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Relation between growth dynamics and diffusional limitations in *Saccharomyces cerevisiae* cells growing as entrapped in an insolubilised gelatin gel

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Abstract

Flow-cytometric analysis was employed to investigate growth dynamics of a yeast cell population immobilised in an insolubilised gelatin gel by means of the quantitative determination of the average protein content per cell. This analysis was carried out on both the immobilised cell population considered as a whole and the subpopulations colonising the gelatin matrix at different depths. The results show that growth of the gelatin-immobilised yeast population was affected by the existence of a gradient of nutrient concentrations through the matrix and are in agreement with the unsteady-state diffusion model employed for the description of glucose transfer in the gel.

Keywords: Yeast immobilization; Flow cytometry; Growth dynamics; Gel diffusivity

1. Introduction

Bioprocesses carried out with viable cells immobilised by entrapment are complex due to simultaneously occurring mass transfer, substrate conversion and growth [1]. Entrapped cells cannot be uniformly bathed in a bulk nutrient bath, but rather fed only diffusibly from a two-dimensional boundary which is the contact surface separating the bulk fluid from the immobilised cells [2].

In order to obtain insight into this complex process, the application of an experimental technique such as flow cytometry can be taken into consideration. Flow cytometry is a well-established technique that allows one to measure the physical and/or chemical characteristics of single cells, yielding the distribution of these properties in the cell population and consequently contributing to the definition of its physiological state [3]. This analytical technique has been applied to growing yeast cells in several different applications, such as growth dynamics [4,5], and analysis of the kinetics of heterologous protein production [6].

In this paper, the quantitative analysis of protein content distribution by means of flow cytometry has been extended to a yeast population immobilised by entrapment in an insolubilised gelatin matrix [7]. Previous works demonstrated that yeast cells entrapped in this gel retain their viability and, once incubated in a nutrient medium, proliferate generating a thick and adherent biofilm on the gel surface [8,9].

In the present investigation, the gelatin-entrapped yeast cells were incubated in a nutrient medium and allowed to grow until cell colonisation of the gel matrix was completed. Biomass formation, budding index, glucose consumption and ethanol production were monitored during the entire time course of growth. Flow-cytometric analysis of the immobilised yeast population during growth was carried out on both the entire immobilised cell population and the subpopulations colonising the matrix at different

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levels. All the experiments were run in parallel with a freely suspended yeast population incubated in the same cultural conditions.

A diffusion model, valid during the initial lag phase when cell growth was absent, was employed for the description of glucose transfer in the gelatin matrix, in order to relate its diffusivity properties to the experimental results obtained.

2. Materials and methods

2.1. Strain

All the experiments were carried out with *Saccharomyces cerevisiae* cells, S288C strain (*MATa, SUC2, Mal, gal2, CUP1*) [10].

2.2. Immobilisation

Yeast cells collected at mid-exponential phase ($OD_{590} = 0.700$) were immobilised by entrapment within oxystarch-hardened gelatin discs according to de Alteriis et al. [7]. The standard cell density was 0.56 mg d.w. cm^{-3} of gel corresponding to 0.113 mg d.w. per disc, the average size of which was 0.8 (diameter) $\times$ 0.4 (length) cm.

2.3. Growth conditions for free and immobilised cell cultures

Both free and immobilised cells were cultivated aerobically at 30°C in 500-cm^3 shake flasks (300 rpm), containing 200 cm^3 of a medium having the following composition (w/v): 1% yeast extract, 0.5% bactopeptone and 1% glucose (YEPG-1), pH 5.0. The flasks of the free cell culture were inoculated with 3.4 mg d.w. of yeast cells from a preculture collected at mid-exponential phase and left at room temperature in sterile saline over the time needed to insolubilise the gel (12 h) during the immobilisation step. This procedure allowed both free and immobilised cell cultures to start with an inoculum characterised by the same budding index (71%). The flasks of the immobilised cell culture were inoculated with 30 discs per flask, corresponding to 3.4 mg d.w. of yeast cells.

