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ARTICLE *in* FOLIA MICROBIOLOGICA · FEBRUARY 2003

Impact Factor: 1 · DOI: 10.1007/BF02930954 · Source: PubMed

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Monitoring the Growth and Stress Responses of Yeast Cells by Two-dimensional Fluorescence Spectroscopy: First Results

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Received 3 March 2002

ABSTRACT. *S. cerevisiae* growth and responses to different treatments were monitored by two-dimensional fluorescence spectroscopy, which simultaneously detects the fluorescence of a number of cells' own fluorophores. Growth curves of cultures of free cells were measured by means of tryptophan fluorescence in nonfluorescent culture medium and a flow-through system at a suitable excitation/emission beam geometry. Fast responses of the cells to anaerobic–aerobic transition or addition of glucose, methanol or cyanide, which could not be measured in this system because of the time delay inherent in transporting the cells from the culture flask to the cuvette, were monitored with cells immobilized in alginate. The major fluorescence changes caused by these treatments belonged to NAD(P)H which is a good indicator of the redox state of the cells.

Two-dimensional fluorescence spectroscopy is a highly efficient optical method that makes it possible to measure simultaneously the fluorescence of many constituents of living cells (NAD(P)H, chlorophyll, amino acids, cofactors, vitamins; Marose *et al.* 1998; Li *et al*. 1991; Horvath *et al*. 1993; Tartakovsky *et al.* 1996; Skibsted *et al.* 2001). Changes in the fluorescence of these indigenous compounds provide information on metabolic and other processes taking place in the cells even in the absence of any added fluorescent dyes. We used this noninvasive method for monitoring growth and the effects of altered culture conditions and stressors on the cells of *S. cerevisiae*. This paper reports on the first results of this study.

MATERIALS AND METHODS

Yeast strain and growth conditions.The experiments were performed with the wild-type *S. cerevisiae* strain SP4 (*α leu arg*) obtained from Prof. T. Biliński (*Rzeszów Pedagogical University*, Poland; cf. Biliński *et al*. 1978). The cells were cultured in a conventional YPD medium (in g/L: D-glucose 20, peptone 10, yeast extract 10) and in a nonfluorescent medium according to Horvath and Spangler (1992) containing (in g/L): $(NH_4)_2SO_4$ 5, KH_2PO_4 1, $CaCl_2$ 1, $MgCl_2$ 0.5 (pH adjusted to 5.5) supplemented with a (4 mL/L) filter-sterilized solution of vitamins and trace elements (in mg/L): *myo*-inositol 500, niacin 100, 4-aminobenzoic acid 100, thiamine 100, pantothenic acid 100, biotin 1, $ZnSO_4$ 100, $FeSO_4$ 50, $CuSO_4$ 10. Other medium components were D-glucose (10 g/L), L-arginine (19 mg/L) and L-leucine (19 mg/L).

Cultivation and immobilization of S. cerevisiae *SP4*. In experiments with free cells, the cells were cultured for 2–3 d in a flask on a shaker at 1–1.3 Hz at 30 °C. The flask was connected by tubes with a flow-through cuvette in which the 2D-fluorescence of the cell culture was measured during growth and following several treatments. In experiments with immobilized cells, the cells were cultured in the same way, harvested, washed with 0.9 % NaCl and resuspended in distilled water. A volume of suspended cells was added to alginate solution and added dropwise to a stirred sterile $CaCl_2$ solution (0.7 g/L) to give regular-shaped beads. The beads were left to stand in the $CaCl_2$ solution for 30 min, washed with sterile distilled water and stored at 4 °C in covered beakers until use. For measurements, they were placed in a special flow-through cuvette packed with the beads that enabled us to perform different interventions with the immobilized cells. The cell wet mass concentration in the alginate beads was 15 g/L.

2-D fluorescence spectroscopy. Fluorescence spectra were measured on a Hitachi F-4500 fluorescence spectrophotometer (*Hitachi*, Japan). The spectra of cellular fluorophores were measured in the range of excitation wavelengths of 250–600 nm and emission wavelengths of 220–550 nm. The instrument measures the whole emission spectrum at one excitation wavelength, the excitation monochromator is adjusted to the next wavelength and the whole spectrum is again measured in the whole range. At a scanning velocity of

30 000 nm/min the whole wavelength range can thus be scanned within 1 min. The data are stored in a computer and evaluated after the measurement.

The measurements were performed in two geometric arrangements of the rectangular measuring cuvette relative to the excitation beam: rectangular (RA) setup in which the cuvette front is perpendicular to the impinging beam and front-face (FF) setup in which the beam impinges on the cuvette front at an angle. Since the RA arrangement is strongly affected by sample turbidity and light scattering, FF geometry was used throughout.

