growth (8–24 days) followed a linear correlation with DCW indicating that the production of NADH was proportional to the increase in dry cell weight (Fig. 2). This relation did not hold when the culture entered into stationary phase.

The potential of NAD(P)H culture fluorescence has been reported for monitoring the metabolic status of plant cell suspension culture of *Catharanthus roseus* [14]. True aerobic and anaerobic states of *C. roseus* culture could be followed from the fluorescence signal. However, on-line monitoring of biomass concentration via fluorescence measurement as reported in this investigation, is important for characterization of growth in bioreactor as it does not involve even sampling and can be applied to even pH stat operating conditions in bioreactor, when conductivity measurement procedure fails.

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