2.4. Digestion of the immobilisate

All the determinations were carried out after release of cells from the matrix following the digestion of the gelatin network with trypsin. The digestion was carried out on discs sampled at fixed time intervals during incubation, in one or multiple steps [9] in order to release the entire immobilised population or the subpopulations colonising the matrix at different levels, respectively. In this work, the multi-step digestion was carried out in three or four steps and performed on discs collected after 3 and 6 or 9, 15 and 24 h incubation, respectively. In the first case, the discs were incubated in the digestion mixture [9] and, after a fixed time interval (1.5 min), the released cells were collected by centrifugation, the digestion mixture was quickly renewed and the same procedure repeated after 12 and 38 min to ensure the complete digestion of the discs. Thus, a separation of the immobilised biomass in three amounts roughly corresponding to three cell layers (L_3, L_2, and L_1 from the outermost layer to the innermost one) was obtained. Cell density of the layers was expressed as mg biomass released per min digestion time. The same procedure was followed in the case of the four-step digestion except for the time intervals of digestion which were 1.5, 4, 5 and 38 min. As a consequence, a further amount of biomass corresponding to an outermost cell layer, namely L_4, was obtained for these samples.

2.5. Biomass and budding index determinations

For biomass determinations, cells were harvested (in the case of immobilised cells after their release from the matrix following trypsin digestion), washed with distilled water and heated at 105°C. The percentage of budded cells or budding index was calculated after sonication of cell suspensions and microscopic examination of at least 600 cells.

2.6. Glucose determination

Residual glucose in the medium was determined by the glucose GOD-Perid kit (Roche Diagnostics GmbH, Mannheim, Germany).

2.7. Diffusional analysis of glucose in the gelatin gel

An unsteady-state diffusion model [11] valid in the absence of cell growth, i.e. during the initial lag phase, was employed for the description of glucose transfer in the gelatin matrix. Let us consider dipping a gelatin disc having the geometrical configuration of a short cylinder (length 0.4 cm, diameter 0.8 cm) and an initial uniform glucose concentration C_0, in a stirred tank containing glucose at a concentration $C_\infty \neq C_0$. We shall assume that the glucose concentration at the external surface of the disc is equal to the glucose concentration in the liquid medium bathing the disc (C_∞) due to perfect mixing. Since the length and the diameter of the disc are comparable, mass transfer by diffusion will be significant for both r and z coordinate directions. Therefore, the concentration within the disc will depend on r, z and time t.

Assuming constant diffusivity throughout the matrix and in the absence of microbial growth, the appropriate form of the mass equation is:

$$\frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial C}{\partial r}\right)+\frac{\partial^2 C}{\partial z^2}=\frac{1}{D}\frac{\partial C}{\partial t}$$

with initial condition:

$$\text{at}\quad t=0\quad \text{for}\begin{cases} 0\le r\le R \\ -L\le z\le L \end{cases} C=C_0$$

and with boundary conditions:

$$\text{at}\quad r=0\quad \text{for}\begin{cases} t>0 \\ -L\le z\le L \end{cases} \frac{\partial C}{\partial r}=0$$

$$\text{at}\quad z=0\quad \text{for}\begin{cases} t>0 \\ 0\le r\le R \end{cases} \frac{\partial C}{\partial z}=0$$

$$\text{at}\quad r=R\quad \text{for}\begin{cases} t>0 \\ -L\le z\le L \end{cases} C=C_\infty$$

$$\text{at}\quad z=\pm L\quad \text{for}\begin{cases} t>0 \\ 0\le r\le R \end{cases} C=C_\infty$$

where r and z are the radial and axial coordinates, respectively; R and L are the radius and semi-length of the short cylinder, respectively; D is the effective diffusion coefficient of glucose in the insolubilised gelatin matrix, 3.23×10^{-6} $cm^2\ s^{-1}$ [12]; C_0 and C are the initial and local glucose concentrations inside the gelatin matrix, respectively; and C_∞ is the glucose concentration in the liquid medium.

A close form solution to this equation may be obtained by the separation of variables method [13] and the end result may be expressed in the following form:

$$\frac{C(r,z,t)-C_\infty}{C_0-C_\infty}=\frac{C(z,t)-C_\infty}{C_0-C_\infty}\Big|_{\text{plane wall}}\frac{C(r,t)-C_\infty}{C_0-C_\infty}\Big|_{\text{infinite cylinder}}$$

that is, the two-dimensional solution may be expressed as a product of one-dimensional solutions that correspond to those for a plane wall of thickness $2L$ and an infinite cylinder of radius R.

