RADOMíRA VANKOVÁ[1]
GABRIELA KUNCOVÁ[2]
ONDREJ PODRAZKY[2]
ALENA GAUDINOVÁ[1]
TOMÁŠ VANEK[3]

[1]Institute of Experimental Botany AS CR, Prague, Czech Republic

[2]Institute of Chemical Process Fundamentals AS CR, Prague, Czech Republic

[3]Institute of Organic Chemistry and Biochemistry AS CR, Prague, Czech Republic

SCIENTIFIC PAPER

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USE OF TWO–DIMENSIONAL FLUORESCENCE SPECTROSCOPY FOR MONITORING OF THE EFFECT OF DIMETHYL SULFOXIDE ON THE GROWTH AND VIABILITY OF IMMOBILIZED PLANT CELLS

The growth and viability of tobacco cells (Nicotiana tabacum L.) immobilized in alginate or pectate were monitored during their cultivation by using two–dimensional fluorescence spectroscopy (2–D FS). The cell growth was followed via the fluorescence of amino acids in proteins. The correlation between the tryptophan fluorescence and the cell biomass inside the alginate beads was verified by comparison with the dry weight of the cells. The determination of biomass content or cell viability by measurement of the intensity of NAD(P)H fluorescence was found unsuitable. Cell viability was estimated by determination of cell esterase activity using fluorescein diacetate as a fluorogenic substrate. The fluorescence intensities of both fluorophores, tryptophan and fluorescein, were determined by scanning a 2–D FS spectrum of intact beads in front face cuvette. Using this technique the effect of organic solvent, dimethyl sulfoxide, on the growth and metabolic activities of cells within the beads was evaluated. While 4% DMSO was tolerated by cells, 6% DMSO led to the cell destruction.

Plant cell amount and viability represent important parameters in both biotechnological and physiological studies. Very sensitive way of viability determination is measurement of esterase activity using fluorescein diacetate (FDA) as substrate. The level of liberated, brightly fluorescent fluorescein may be estimated with microscope [1]. To avoid microscopic evaluation, which is very tedious, time–consuming and more–or–less based on yes/no principle, the amount of fluorescein could be quantified by phospho–imager analysis [2]. Esterase activity can be also measured *in vitro* after enzyme extraction from the cells [3]. Recently we reported quantification of fluorescein in free tobacco cells by two–dimensional fluorescence spectroscopy (2–D FS) [4].

2–D FS is a fast method, which allows to follow easily all three parameters of fluorescence (excitation and emission wavelengths and emission intensity) and to obtain the excitation–emission data matrix within few minutes. Fluorescence maxima of biogenic fluorophores from proteins, such as tryptophan (Trp), tyrosine and phenylalanine, may be measured in the range of excitation wavelengths λ_{EX} = 220–350 nm and emission wavelengths λ_{EM} = 270–400. In the range λ_{EX} = 300–500 nm and λ_{EM} = 350–600 nm vitamins, e.g. pyridoxine, riboflavin, and coenzymes NADH, NADPH, FAD and FMN, have fluorescence maxima.

Measurement of fluorescence intensity of biogenic fluorophores was applied for on–line determination of biomass concentration during bioprocesses in bioreactors [5,6,7]. In a broth, biomass amount was most often determined by measurement of the intensity of Trp fluorescence [8], which corresponds to the protein content, or of the fluorescence intensity of nicotinamide coenzymes NAD(P)H. The fluorescence techniques can be applied also for measurement in bioreactors with immobilized cells, under conditions that the carrier is transparent or at least translucent, for example alginate or silica, and medium has low absorbance and fluorescence [9,10].

Podrazký correlated 2–D fluorescence spectra of *Saccharomyces cerevisiae* immobilized in alginate beads using the function CORREL (Microsoft Excel 97) [11]. The highest correlation coefficients that corresponded with the best agreement between concentration and fluorescence intensity were found at wavelengths λ_{EX}=330 nm, λ_{EM}=440 nm for living cells and for total content of biomass at λ_{EX}=320 nm, λ_{EM}=360. Thus the best correlation for concentration of living cells was achieved at the range of maxima fluorescence of NAD(P)H, while for total yeast biomass concentration no particular fluorescence maxima were identified.

