
PHYSIOLOGICAL PECULIARITIES OF *LAVANDULA VERA* MM CELL SUSPENSION CULTURE IN STIRRED TANK REACTOR

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ABSTRACT

Plants are an immense source of valuable secondary metabolites used as pharmaceuticals, food additives, fragrances, dyes and agrochemicals. Production of plant derived metabolites using classical technologies is connected with several difficulties, resulting from seasonal, geographical and soil features. The isolation of such metabolites (usually in very low amounts) from huge plant mass is labour-, time-consuming and makes the process more expensive. Biotechnology offers an opportunity to exploit the plant cells, tissues, organs or entire organisms by growing them *in vitro* to get desired compounds.

Cell suspension culture of *Lavandula vera*, an attractive producer of rosmarinic acid (naturally occurring compound with a wide spectrum of biological activities), was cultivated in 3 L stirred tank reactor under the previously optimized culture conditions. The utilization of carbon source (sucrose, glucose and fructose), nitrogen and phosphorus sources were followed during the batch cultivation. The physiological peculiarities of the Lavender cells as well as the high content of rosmarinic acid (132.3 mg/g dry weight) were discussed with the respective stress levels (created from the mechanical agitation). A strong correlation (correlation coefficient 0.96) between growth of the cells and the medium conductivity changes has been observed. These findings are discussed with respect to the possibility to use such relation for biomonitoring purposes.

This paper is dedicated to the 70th birthday of Prof. Dr. Mladenka Ilieva

Keywords: bioreactor, cell suspension, *Lavandula vera* MM, rosmarinic acid

Introduction

Plants are the natural source of vast array of valuable secondary metabolites used from centuries as food additives, fragrances, dyes, pharmaceuticals and agrochemicals. Currently, more than 25% of all prescribed medicines used in industrialized countries are derived either directly or indirectly from plants, and annual sales of these products in the USA alone exceeded 30 billion dollars in 2002 (14). However, the production of plant derived metabolites using classical technologies is connected with several difficulties, resulting from seasonal, geographical and soil features. Plant biotechnology offers an attractive alternative of the classical technologies for production of plant-derived metabolites. Furthermore, the different plant *in vitro* systems appear to be the only way for production of high value metabolites from rare and threatened plants (3, 11). Although several commercial technologies have been established (e.g. paclitaxel, shikonin, berberine and etc) so far (8, 12), the

significant problem, which hampered the commercialization in most cases, is the genetic instability and heterogeneity of plant cells, grown *in vitro*. The development of on-/off-line methods for the determination of cell growth as well as the physiological behavior of the cell during their cultivation in different systems is of high importance (4).

Rosmarinic acid (ester of caffeic acid and 3,4-dihydroxyphenyllactic acid) is a naturally-occurring plant secondary metabolite, which exhibit a wide spectrum of biological activities, such anti-inflammatory, antibacterial, antiviral and antioxidant (6, 13). The previous investigations with cell suspension culture of *Lavandula vera* MM revealed it high potential for bioproduction of rosmarinic acid (5, 9). As a result of cell lines selection (1), elicitation with abiotic elicitors (2) and optimization of the bioreactor internal environment (10) the volumetric yields of rosmarinic acid significantly exceeded those reported for naturally-grown plants. The aim of the study presented here was to investigate the physiological performance of *Lavender* cells during their batch cultivation in 3-L stirred tank reactor, under the previously optimized culture conditions (10). A special

emphasis was given for the examination of method for estimation of cells' growth.

Materials and methods

Plant cell culture

The *Lavandula vera* MM plant cell suspension, was maintained in Linsmayer-Skoog (LS) medium (7), supplemented with 30 g/L sucrose and 0.2 mg/L 2,4-dichlorophenoxyacetic acid. The liquid culture was cultivated in 500 mL Erlenmeyer flasks with 1/5 net volume on shaker (11.6 rad/s) at 26 °C, in the dark. Sub cultivation was performed every 7 days using 20% (v/v) inoculum.

Bioreactor stage

The experiments were performed in a 3-L bioreactor (BioFlo 110, New Brunswick), equipped with a propeller impeller and "four-gas mix device" (New Brunswick, M1273-0055). For cultivations 1.8-L LS-modified nutrient medium (9) was used and the operation conditions were adjusted as follow 29.9 °C, 400 rpm and 50% of air saturation (10).

Cell growth

The growth of cell suspension was monitored by gravimetric determination of dry cell weight at 60°C.