2.8. Flow cytometry

Samples of cells from both free and immobilised cell cultures were sonicated, collected by centrifugation, washed and suspended in 70% (v/v) ethanol. The fixed cells were centrifuged, washed once with phosphate buffer, pH 7.4, then resuspended in 0.5 M sodium bicarbonate containing 50 μg cm^{-3} of fluorescein isothiocyanate [14]. After 30 min, cells were recovered by centrifugation and washed three times with phosphate buffer. The intensity of green (>520 nm) fluorescence (i.e. the cell protein content) was determined on at least 100 000 cells with a fluorescence-activated cell sorter (FACS IV and/or FACStar^{plus}, Becton and Dickinson) equipped with an argon ion laser yielding 200 mW at 488 nm.

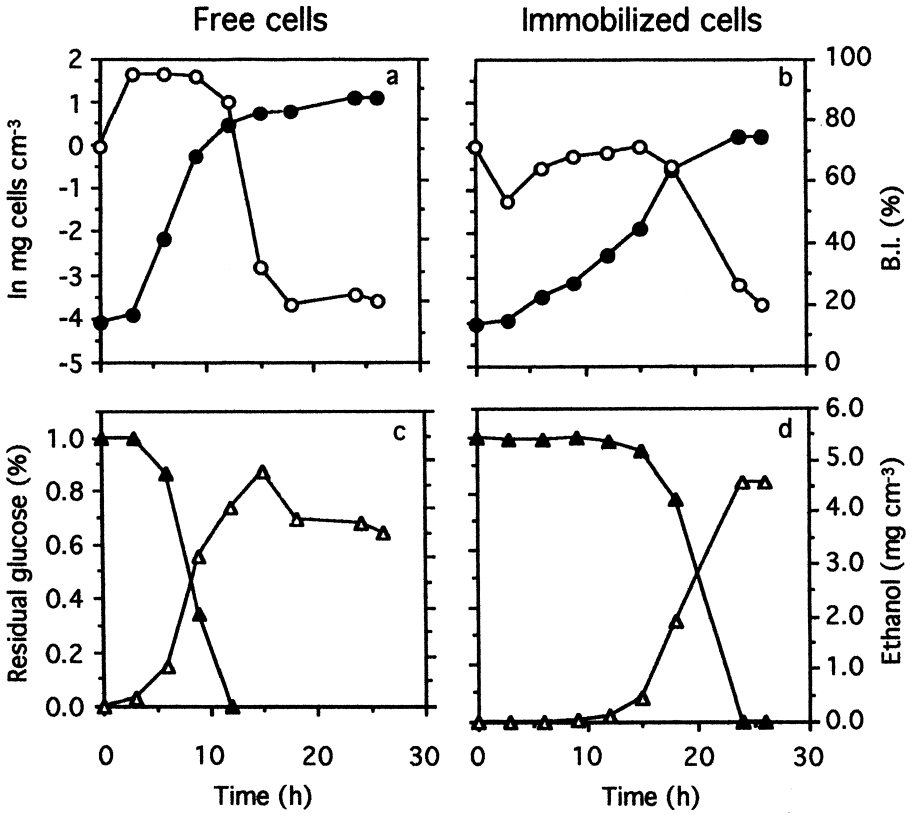

Fig. 1. Biomass (●), budding index (○), residual glucose in the bulk (▲) and ethanol (△) of free (a, c) and immobilised (b, d) cells of strain S288C cultured in YEPG-1.

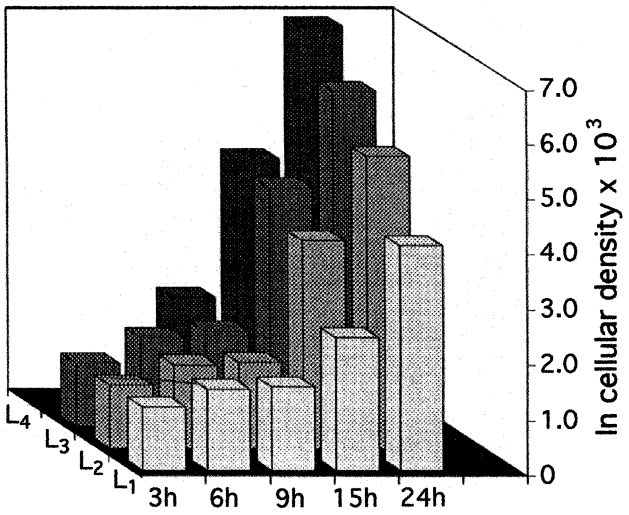

Fig. 2. Cellular density (mg of biomass released min^{-1} of digestion time) of different layers (L_1, L_2, L_3, L_4, from the innermost to the outermost one) in the discs, obtained by a multi-step digestion procedure (for details see Section 2). Discs were collected after 3, 6, 9, 15 and 24 h along the time course of growth.