Using 2–D FS we followed the effect of dimethyl sulfoxide (DMSO) on viability of free tobacco BY–2 cells [4]. This organic solvent has been used for permeabilization of free or immobilized plant cells to achieve a controlled release of cell stored secondary metabolites into the medium [12]. To ensure the repeated use of immobilized cells the concentration of DMSO should enable the release of secondary metabolites, however, should not be detrimental to cells, to allow their subsequent recovery [13]. DMSO is also used as a cryoprotectant, usually in the mixture with sucrose and glycerol [14]. Cryopreservation has become of special importance for long–term storing of either high–producing strains or unique transformed lines. In this contribution we report on the use of 2–D FS for the determination of biomass amount and viability of plant cells inside intact alginate or pectate beads, as well as for the evaluation of stress conditions (the presence of DMSO).

Author address: Institute of Experimental Botany AS CR Rozvojova 135, 165 02 Prague 6, Czech Republic
E–mail: vankova@ueb.cas.cz
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EXPERIMENTAL

Cell culture. Tobacco BY–2 cell suspension culture was maintained in modified Linsmaier and Skoog medium supplemented with 0.2 mg/l of 2,4–dichlorophenoxyacetic acid. The suspension was cultivated in dark at 27°C on an orbitary shaker (155 rpm) and subcultured weekly by 40–fold dilution.

Cell encapsulation into alginate. Autoclaved (120°C, 18 min) alginate (Sigma–Aldrich, Type IV, Practical Grade) was dissolved in distilled water (3.2%, w/v), 20 ml of viscous alginate solution was mixed with 17 ml of water and 3 ml of 7–day–old cell suspension [late exponential phase, 1 g fresh weight (FW)]. The mixture was extruded into continuously stirred $CaCl_2$ solution (1%, w/v). Formed beads (diameter 3–4 mm) containing encapsulated cells were stirred in $CaCl_2$ solution for 1 h and then washed with water. The full culture medium was used for their further cultivation. DMSO was added to the part of alginate beads at a final concentration 4% (v/v) and 6% (v/v), respectively.

Cell encapsulation into pectate. Autoclaved (120°C, 18 min) pectate (Roth, Pektin N, Na–salt) was dissolved in distilled water (6%, w/v), 20 ml of solution was mixed with 17 ml of water and 3 ml of 7–day–old tobacco cell suspension (1 g FW). The mixture was extruded through the syringe tip needle (0.84 mm internal diameter) into continuously stirred $CaCl_2$ solution (1%, w/v). Formed irregular beads, having shape more like lens than spheres, containing encapsulated cells were stirred for 1 h and then washed with water. The full culture medium was used for their further cultivation. DMSO was added to the part of pectate beads. The final DMSO concentrations were 4% (v/v) and 6% (v/v), respectively.

The esterase activity determination. The esterase activity of the cells was estimated as the intensity of fluorescence of fluorescein released from FDA. Thirty beads washed with water were placed on a sieve and immersed into a flask with 10 ml of 0.005% (w/v) FDA solution [stock in acetone (0.5%, w/v, stored at –20°C) was diluted with water prior the use]. The solution with the beads was stirred for 15 min with a magnetic stirrer placed bellow the sieve. The beads were washed with distilled water and 2–D FS spectrum was recorded.

Biomass determination. The cell growth was followed for 7 days. Each day 100 alginate beads (the content of one flask) were washed three times with distilled water and the fluorescence spectra were measured. The beads were dissolved in 0.5 M citrate buffer (pH 5.3). The cells were retained on the filter, washed and weighed after drying 24 h at 80°C.

2–D FS. Fluorescence spectra were measured with fluorescence spectrophotometer Hitachi F–4500. Standard 1 cm wide fluorescence cuvette, filled with 1 layer of tightly packed 30 beads, was placed into a solid sample holder.

RESULTS AND DISCUSSION

Typical fluorescence spectrum of immobilized tobacco BY–2 cells after their treatment with FDA is shown in Fig. 1. Left upper part of the picture represents a non–fluorescent area. The diagonal area corresponds to scattered light. The fluorescence of Trp can be found at λ_{ex} 280–290 nm/λ_{em} 340–350 nm, fluorescence of NAD(P)H at λ_{ex} 340–350 nm/λ_{em} 450–460 nm and that of fluorescein at λ_{ex} 460–490 nm/λ_{em} 510–520 nm.

