

COMBINED CONTROL OF THE BIOPROCESS FOR
PRODUCTION OF C-GLYCOSYLFLAVONES FROM
LINUM THRACICUM IN BIOREACTOR

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(Submitted by Academician E. Golovinsky on July 19, 2012)

Abstract

The high intake of foods and beverages rich in polyphenols, especially in flavonoids, has been associated with decreased risk of neoplasm. Luteolin shows therapeutic potential as a lead anticancer compound because it induces topoisomerase II-mediated apoptosis. The objective of this study is to develop cost-effective laboratory bio-technology for production of anticancer C-glycosylflavones. For the first time, callus and suspension cultures of *Linum thracicum* ssp. *thracicum* were initiated. The C-glycosylflavones were identified in plant and cell extracts on grounds of their characteristic LC-PDA-ESI-MS spectra and retention data in comparison with authentic reference samples. The main flavonoids were identified as an isoorientin (luteolin 6-C-glucoside) and isovitexin (apigenin 6-C-glucoside).

Combining two fundamentally different control laws – the sliding nonlinear control and the linear controller, we are able to control a bioreactor in production of flavonoids in cell suspension of sensitive *L. thracicum* ssp. *thracicum* with a high concentration. After 24 days of sterile run cultivation of suspension in bioreactor, 22.8 g.l⁻¹ dry wt of cell biomass was harvested from the bioreactor culture vessel, recording an increase of about 8 times over initial inoculum, with 2.47% ± 0.11 total flavonoids, which is with 1.18% ± 0.09 greater than yields from 300 ml flasks. Our findings are the first work on large cultivation of suspension of *L. thracicum* and bioreactor production of plant anticancer C-glycosylflavones.

Financial support from the Ministry of Education, Youth and Science, Sofia, Bulgaria (D002-128/2008 and DTK 02-1/2009) is acknowledged.

Key words: *L. thracicum* ssp. *thracicum*, in vitro cell cultures, C-glycosylflavones, stirred-tank bioreactor, combined bioreactor control

Introduction. Flavonoids are phenolic substances isolated from a wide range of vascular plants. The role of dietary flavonoids in cancer prevention is widely discussed. They inhibit the proliferation of various cancer cells and tumour growth at nontoxic concentrations in organisms [1]. The high intake of foods and beverages rich in polyphenols, especially in flavonoids, has been associated with decreased risk of neoplasm. Chemoprevention has the potential to be a major component of colon, lung, prostate and bladder cancer control. These aspects made flavonoids an interesting object for industrial production. Luteolin shows therapeutic potential as a lead anti-cancer compound because it induces topoisomerase II-mediated apoptosis [5]. These aspects made luteolin and its glycosides an interesting object for pharmaceutical production.

Different *Linum* species are shown to produce considerable amounts of lignans [2]. Only a few species from genus *Linum* are investigated for flavonoids [3, 4]. In this genus, C-glycosylflavones were found only in the leaves of *L. usitatissimum* [4]. South-eastern Europe and the Balkan Peninsula in particular is a region rich in representatives of genus *Linum*, which could be a source of valuable molecules. *Linum thracicum* (Linaceae) – endemic species, native to the Balkan region, was not investigated in vitro up to now.

For the first time, cell culture of *Linum thracicum* ssp. *thracicum* was established, and flavonoid production was determined and optimized. All in vitro cultures of *Linum thracicum* produced flavonoids. A rapidly-growing cell line of *Linum thracicum* was selected for cultivation in 2-L stirred-tank bioreactor, by batch mode of cultivation for 24 days of culturing. This is the first report on the production of flavonoids from cell cultures of *L. thracicum* – an endemic rare plant species, by model-based control of the bioprocess in stirred tank bioreactor.

The objective of this study is to develop cost-effective laboratory bio-technology for production of these anticancer C-glycosylflavones. So we are paying attention not only to fundamental scientific tasks – to determine the flavonoid content in the different cell cultures from *L. thracicum* ssp. *thracicum*, but also to those related to some of the technological problems associated with bioreactor production of flavonoids and their optimization. These results could provide practical means of in vitro cultivation of this medicinally-important plant and for further biotechnological applications.

Materials and methods. Plant material of *L. thracicum* ssp. *thracicum* was collected near the town of Pazardzhik in 2008. The plant material – a voucher specimen, was deposited at the Faculty of Pharmacy, Sofia.