Cell protein content profiles are expressed as channel number (abscissa) against cell frequency (ordinate).

3. Results

3.1. Growth of free and immobilised yeast cells in YEPG-1

Aerobic free cell flask culture displayed an exponential growth ($\mu_{max} = 0.5\ h^{-1}$) in correspondence to which a maximum budding index of 95% was observed (Fig. 1a); glucose in the bulk was rapidly consumed with production of ethanol (Fig. 1c). Differently from free cell culture, growth of the entrapped cells did not occur at the maximal rate and the budding index never exceeded 70% (Fig. 1b). Maximum ethanol produced during growth (Fig. 1d) was higher than that observed for the free culture, similarly to what already observed in the case of immobilised baker's yeast [15]. Glucose in the bulk (Fig. 1d) was consumed much more slowly, being exhausted only after 24 h incubation, when maximal cell loading of the matrix (8 mg d.w. per disc) was achieved. Until this time cell release from the matrix was negligible (data not shown).

Progressive colonisation of the matrix during incubation led to a more and more heterogeneous biomass distribution in the gel, as evidenced by the multi-step digestion carried out on discs collected after 3, 6, 9, 15 and 24 h incubation, respectively (Fig. 2). This allowed us to identify three cell layers (namely L_3, L_2, L_1 from the outside to the inside of the immobilisate) in the discs collected after 3 and 6 h incubation, and an outermost layer (L_4) in the discs collected from 9 h onwards, i.e. when growth in the subsuperficial regions had actively started, as observable to the naked eye. These layers were characterised by a progressively enhanced cellular density from the innermost layer (L_1) to the outermost one (L_4) with the exception of the sample collected after 3 h incubation, which exhibited the initial cellular density at any level.

3.2. Mass transfer in the gelatin matrix

Employing the diffusion model described in Section 2, it is possible to evaluate that a sharp gradient of glucose established throughout the gelatin matrix after 1 h incubation in YEPG-1 (Fig. 3). Indeed, the glucose concentration in the centre of the disc was 4161 vs. 10 mg cm^{-3} of gel at the interface with the bulk. Considering that after 1 h incubation cell density in the gel was still that of the inoculum, i.e. 0.56 mg d.w. cm^{-3}, the average glucose transfer rate per biomass unit was 0.124 mg min^{-1} mg^{-1} cell d.w. in the centre of the disc.

If Ca-alginate was employed instead of gelatin to entrap yeast cells in discs having the same geometrical configuration and initial cellular density of the gelatin gel, it was possible to evaluate, by the model previously described and on the basis of the glucose diffusion coefficient in Ca-alginate (6.67×10^{-6} cm^2 s^{-1}) [16], that the glucose concentration in the centre of the disc was 8114 mg cm^{-3} of gel after 1 h incubation in YEPG-1. This resulted in an average glucose transfer rate per biomass unit of 0.241 mg min^{-1} mg^{-1} cell in the centre of the disc.

In Ca-alginate, yeast cells proliferated at a μ value (data not shown) very close to μ_{max}, differently from what occurred in the case of gelatin-entrapped cells (Fig. 1b). Apparently, the higher glucose transfer rate in Ca-alginate was able to supply all the entrapped cells with saturating glucose. Conversely, in the gelatin gel the inner cells were not adequately supplied with glucose from the beginning of incubation so that their growth could not occur at the maximal rate. This fact limited the growth rate of the entire immobilised yeast population (Fig. 1b).

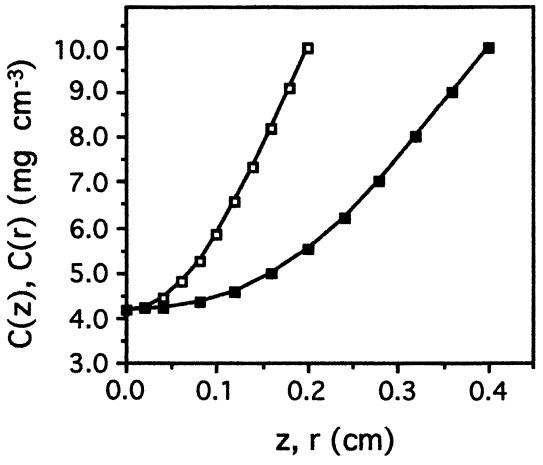

Fig. 3. Glucose concentration profiles along the semi-axis, z (□) for $r = 0$ and along the radius, r (■) for $z = 0$ of the insolubilised gelatin disc after 1 h incubation in YEPG-1.