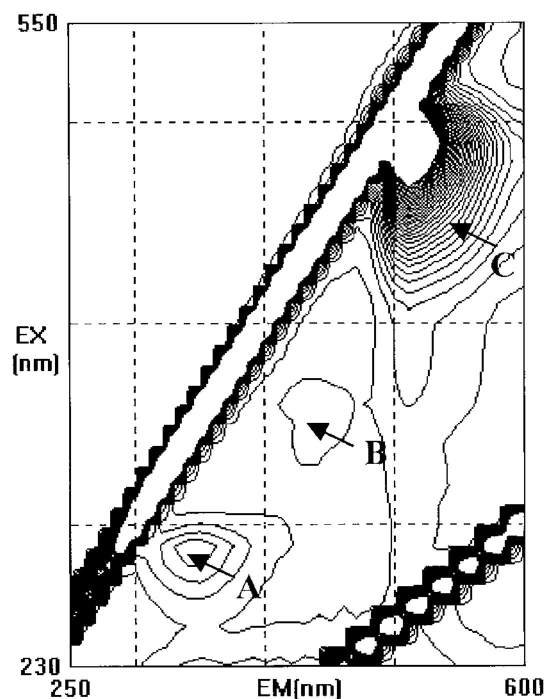

Figure 1. 2–D Fluorescence spectrum of living tobacco BY–2 cells immobilized in alginate beads after treatment with fluorescein diacetate. A – peak of tryptophan, B – peak of NAD(P)H, C – peak of fluorescein.

The potential correlation of biomass amount and fluorescence of biogenic fluorophores Trp and NAD(P)H was tested by comparison with the dry mass of cells liberated from the alginate beads with citrate (Fig. 2). The Trp fluorescence was proportional to the amount of biomass. In the same spectrum the intensity of fluorescence of NAD(P)H was more than five times lower as compared to fluorescence of Trp. The changes in medium composition during cultivation might influence NAD(P)H fluorescence intensity, which was found unsuitable for monitoring of biomass amount.

In our previous experiments we obtained very good correlation between viability of free cells and fluorescein fluorescence, as verified by comparison with dye exclusion test [4]. In experiments where both cell amount and their metabolic activity may be changed, it is necessary to estimate the biomass amount (in case of BY–2 cells via the Trp fluorescence). Then the esterase activity can be related to the cell number, which allows evaluation of the cell metabolic activity.

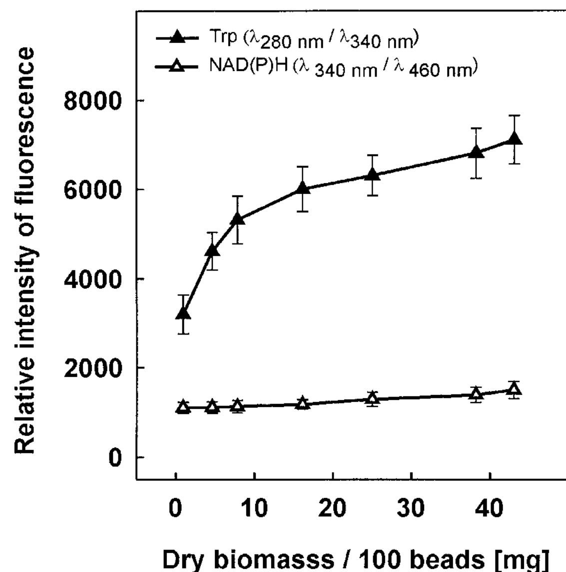

Figure 2. Correlation of the dry weight and fluorescence intensity of tryptophan (λ_{ex} 280 nm/λ_{em} 340 nm) and of NAD(P)H (λ_{ex} 340 nm/λ_{em} 460 nm) in immobilized tobacco BY-2 cells.

When the fluorescein fluorescence was followed during the cultivation of alginate beads, its intensities were proportional to the amount of dry biomass (in the region above 15 mg dry weight/100 beads) (Fig. 3). All 2-D FS spectra of intact beads were scanned immediately after 15 min incubation with fluorescein diacetate. While fluorescein fluorescence measured with free cells was stable for one hour [4], in alginate beads we observed during an hour a drop by one half. The reason might be slow diffusion of fluorescein into the centres of beads.

