Intracellular pH determination of pristinamycin-producing *Streptomyces pristinaespiralis* by image analysis

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Intracellular pH (pH_i) is an essential parameter in the regulation of intracellular processes. Thus, its measurement might provide clues regarding the physiological state of cells cultivated *in vitro*. pH_i of the filamentous, pristinamycin-producing *Streptomyces pristinaespiralis* was determined by epifluorescence microscopy and image analysis using the pH-sensitive fluorescent probe BCECF-AM [2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester]. Staining cell culture samples (OD₆₆₀ = 1) of *S. pristinaespiralis* with 20 µM BCECF at 28 °C for 30 min yielded a green/red fluorescence ratio (R_{527/600}) that correlated with the pH_i of the cells for values ranging from 6.5 to 8.5. When *S. pristinaespiralis* was cultivated in pristinamycin-producing conditions (in batch mode, with a constant external pH of 6.8), the measured pH_i varied between 6.3 and 8.7. In fact, pH_i correlated with the excretion of pristinamycins and glucose consumption during the production process.

Keywords: intracellular pH, *Streptomyces pristinaespiralis*, epifluorescence microscopy, image analysis

INTRODUCTION

Pristinamycins belong to the synergistin family of antibiotics and are composed of two distinct groups of molecules, pristinamycins I (P_I) and pristinamycins II (P_{II}) (Preud'homme *et al.*, 1968), which act synergistically to exert their bactericidal action. For example in therapy against *Staphylococcus aureus* infection it has been found that the most efficient ratio is 60% P_{II}/40% P_I (Videau, 1982). As for two-thirds of all antibiotics, pristinamycins are produced, in batch process, by a filamentous bacterium of the genus *Streptomyces* (Demain, 1999), namely *Streptomyces pristinaespiralis* (Rhône-Poulenc, 1961).

Because there has been an ever-increasing demand for antibiotics (new molecules and higher doses) over the last decade (Craig, 1996), the optimization of the existing processes and the modification (improvement) of bacterial strains by genetic engineering (Baltz, 1998) are of great interest. Fine optimization of the pristin-

amycin production process requires good knowledge of cell physiology during the fermentation. In the context of studies on the physiology of actinomycetes, research teams have focused on metabolic changes occurring when antibiotic biosynthesis takes place under the influence of numerous external factors (Paquet, 1990; Demain, 1972). Assuming the existence of a close link between morphology and secondary metabolite production, most reports on image analysis of filamentous micro-organisms, including *Streptomyces*, present their morphology (Paul & Thomas, 1998; Cox *et al.*, 1998; Yang *et al.*, 1996). Because *Streptomyces* filaments can have different morphologies (filaments, clumps and pellets), depending on culture conditions and the physiological state of cells, separate studies have been required to investigate the relationship between each morphological type and metabolite production by image analysis (Drouin *et al.*, 1997; Durant *et al.*, 1994a, b; Cox & Thomas, 1992). In addition, image analysis has been used to determine the location of respiration activity in *Streptomyces ambofaciens* filaments (Mauss *et al.*, 1997) and the leakage of cellular components through the membrane of *S. ambofaciens* using carbon gentian violet staining (Pons *et al.*, 1998).

Abbreviations: BCECF-AM, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester; pH_i, intracellular pH; R_{527/600}, green/red fluorescence ratio (527/600 nm).

Among the parameters controlling metabolic activities, intracellular pH (pH_i) plays a major role in the regulation of enzyme activities and transport kinetics of nutrients and metabolites. Furthermore, the pH gradient across cell membranes (ΔpH) is related to cellular energetic mechanisms such as ATP generation (Leyval *et al.*, 1997; Imai & Ohno, 1995). pH_i assessment in eukaryotic cells is now well documented in the literature; fluorescent probes coupled to spectrofluorometric methods and image analysis techniques have been successfully used to investigate pH_i of several cell lines (Heiple & Taylor, 1980; Slavik, 1983; Paradiso *et al.*, 1987; Dix & Verkman, 1990; Cherlet *et al.*, 1999). On the other hand, pH_i determination in prokaryotic cells by means of flow cytometry (Leyval *et al.*, 1997) or microscopy coupled to image analysis tools (Siegumfeldt *et al.*, 1999) remains sparse and pH_i assessment in filamentous bacteria, such as *S. pristinaespiralis*, has not been reported so far. Due to a limited resolution time for distribution measurement of labelled weak acids or bases and NMR methods, other techniques must be considered for such studies. However, because most *Streptomyces* species tend to grow as pellet and filamentous forms in submerged culture (Whitaker, 1991), flow cytometry cannot be used. Therefore, epifluorescence microscopy combined with image analysis has emerged as an alternative method.