2D-spectra of biogenic fluorophores. Solutions of (in μmol/L) phenylalanine 1250, tyrosine 100, tryptophan 50, FAD 500, NADH 500, pyridoxine 50, riboflavine 12.5 were used as standards and their 2D-spectra were measured. The concentrations were such as to give about equal peak response.

RESULTS AND DISCUSSION

Characteristics of 2D-fluorescence spectra. The 2D-fluorescence spectra obtained were graphically presented as 3-D graphs where the *x* and *y* axes represent the emission and excitation wavelength ranges, respectively, and the *z* axis represents the detector response. The commonly used imaging is the so-called contour plot – a bird's-eye view of the spectral "landscape". A 2D-fluorescence spectrum of immobilized SP4 cells is given in Fig. 1. Analytically important is only the part of the spectrum situated between the scattered light regions. Difference spectra obtained by subtracting a spectrum background from an experimental spectrum provide information on changes in the fluorescence characteristics of individual fluorophores.

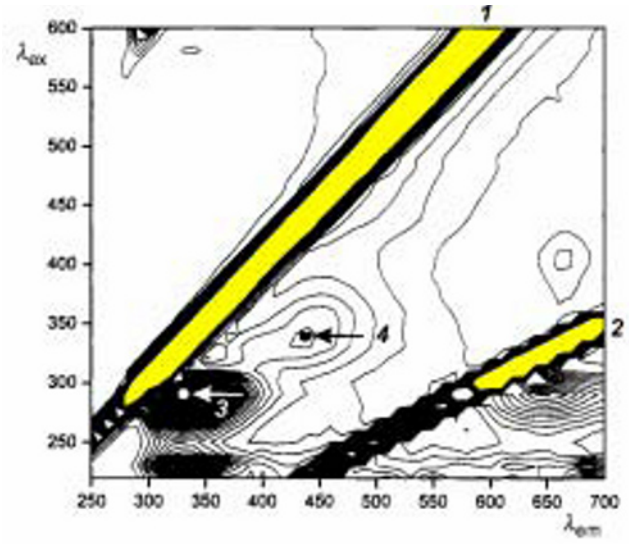

Fig. 1. 2D-fluorescence spectrum of *S. cerevisiae* SP4 cells immobilized in alginate; relevant part of the spectrum is situated between the two diagonal scattered light regions ***1*** and ***2*** (*yellow area*); main peaks: ***3*** – tryptophan ($\lambda_{ex}/\lambda_{em}$ = 290/330 nm; *white arrow*), ***4*** – NAD(P)H ($\lambda_{ex}/\lambda_{em}$ = 340/440 nm; *black arrow*).

Fluorescence assessment of growth of free cells and their response to different treatments. Measurements performed in the flow-through system with free cells in YPD medium showed that fluorescent medium components masked the fluorescence response of cellular fluorophores to different interventions. Subsequent experiments were therefore done in the nonfluorescent medium according to Horvath ad Spangler (1992). The flow-through arrangement with the cuvette in a FF setup was used with good results for measuring the growth curve of the yeast culture. The best indicator of increasing cell number in the culture was tryptophan fluorescence (Fig. 2), the fluorescence of amino acids or pyridoxine being much weaker. This method was unsuitable for measuring fast responses of the cells to different challenges (anaerobic–aerobic transition accomplished by N_2 and subsequent O_2 flushing, addition of glucose, methanol or cyanide) because of the time delay inherent in transporting the cells from the culture flask to the cuvette. Subsequent experiments involving exposure of the cells to various stressors were therefore carried out only with immobilized cells.

Fluorescence response of alginate-immobilized cells to aerobiosis, anaerobiosis and glucose addition. Immobilized cells in a flow-through cuvette responded to anaerobiosis–aerobiosis transition as expected, *i.e*. the fluorescence measured at 340/450 nm and corresponding to NAD(P)H rose on introducing N_2 into the cuvette and decreased on replacing it with air (cf. Marose *et al*. 1998; Siano and Mutharasan 1989,

1991) (Fig. 3A). Tryptophan fluorescence detected at 290/330 nm did not show any clear response to the treatment. Its initial level was found to reflect in part the presence of extracellular substances released from lysed bead-immobilized cells while its continuous decrease during the measurement obviously corresponds to the consumption of these substances by surviving cells.

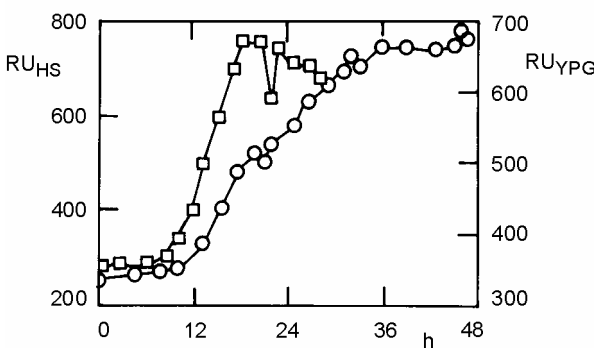

Fig. 2. Time course of tryptophan fluorescence of free *S. cerevisiae* SP4 cells during cultivation in YPG medium and nonfluorescent Horvath-Spangler (HS) medium; RU – fluorescence intensity (relative units) in YPG (*squares*) and HS medium (*circles*).