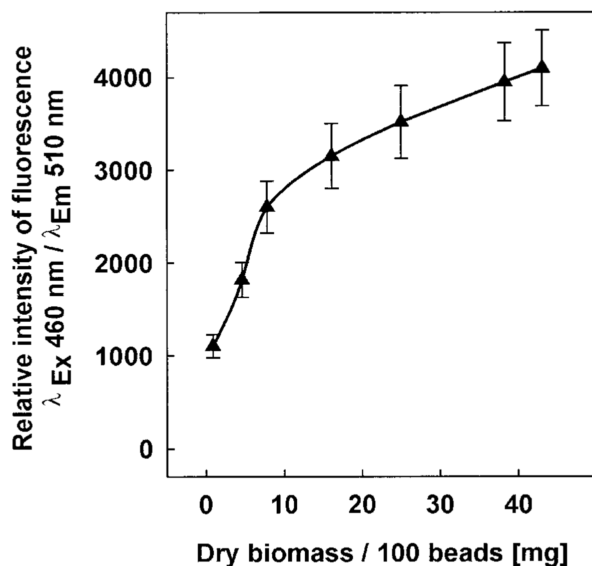

Figure 3. Correlation of the dry weight and fluorescence intensity of fluorescein (λ_{ex} 460 nm/λ_{em} 510 nm) released from fluorescein diacetate by living tobacco BY-2 cells in alginate beads.

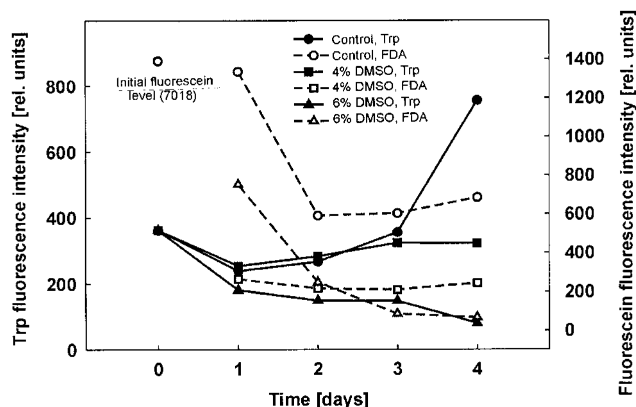

Figure 4. The tryptophan and fluorescein fluorescence in alginate beads incubated either in cultivation medium (control) or in the medium supplemented with 4 % or 6 % DMSO.

In alginate beads the biomass amount estimated by Trp fluorescence (λ_{ex} 280nm/λ_{em} 330 nm) revealed prolonged lag phase – ca 2 days in comparison with 1 day in case of free cells (Fig. 4). The mild decrease of biomass amount within first 24 h after immobilization might be caused by the leakage of the cells from the surface of the beads during cultivation and/or by release of disintegrated cells that had been damaged during the immobilization procedure. After 3 days the cell number started increasing exponentially.

When esterase activity of tobacco cells (λ_{ex} 460 nm/λ_{em} 510 nm) was determined after cell immobilization into alginate and 1 h hardening in 1% (w/v) $CaCl_2$, relatively high level of esterase activity, comparable to that of free cells, was found. After 24 h incubation in full cultivation medium considerable decrease of esterase activity was detected (Fig. 4). The suppression of metabolic activity might be caused by oxygen limitation as BY-2 cell suspension, which is very fast growing, has rather high oxygen demands. The diameter of alginate beads (ca 4 mm) thus might not ensure sufficient oxygen supply.

DMSO at 4% concentration diminished, or at least postponed, the growth of the cells. Nevertheless the increase of esterase activity after 3 days indicates that BY-2 cells are able to tolerate DMSO in this concentration. On the contrary 6% DMSO caused irreversible damage of cells. When FW of liberated cells was determined after 4 day exposure to 6% DMSO, damage of BY-2 cells was so severe that the vacuum applied during washing out the dissolved alginate led to their complete destruction (results not shown).