In this study a protocol was developed for pH_i measurement in the pristinamycin-producing species *S. pristinaespiralis* by epifluorescence microscopy and image analysis using the fluorochrome BCECF-AM [2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxy-methyl ester]. A second consideration concerns the methodology applied to monitor the variations of pH_i during pristinamycin-producing batch culture of *S. pristinaespiralis*.

METHODS

Bacterial strain. *Streptomyces pristinaespiralis* mutant strain Pr11 was used for all experiments and was obtained from the Rhône-Poulenc-Rorer research centre, Vitry sur Seine, France.

Media. The following media were used in this study. Complex medium (g l^{-1}): sucrose, 15; corn steep (Roquette), 5; $(\text{NH}_4)_2\text{SO}_4$, 10; K_2HPO_4 , 1; NaCl, 3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; CaCO_3 , 1.25; pH 6.8; sterilized for 20 min at 120 °C. Synthetic medium (g l^{-1}): glucose, 30; malonic acid, 7.5; L-arginine, 3; L-glutamate, 1.5; K_2HPO_4 , 0.75; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 15; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.55; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.4; 3-morpholinopropanesulfonic acid, 20; pH 6.8; sterilized for 20 min at 110 °C. K_2HPO_4 and CaCl_2 in concentrated solutions were sterilized by filtration on 0.22 μm filters (Millipore) and added separately to avoid precipitation. All chemicals were analytical grade.

Culture conditions. Inocula were prepared by pouring 0.8 ml of a thawed spore solution calibrated at 3×10^8 c.f.u. l^{-1} into 500 ml baffled shake flasks containing 80 ml complex medium. Flasks were shaken at 250 r.p.m. and 28 °C for 44 h using an orbital shaker (Ika).

Bacteria used during the experiments dedicated to method development were grown in 300 ml Erlenmeyer flasks, whereas monitoring of pH_i of pristinamycin-producing *S. pristinaespiralis*

was performed on cultures grown in a 2 l bioreactor (CMF 100; Alpha-Laval-Chemap). Inocula (2 ml) were poured into 250 ml Erlenmeyer flasks containing 30 ml synthetic medium. Cells were grown with agitation at 250 r.p.m. and 28 °C. For bioreactor batch cultures, 80 ml preculture was inoculated into 2 l synthetic medium at 28 °C. The oxygen level was maintained at a minimum of 30% of air saturation and controlled through stirring conditions. Agitation with Rushton impellers ranged between 300 and 1500 r.p.m.

Cell staining. All experiments were carried out at 4 °C in a dark room. Stock solutions (50 μl samples) of 1 mM BCECF-AM (Molecular Probes) in DMSO were stored in the dark at -20 °C. Culture samples were directly diluted in PBS to obtain a final OD_{660} of 1.0. Cells were incubated at 28 °C on a rotary shaker with different concentrations of BCECF-AM (2.5–50 μM) for incubation times ranging from 0 to 40 min.

In vivo calibration. The $[\text{H}^+ - \text{K}^+]$ carboxylic ionophore nigericin and neutral ionophore valinomycin (Sigma) were used for pH_i calibration as described elsewhere (Musgrove *et al.*, 1986; Pressman, 1976). Nigericin was dissolved in absolute ethanol and valinomycin in DMSO at final concentrations of 1 and 10 mM, respectively. Both solutions were stored at -20 °C. The cells used for the establishment of calibration curves were stained as described above. After a 30 s centrifugation at 6000 g and the removal of supernatant, cells were resuspended in high $[\text{K}^+]$ buffers at different pH values (6.5–8.5). High $[\text{K}^+]$ buffers were obtained by mixing appropriate quantities of 135 mM KH_2PO_4 /20 mM NaOH and 110 mM K_2HPO_4 /20 mM NaOH. Buffer solutions were filtered before use through 0.22 μm filters and stored at 4 °C. When stated, osmolality of each buffered solution (measured with a Roebing osmometer) was adjusted to that of the fermentation broth by adding xylose. Nigericin was added at a final concentration ranging from 5 to 40 μM for 10 min. Valinomycin was added simultaneously to 20 μM nigericin at concentrations varying from 0 to 10 μM for different incubation times (5–30 min). Cells were then centrifuged again as described above, resuspended in the same high $[\text{K}^+]$ buffer and kept on ice.