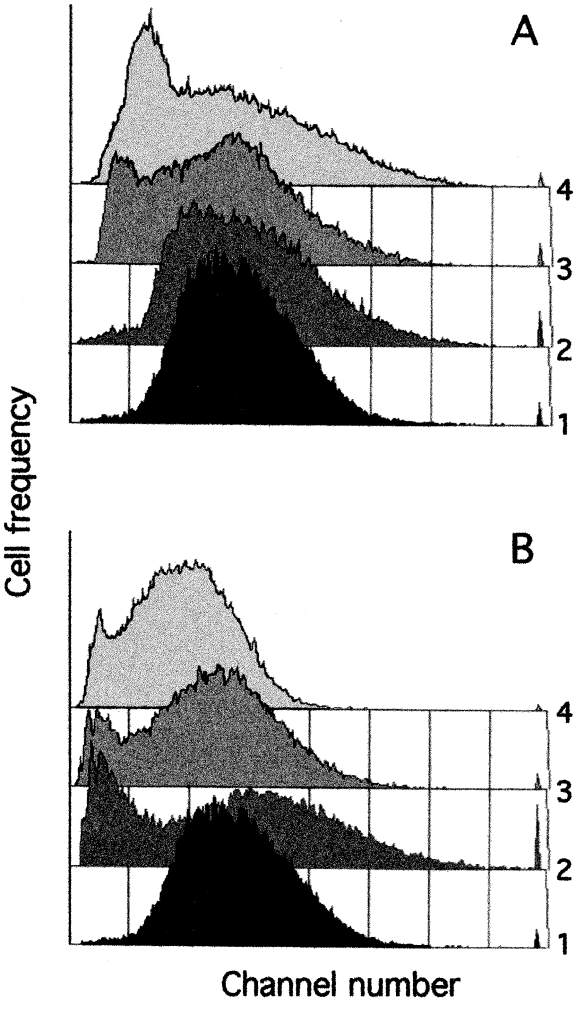

Fig. 4. Experimental protein content distributions obtained by flow-cytometric analysis of free (A) and immobilised (B) yeast populations. Samples of the culture were withdrawn at $t=0$ (1; inoculum), $t=9$ (2), $t=15$ (3) and $t=24$ h (4) of growth, total cell proteins stained with FITC and analysed.

3.3. Quantitative analysis of protein distributions by flow cytometry in free and immobilised cell cultures

We determined and compared the protein content distributions for both free and immobilised growing yeast cell populations over time. Fig. 4 shows protein content distributions after 9, 15 and 24 h of growth, compared to that of the inoculum (0 h incubation) in the case of the free (panel A) and immobilised (panel B) cell populations (the latter considered as a whole, i.e. after one-step digestion of the matrix).

The analysis of the protein content profiles of free and immobilised cells shows that the immobilised cell population is characterised by a lower protein content during the entire time course of growth. Further, protein content distribution of the free cell culture was unimodal until 9 h of growth when glucose was still present in the medium (Fig. 1c); from 15 h onwards (that is when glucose was completely exhausted in the medium) it became bimodal and shifted towards to the left with respect to the distribution of the inoculum. Conversely, protein content distribution of the immobilised cell population was already bimodal and shifted towards to the left with respect to the distribution of the inoculum from 9 h onwards, when glucose was still present in the medium (Fig. 1d).

The analysis of the protein content in the immobilised yeast culture was also performed on the subpopulations colonising the matrix at different levels and obtained by a multi-step digestion of discs collected after 3, 6, 9, 15 and 24 h incubation, respectively (Fig. 5). All the protein content distribution profiles of the subpopulations of the discs collected after 3 h (Fig. 5A) were similar to that of the inoculum, due to the fact that growth had not started yet (see also Fig. 1b), whereas those of the subpopulations of the discs collected after 6 (Fig. 5B), 9 (Fig. 5C), 15 (Fig. 5D) and 24 h (Fig. 5E) incubation were unimodal and strongly shifted towards to the left from the outermost layer to the innermost one. Only the deepest layer of samples collected after 15 and 24 h incubation was characterised by a bimodal protein distribution profile.