Germination of seeds and callus induction. Seeds from *L. thracicum* ssp. *thracicum* were surface-sterilized in 80% alcohol for 1 min, then in 10%

commercial bleach (Domestos) for 10 min, followed by three rinses with sterile distilled water. Seeds were germinated aseptically in Petri dishes containing 25 ml of growth regulator free MS medium [7], supplemented with 3% (w/v) sucrose and solidified with 0.9% agar (w/v). The pH of MS medium was adjusted to 5.6 before sterilization by autoclaving. For callus induction, mesocotyl parts of seedlings were used. Seedlings were grown on MS medium supplemented with 1.1 mg/l 1-naphthaleneacetic acid (NAA), and 1.0 mg/l 6-benzylaminopurine (BAP). Callus proliferation was obtained on MS medium supplemented with 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/l indole-3-acetic acid (IAA) and subculture was performed at three week intervals. Twenty-one-day-old calli were used to establish cell suspension cultures after the first subculture. Seed germination, callus induction and subculture were carried out in a growth chamber illuminated with fluorescent light.

Cell suspension culture. Twenty-one-day-old calli were transferred to 300 ml Erlenmeyer flasks (0.5 g fresh mass) containing 50 ml of MS liquid medium enriched with 1.0 mg/l concentrations of 2,4-D and 1.0 mg/l 6-benzylaminopurine (BAP) and agitated on a gyratory shaker (120 rpm, $25 \pm 2^\circ\text{C}$, in light 2000 Lx). Cell suspensions were subcultured at two week intervals. Cultures were maintained in a growth chamber illuminated with fluorescent light and temperature conditions as mentioned above. Two replicates were used for each treatment, and the experiment was repeated twice.

Extraction and isolation of flavonoids. The methanolic extract of the cell mass was dissolved in water. The aqueous remnant was extracted twice with equal volumes of ethylacetate p.a. After the ethylacetate phases were combined and evaporated, the remains were dissolved in methanol p.a. The ethyl acetate extract was fractionated by semi-preparative HPLC. Column: Lichrospher 100 RP-18, 250×10 mm, $10 \mu\text{m}$ (Merck). Mobile phase: acetonitrile (AcCN) – water gradient elution starting with 20% acetonitrile, increasing to 80% AcCN within 40 min, injection volume of 200 μl , flow of 2 ml/min, column temperature of 23°C . HPLC instrument: L-6200 A Intelligent Pump, L-4500 Diode array detector (Merck Hitachi); retention times: cpd. 1, 11.3 min; cpd.2, 13.3 min.

LC-PDA-ESI-MS analysis of compounds 1 and 2. Mass spectra were further obtained by LC-PDA-ESI-MS analysis on a Thermo Finnigan LCQ Deca XP Plus mass spectrometer connected to a Surveyor LC-system (Thermo Finnigan). HPLC conditions: Synergi Polar RP, 150×2 mm, $4 \mu\text{m}$ (Phenomenex); mobile phase: 0.1% formic acid in water (A), acetonitrile (B), gradient: 9% B to 22% B in 13.5 min, flow of 300 $\mu\text{l}/\text{min}$; column temperature of 30°C ; PDA detection 200–700 nm; ESI-MS conditions: negative polarity; 3.5 kV source voltage, -20 V capillary voltage, 300°C capillary temperature, 50 arb sheath gas flow, full scan 150–1000 amu, source fragmentation of 20 V; 35% normalized collision energy, 3 dependent scans using the most prominent peak in the parent spectra.

Bioreactor equipment. Fermentations were carried out in 2-L jacketed glass vessel applying cultivation. The equipment of the vessel includes: sensors for temperature, pH, dissolved oxygen concentration, foam, and speed of stirrer drive system; four integrated peristaltic pumps and one external pump for flow control and feeding. Bioreactor conditions: temperature – 26 °C, batch mode of cultivation, dissolved oxygen saturation (DO at 60%), marine-type impeller design with low-shear stress, speed of 140 rpm. This impeller provides mixing and creates a higher oxygen mass transfer rate (K_{la}).

Control system. Control system has the following functions: display of all process values via schematic P&ID algorithms, digital calibration of sensors and pump dosing counters indication of sensor parameters, recalibration function of pH-probe, control loops for temperature, stirrer speed, pH, foam, level, substrate, pO₂ with two stage cascade control, set point profile for substrate pumps.

Data acquisition. Data acquisition system includes the following functions: data collection, data base maintenance, visualization of the process variables by several plotting functions. This software allows starting or finished process batches, exporting database in appropriate data formants and sample data configuration.

Statistical analysis. All statistical analyses were performed by one way ANOVA test, $p < 0.05$ and $n = 3$.