For cell wet mass concentrations below 17 g/L the intensity of NAD(P)H fluorescence was found to correlate very well with the number of immobilized live cells (correlation coefficient 0.982).

Addition of 1 % D-glucose to the flow-through medium under aerobic conditions caused a rise in NAD(P)H fluorescence during the first minute after glucose addition, followed by a sharp drop and then gradual rise (Fig. 3B). A similar pattern was described by Scheper and Schügerl (1987) during fermentative glucose metabolism while other authors (*e.g.*, Marose *et al.* 1998; Li *et al.* 1991) observed a mere fluorescence rise. The inverse time course of tryptophan fluorescence (290/330 nm) reflects a strong internal filter effect (light emitted by tryptophan molecules is used for excitation of NAD(P)H). No significant changes were found in pyridoxine fluorescence (330/390 nm). Similar pattern was found also under anaerobic conditions.

Fluorescence response of alginate-immobilized cells to methanol and cyanide. Addition of methanol (4 %) brought about a transient drop in NAD(P)H fluorescence and a transient rise in tryptophan fluorescence (Fig. 3C) mirroring again the effect of internal filter, while addition of 1 mmol/L KCN inhibiting cytochrome oxidase caused a rapid transient rise in NAD(P)H and a concomitant mirror-like change in tryptophan fluorescence (Fig. 3D).

Our data showed that 2D-fluorescence spectroscopy can be conveniently used for continuous monitoring of yeast cell growth and, in a suitable arrangement, for real-time monitoring of cell responses to various interventions altering the redox state of the cells. Studies are in progress that should extend the application possibilities of the method.

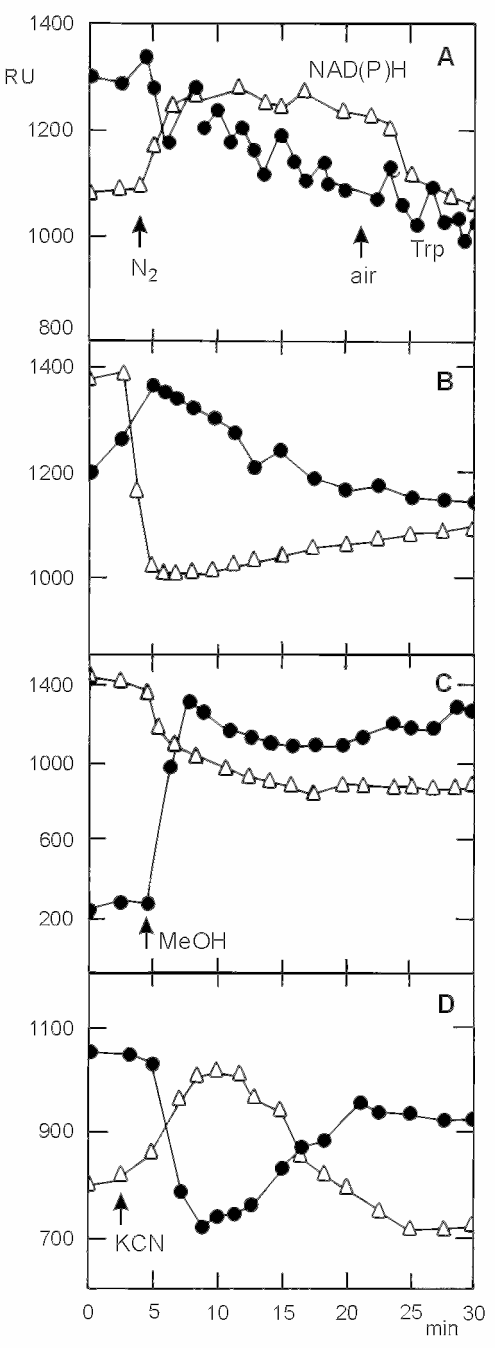

Fig. 3. Changes in NAD(P)H (*open triangles*) and tryptophan (*closed circles*) fluorescence of alginate-immobilized *S. cerevisiae* SP4 cells during different short-time treatments; **A** – anaerobic/aerobic transition (flushing with N_2/air); **B** – addition of 1 % glucose at time 0; **C** – addition of 4 % methanol (MeOH); **D** – addition of 1 mmol/L KCN; RU – fluorescence intensity, relative units.

The work was supported by grant S 502 0202 of the *Grant Agency of the Academy of Sciences of the Czech Republic*, by grant ME 577 of the *Ministry of Education, Youth and Sports of the Czech Republic* and by *Institutional Research Concept* no. AV 0Z 502 0903.

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