4. Discussion

Oxystarch-hardened gelatin has proved to be a convenient matrix for entrapping viable yeast cells to be employed in fermentation processes [8,17]. It is superior to the most commonly used natural polymeric gels (Ca-alginate, *k*-carrageenan, agar) as regards mechanical strength, though less permeable towards solutes. Indeed, it is characterised by a markedly reduced diffusion coefficient for glucose (3.23×10^{-6} cm^2 s^{-1}) [12], if compared to that of Ca-alginate, which nearly equals the glucose diffusion coefficient in water (6.67×10^{-6} cm^2 s^{-1}) [16]. As a consequence, when the gelatin-entrapped cells are incubated in the nutrient medium, a gradient of glucose concentration is established throughout the matrix determining a glucose transfer rate insufficient to ensure maximal growth in the inner layers. This affects the growth of the entire entrapped cell population and the immobilised biomass, initially homogeneously distributed in the gel, colonises the matrix generating a spatially organised microenvironment with a cellular density progressively higher from the innermost layer to the outermost one.

The existence of a gradient of nutrient concentration throughout the matrix accounts for the departure of the specific growth rate of the immobilised culture from μ_{max}, the lowered budding index and the delayed consumption of glucose in the medium with respect to that observed in the free cell culture.

The results of flow-cytometric analysis represent further evidence that growth of immobilised yeast is nutrient-limited.

A budding yeast divides with an asymmetrical process.