The behaviour of cells immobilized into pectate was similar to that in alginate (Fig. 5). Shorter lag phase (as determined via cell amount) and subsequent higher increase of esterase activity in case of flat pectate particles support the presumption that limitations in oxygen diffusion might occurred in large alginate beads, which slows down the cell growth.

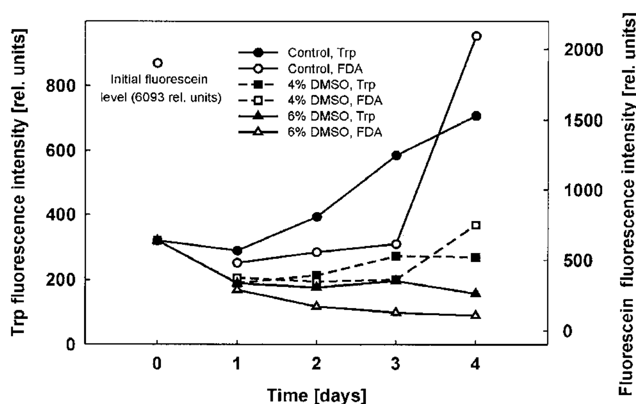

Figure 5. The tryptophan and fluorescein fluorescence in pectate beads incubated either in cultivation medium (control) or in the medium supplemented with 4 % or 6 % DMSO.

CONCLUSION

The growth of tobacco BY–2 cells immobilized in alginate and pectate beads was monitored by the measurement of intensity of fluorescence of amino acids and fluorescein released from fluorescein diacetate. The fluorescence measurements were conducted on intact beads in front face arrangement by scanning 2–D FS spectra. Correlation of Trp fluorescence intensity with cell dry weight confirmed the applicability of this optical method for immobilized biomass determination.

Fluorescein diacetate treatment allowed to measure the metabolic activity of cells simultaneously with biomass content.

The effect of DMSO on tobacco cells immobilized in alginate and pectate was determined in the interval of 4 days. DMSO at 4 % concentration was tolerated by cells, while 6 % DMSO led to the cell destruction.

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IZVOD

KORIŠĆENJE DVODIMENZIONE FLUORESCENTNE SPEKTROSKOPIJE ZA PRAĆENJE EFEKTA DIMETIL SULFOKSIDA NA RAST I PREŽIVLJAVANJE IMOBILISANIH BILJNIH ĆELIJA

Radomira Vankova[1], Gabriela Kuncova[2], Ondrej Podrazky[2], Alena Gaudinova[1], Tomas Vanek[3]

[1]Institute of Experimental Botany AS CR, Prague, Czech Republic
[2]Institute of Chemical Process Fundamentals AS CR, Prague, Czech Republic
[3]Institute of Organic Chemistry and Biochemistry AS CR, Prague, Czech Republic

Rast i preživljavanje (vijabilnost) biljnih ćelija duvana (Nicotiana tabacum L.) imobilisanog u alginat ili pektat u toku njihove kultivacije praćeno je korišćenjem dvodimenzione fluorescentne spektroskopije (2–D FS). Ćelijski rast je praćen preko fluorescencije amino kiselina u proteinima. U radu je utvrđena korelacija između fluorescencije triptofana i ćelijske biomase unutar alginatnih čestica. Metoda za dređivanje sadržaja biomase i vijabilnosti ćelija merenjem intenziteta NAD(P)H fluorescencije se pokazala kao nepodobna. Vijabilnost ćelija je procenjivana utvrđivanjem aktivnosti ćelijske esteraze korišćenjem fluorescin diacetata kao fluorogenog substrata. Intenzitet fluorescencije oba fluoroforna jedinjenja: triptofana i fluorescina je utvrđen snimanjem 2–D FS spektra čestica u kiveti. U toku korišćenja ove tehnike, praćen je efekat organskog rastvarača, dimetil sulfoksida na rast i na metaboličku aktivnost ćelija u česticama. Dok su ćelije tolerisale 4% DMSO, 6%–tni DMSO je vodio destrukciji ćelija.

Ključne reči: 2–D fluorescencija • Inkapsulacija • Alginat • Pektat • Ćelije duvana • Fluorescin diacetat • Dimetil sulfoksid •
Key words: 2–D fluorescence • Encapsulation • Alginate, Pectate • Tobacco cells • Fluorescein diacetate • Dimethyl sulfoxide •