Determination of sugars and inorganic salts

Sucrose, glucose and fructose content in the culture medium were determined using an enzyme test kit (R-Pharm, Germany, Cat. No. 10716260035), while phosphate, ammonium and nitrate ions were determined using test kits supplied by Merck (Germany, Cats. Nos. 1.00798.0001, 1.00683.0001, 1.09713.0001, respectively).

The results presented in this paper have been summarized from two independent experiments. All determinations were performed in three replicates.

Results and Discussion

Cultivation of plant *in vitro* systems in bioreactors represents the final step in the development of techniques for producing desired metabolites (3). In our previous work optimization of the bioreactor internal environment was made (by applying modified Simplex method) and the optimal culture conditions were found to be 29.9 °C, 400 rpm, and dissolved oxygen-50% of air saturation (10). The outcome of the optimization procedure was the significant enhancement of rosmarinic acid accumulation (132.2 mg/g dry weight) in the *Lavender* cells, most probably as a response of the plant cells to the elevated stress levels. With the aim to obtain better information about

the physiological performance of the *L. vera* cells in the stirred bioreactor under the above described conditions the present study was taken.

It was found the *L. vera* cells showed stable growth at such extreme culture conditions with a maximum at 10th day from the beginning of the cultivation (**Fig. 1**). The time course of pH changes during the cultivation of plant cell suspension is generally range between 4 and 7. The sharp changes in pH during the cultivation process are a signal for microbial contamination and/or for the beginning of lyses processes (8).

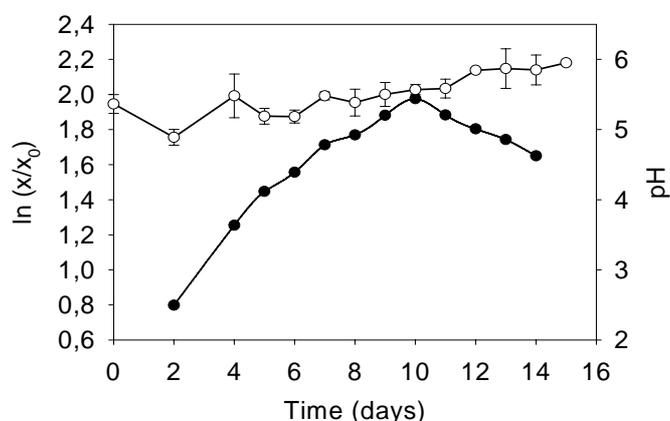


Fig. 1. Time courses of *Lavender* cells growth, presented as $\ln(x/x_0)$ (closed circle) and pH changes (open circles) during batch cultivation in 3-L bioreactor. Error bars represent standard deviations.

The physiological behavior of *Lavender* cells, with respect to utilization of major nutrients, during their cultivation in 3-L stirred tank reactor is presented in **Fig. 2**. The sucrose consumption began with rapid hydrolysis in the culture medium (**Fig. 2A**), catalyzed most probably by cell wall invertase (16), as at 6th day from the beginning of cultivation it has been completely exhausted. The formed glucose and fructose were utilized in different temporal patterns, as till 8th day of cultivation glucose was completely depleted, while utilization of fructose was extended till day 12. This is a typical feature of *L. vera* MM plant cell suspension (9), in contrast to cell suspension of *Nicotiana tabacum* 1507, where fructose has been utilized faster than glucose (5). It should be also noted that intensive consumption of glucose and fructose coincided with the exponential phase of cells growth (**Fig. 1**).

The nitrogen sources, added to the medium as combination of NH_4^+ cations and NO_3^- anions, were intensively utilized, and at 7th day of cultivation NH_4^+ ions were completely exhausted, while the utilization of NO_3^- ions continue till the end of cultivation process (**Fig. 2B**). The

reduction of pH levels at the beginning of the cultivation (**Fig. 1**) is due to the fast utilization of the NH_4^+ ions, followed by the raise in pH levels in consequence of NO_3^- ions utilization (**Fig. 2B**). Most biochemical processes in plant cells require phosphorus, as it is a constituent of many substances, and thus is involved in the energy transfer, activation of proteins and regulation of metabolic pathways (11). The phosphorous source was metabolized with high velocity from the cells of *L. vera* MM from the beginning of the cultivation, and between 10th and 12th day of cultivation it has been completely depleted (**Fig. 1B**).