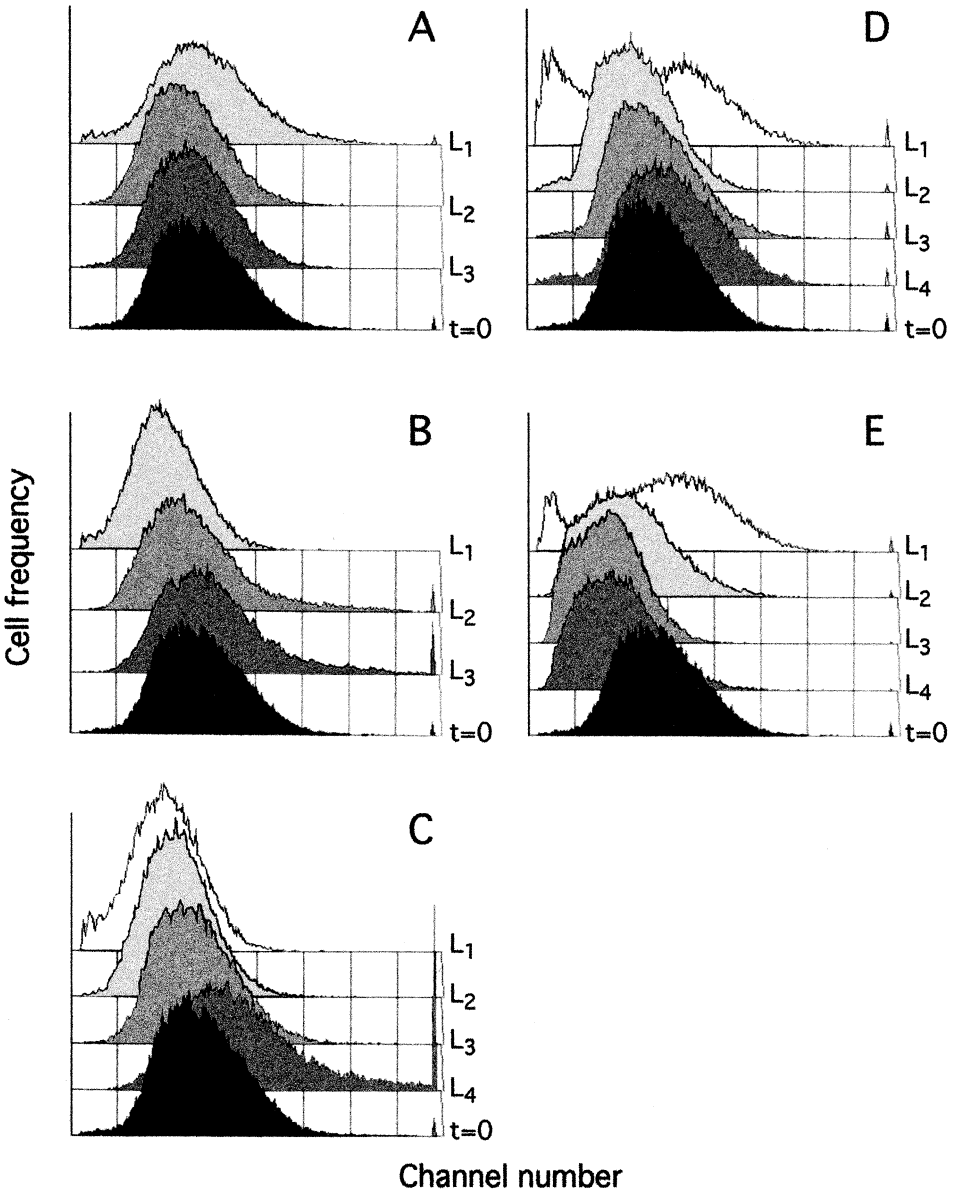

Fig. 5. Experimental protein content distributions obtained by flow cytometric analysis of immobilised yeast subpopulations. Samples of yeast subpopulations colonising the matrix at different levels were obtained by a multi-step digestion of discs collected at $t = 3$ (A), $t = 6$ (B), $t = 9$ (C), $t = 15$ (D) and $t = 24$ h (E) of growth. Total cell proteins were stained with FITC and analysed. Each panel compares the cell protein content distribution obtained from the whole yeast population employed for inoculum ($t = 0$) with those obtained from yeast subpopulations colonising the matrix at different levels (L_1, L_2, L_3 and L_4, see also text) from the innermost to the outermost layer, respectively. All distributions were acquired with the same flow-cytometric settings with the exceptions of the protein content distributions shown in panels D (L_1) and E (L_1 and L_2). In fact, since the size of these yeast cells from the inner layers is much smaller, their FITC signals were amplified of a factor of two compared to all the other signal intensities.

The degree of asymmetry at division depends on the growth rate, with slow-growing cells dividing more asymmetrically. In an asynchronously growing *S. cerevisiae* population, individual cells differ in their position within the cell division cycle, their genealogical age (i.e. daughters, parents of first generation, parents of second generation, etc.) and their size, although cells which have the same size do not necessarily share the same age or the same cell cycle position. All these differences yield the cell size distribution (i.e. the cell protein content distribution) of the growing population. It has been shown that the protein content distribution of a given population in balanced exponential growth is stable and characteristic of each growth condition (for a review see [4]). The analysis of the protein content profiles of free and immobilised cells (the latter after one-step digestion) shows that the immobilised cell population as a whole is characterised by a lower protein content during the entire time course of growth.

The protein content distribution of free yeast cells exponentially growing on glucose is unimodal. A bimodal protein content distribution is obtained after exhaustion

of the substrate; the bimodal distribution is determined by a group of cells with small mass that emerge on the left side of the distribution [4]. Differently from free culture, the immobilised culture showed a bimodal protein content distribution of the population considered as a whole already after 9 h incubation. However, the meaning of these profiles strongly differs from those observed for free-growing yeast cells. First, for the immobilised culture, a strong change of the protein content profiles takes place when glucose (i.e. the main nutrient) is still available in the medium. Secondly, if the flow-cytometric analysis is carried out on the subpopulations of the immobilisate rather than on the entire immobilised population, it appears that, except for the protein distribution profile of the outermost layer which is the same as that of the inoculum, protein distribution profiles of the deepest layers are shifted towards to the left already after 6 h incubation. Therefore, this shift (responsible for the apparent bimodal protein distribution profile of the immobilised cell population considered as a whole after 9 h incubation) is indicative of the fact that the immobilised cells have different physiological states which depend on their position inside the gel. This difference could be determined by a gradient of glucose (as well as other nutrients) concentrations established throughout the disc. The cells of the deepest layers precociously suffer from this nutrient limitation. In contrast, cells of the outermost layer, characterised by active growth, will give rise to the formation of a dense biofilm perfectly adherent on the gel surface. This active biofilm is exactly what works when a bioprocess is performed employing viable yeast cells entrapped in an insolubilised gelatin gel [9,17].

The results of this work demonstrate that flow-cytometric analysis of protein distribution is very informative in immobilised yeast growth dynamics. It presents an indirect demonstration of the existence of a gradient of nutrient concentrations in the immobilisate. In this regard, flow-cytometric analysis is revealed to be a useful tool to show the different physiological conditions which characterise a heterogeneously distributed cell population and may affect productivity and reliability of an immobilised cell bioprocess.

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