Results and discussion. The chemical investigation of cell extracts from *Linum thracicum* ssp. *thracicum* led to the isolation of different flavonoids by means of HPLC and TLC. Two major flavone C-glucosides: isoorientin (1) and isovitexin (2) and phenolic acids (p-hydroxybenzoic, syringic, ferulic and vanillic acid) were isolated for the first time from suspension cultures of *Linum thracicum* ssp. *thracicum* by fractionation with various analytical techniques including TLC, column and HPLC. Their structures were elucidated by LC-ESI-MS analysis as isoorientin (luteolin 6-C-glucoside) and isovitexin (apigenin 6-C-glucoside). They afforded quasimolecular ions [M-H]⁻ at m/z 447 and m/z 431 respectively. Contrary to the C-8 isomers – vitexin and orientin, the MS spectra compounds 1 and 2 clearly showed a loss of 1 and 2 molecules of water [M-H-18]⁻, [M-H-36]⁻ respectively. In detail: MS² compound 1 (isoorientin) 429 (30), 411 (3), 357 (100), 327 (90); MS² orientin 429 (2), 357 (55), 327 (100); MS² compound 2 (isovitexin) 413 (8), 341 (30), 311 (100); MS² vitexin 341 (8), 311 (100). This was in accordance with [6]. With respect to the potential use of isoorientin (luteolin 6-C-glucoside) and isovitexin (apigenin 6-C-glucoside) as cancer-preventive or chemotherapeutic agents [1, 5], it is worth mentioning that these aspects made these glycosides an interesting object for industrial production.

Bioreactor strategy for production of C-glycosylflavones by suspension culture of *L. thracicum* ssp. *thracicum*. The aim of this study was to establish a suspension culture of *L. thracicum* ssp. *thracicum* for bioreactor cultivation as a renewable source of potent anticancer C-glycosylflavones and to

improve the bioreactor-based production of flavonoids by model-based control of the bioprocess. A rapidly-growing selected cell line of *L. thracicum* ssp. *thracicum* was grown in 2-L air-lift bioreactor for a period of 24 days. In order to reach effective production, we had to combine two factors – both flavonoid content and biomass growth. The manipulation of environmental factors such as pH, temperature, shear stress and O₂ supply is the key to production of plant cell culture processes. This research presents the implementation of optimal control strategy to control a reactor in production of flavonoids in cell suspension of *L. thracicum*, combining two fundamentally-different control laws to achieve satisfactory transient performance. Combining the sliding nonlinear control and the linear controller, we are able to control a reactor in production of flavonoids in cell suspension of sensitive *L. thracicum* ssp. *thracicum* with a high concentration.

Bioreactor control algorithm. An algorithm is proposed combining a nonlinear controller – f_2 , and a linear controller – f_1 , for disturbance rejection. The dynamics of the bioreactor system in the case of substantial fluctuations caused by the main regulators or external disturbances can be described by

$$a_1\ddot{p} + a_2(\xi) \sin(p) = u,$$

where p is an integrated value – sensitivity of the plant cells to disturbances of the pH and the O₂, u is a multidimensional control vector, ξ is a disturbances vector.

The structure of the control system is shown in Fig. 1

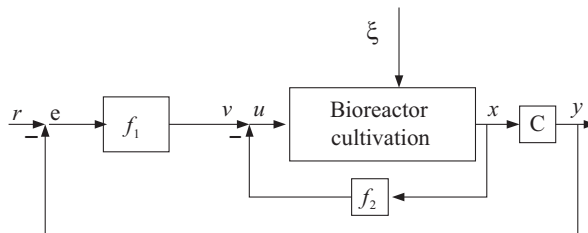


Fig. 1. Bioreactor control system

Case 1. Only the linear, not optimized controller f_1 contains a single integrator on. Graphs shown respectively in Fig. 2 represent the system transient response and control signal. Disturbance amplitude is 0.1 and is included in 2.5 s after the beginning of the transition process.

Case 2. Only the linear optimized controller f_1 contains a single integrator on. A reduction in the overshoot is obtained (Fig. 3 presents the system transient response and control signal).

Case 3. Achieve full compensation for fluctuations by implementation of an additional nonlinear controller f_2 in parallel with the linear controller f_1 . Figure 4 presents the system transient response and control signal to bioreactor.