Accurate and speedy measurement of cell growth and assessment of growth-related bioprocess kinetics are essential to the efficient and rational development of plant cell bioprocess engineering (15). It is known that the relation between changes of medium conductivity and growth of plant

cell exist (4, 11, 15). Such linear relationship between decrease in conductivity ($\Delta \sigma$) and increase of cell mass (ΔX) was observed during batch culture of *Lavandula vera* MM as shown in **Fig. 3**. Here ΔX is $(X - X_0)$ where X_0 and X are the dry biomasses at the beginning of cultivation and at any given time t (g/L) and $\Delta \sigma$ is $(\sigma_0 - \sigma)$ where σ_0 is initial conductivity of the medium (mS/cm) and σ is the conductivity at time t . The correlation coefficient of the dependence was calculated as 0.98 (**Fig. 3A**), which showed that the changes of the medium conductivity could be used for indirect estimation of cells growth (**Fig. 3B**). This dependence is of great significance for the control and management of the biosynthetic process during the cultivation of *L. vera* MM in bioreactors with higher volumes.

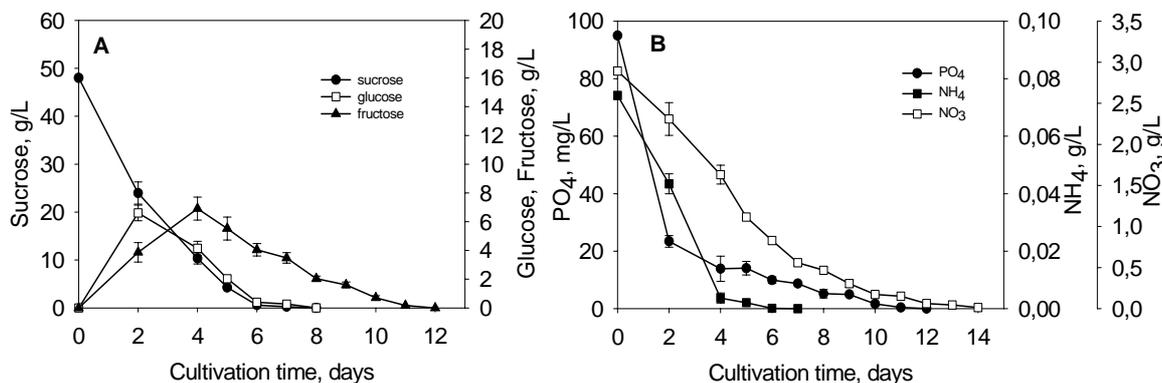


Fig. 2. Time courses of (A) carbon source and (B) main nutrients utilization during batch cultivation of *Lavandula vera* MM cell suspension culture in 3-L bioreactor. Error bars represent standard deviations.

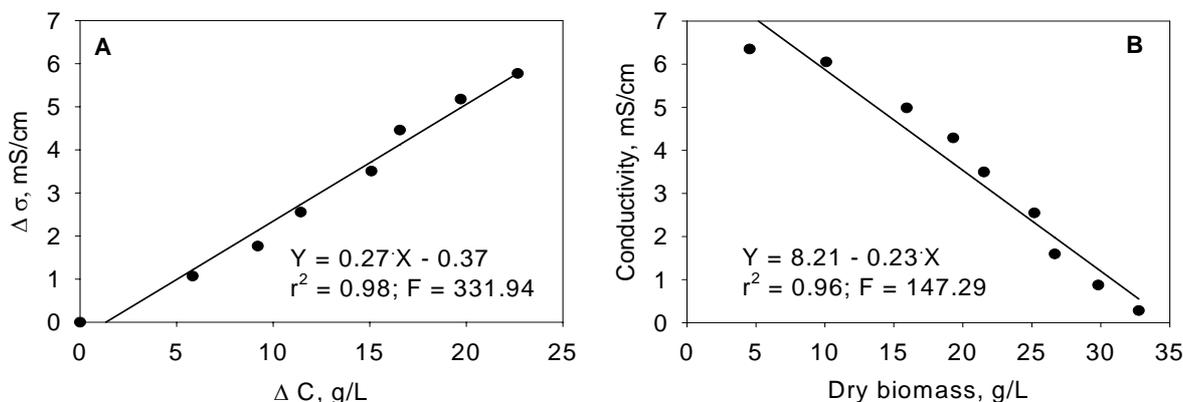


Fig. 3. (A) Increase in biomass concentration (ΔC) as a function of decrease in conductivity ($\Delta \sigma$) and (B) relationship between conductivity and biomass concentration during batch cultivation of *Lavandula vera* MM cell suspension culture in 3-L bioreactor

Based on the obtained results, it could be concluded that the cells of *Lavandula vera* MM showed stable growth and a

good physiological performance during their batch cultivation in 3-L stirred tank reactor. The conductivity method could be successfully applied for speedy

measurement of growth kinetics of *Lavandula vera* MM cell culture during the bioreactor cultivation.

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