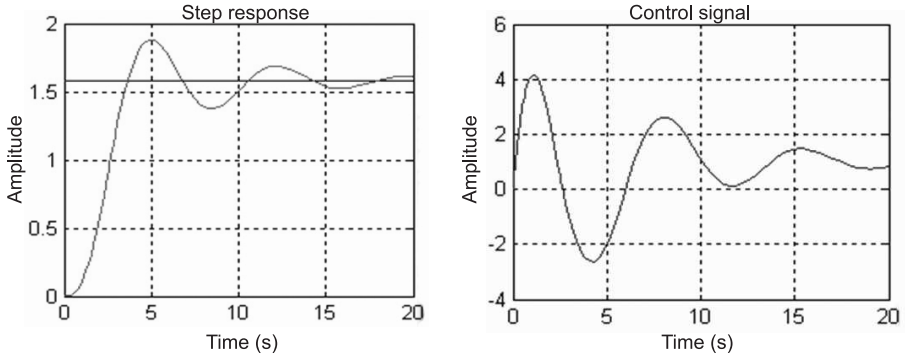


Fig. 2. *Case 1*: system transient response and control signal

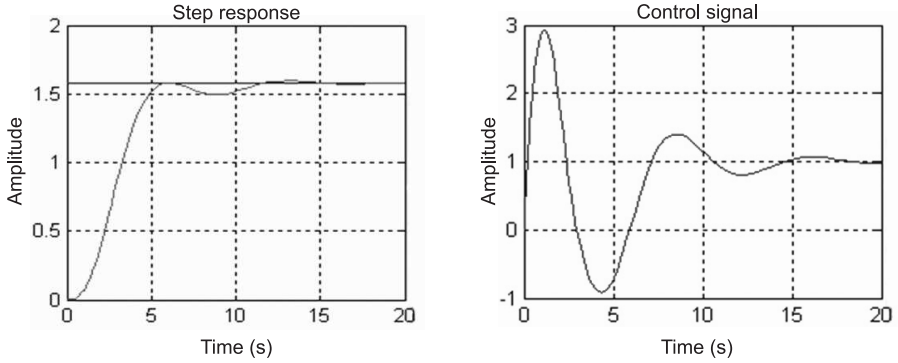


Fig. 3. *Case 2*: system transient response and control signal

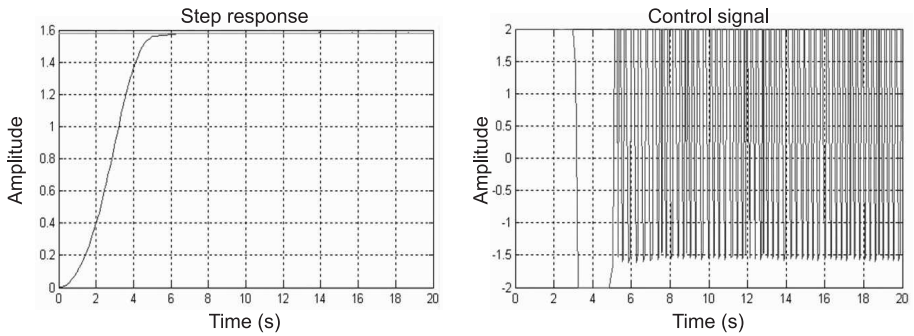


Fig. 4. *Case 3*: system transient response and control signal

In this article, it is shown how to connect two fundamentally-different control laws to achieve satisfactory transient performance. Combining the sliding nonlinear control algorithm and the linear controller (PI, PI², PID), one can use a control action of high authority to force the system to approach the target states in a short transient, and to maintain a steady response of zero regulation errors with smooth control input. The evaluation of this strategy shows that this method can successfully improve the cell yield of the bioreactor.

The maximum total amount of flavonoids of 1.29% was achieved by production in suspension cultures in the flasks. The highest amount of isoorientin (luteolin 6-C-glucoside) was 2.3 mg/g DW and isovitexin (apigenin 6-C-glucoside) – 4.1 mg/g DW. After 24 days of sterile run cultivation of suspension in bioreactor, 22.8 g.l⁻¹ dry wt of cell biomass was harvested from the bioreactor culture vessel, recording about 8 times increase over initial inoculum (5.0 g), with 2.47% ± 0.11 total flavonoids. The obtained flavonoid content, after improving the bioreactor-based production, combining the sliding nonlinear control and the linear controller is with 1.18% ± 0.09 greater than the yields from the 300 ml flasks. Our findings are the first work on large cultivation of suspension of *L. thracicum* and bioreactor production of plant anticancer C-glycosylflavones, employing bioreactors for high biomass production to meet the industrial requirement, leading to the successful commercialization of plant cell bioprocesses.

Conclusion. In the current study, for the first time a rapidly-growing cell line of *L. thracicum* was selected for cultivation in 2-L stirred-tank bioreactor, by batch mode of cultivation. Our goal here was to improve the bioreactor-based production of flavonoids in suspension cultures of *L. thracicum*, by model-based control of the bioprocess. Our results indicate that suspension culture is a valuable alternative approach for obtaining flavone C-glucosides with therapeutic potential as a leading anti-cancer compound from *Linum thracicum* ssp. *thracicum*.

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