Double emission ratio technique. An epifluorescent microscope (DMRB; Leica Leitz) with $\times 10$ magnification and a 0.30 numerical aperture PL Fluotar objective was used. The final magnification was $\times 100$, because of the camera and the $\times 10$ magnification of the objective. The light source was a 50 W Hg lamp (Osram). Excitation and emission band pass filters were assembled. Whereas the excitation filter was the same for both modules (480/40 nm), two different emission filters were used: 527/30 nm (green) and 600/40 nm (red). Stained cells (20 μl) were carefully spread onto a glass slide. After the 527 nm image (green image) was taken and saved, another image of the same field (same pellet) was acquired at 600 nm (red image). Photobleaching of the stained samples was considered as negligible because only a 5% decrease in fluorescence intensity was registered after five ratio measurements on the same field (10 successive excitations). Autofluorescence of the cells was shown to have no influence on the calculated ratio, since the mean fluorescence ratio at 527/600 nm ($R_{527/600}$) was 1.00 ($\text{SD} < 0.01$) and pixel level intensity was inferior to image processing threshold values. No correlation could be found between ratio values and pellet size, suggesting that the methodology can be used for pellets of different sizes. For each experiment, 20–30 images were recorded at each emission wavelength. Error bars on the graphs represent the SD of mean $R_{527/600}$ values.

Image processing. An integrated controlled CCD monochrome camera (Cohu) was fitted onto the epifluorescence

microscope. It was controlled via a PC through an acquisition card (Secad Vision) which allowed capture of 768×576 pixel TIFF images coded on 256 grey levels. The integration time was set to 25 ms for each image. Image treatment was carried out on a PC using professional image analysis software (Visilog; Noesis).

The aim was to obtain images with pixel values directly connected to the pH_i value (pH image) by using the double ratio technique described. The first part of the treatment deals with the segmentation of these images. This operation was carried out by an automatic thresholding technique based on image entropy. Concerning the image, entropy is maximum for an equally distributed grey level histogram and equal to zero for an image with only one grey level. This technique is used to segment images into two regions by maximizing the total entropy (Coster & Chermant, 1989). It has been shown that such a segmentation best fits images containing pellets (Pons & Vivier, 1999). Consequently, two binary images were obtained, one for each initial grey level image. The binary image only contains the relevant information (object), with pixels set to 1 and background pixels set to 0. Then, a logical operation (intersection) between these images gives a single binary image representing the common part of the initial images. After morphological operations (dilation and reconstruction) to eliminate artefacts due to the thresholding, a final binary image is obtained and used as a mask for further operations. Masking is a point operation between a grey level and a binary image that produces a grey level image. In such an image, only pixel parts of the object (i.e. the pellet) are visible in grey level, the background pixels being set to black (0). Then, both grey level images (527 and 600 nm) were masked and the mean grey levels of the pellets and the SD values were computed. These values are good indicators of the quality of calibration images and they were used as criteria for the final pH calculation. Finally, after transformation in floating point images to allow arithmetic operations, a pixel to pixel division between images was performed to get the pH image. Mean grey level and SD values were also calculated on this final image to give values related to pH_i .

Additional analytical methods. During batch fermentations samples were collected to determine glucose, pristinamycin and dry weight biomass levels.

OD_{660} was measured according to the method of Lubbe *et al.* (1985) by means of a Beckman spectrophotometer, DU 7500.

Dry weight biomass was determined gravimetrically by filtering 10 ml fermentation broth on pre-weighed $0.45 \mu\text{m}$ cellulose acetate membranes (Sartorius). Membranes were washed twice with 0.85% NaCl and placed in an oven at 100°C for 12 h before being weighed again. An OD_{660} vs dry weight correlation curve allowed dry weight biomass determination.

Pristinamycins were extracted and analysed by HPLC as described by Thibaut *et al.* (1995) with a Spectra-Physics HPLC system.

Glucose concentration was determined by HPLC according to the protocol described by Rondags *et al.* (1998).

RESULTS

Method development

Microscopic and morphological parameters. Due to the characteristics of the genus *Streptomyces*, *S. pristinaespiralis* tends to grow as a pellet of several hundred micrometres in submerged culture. Moreover, during

Fig. 1. $R_{527/600}$ of stained pellets (30 min) of *S. pristinaespiralis* as a function of BCECF-AM concentration.

Fig. 2. $R_{527/600}$ of stained pellets of *S. pristinaespiralis* as a function of incubation time with $20 \mu\text{M}$ BCECF-AM.

the pristinamycin production process, most of the cells are aggregated and form small pellets. Consequently, pH_i was determined only on pellets, the predominant morphological form in this study.

Because magnifications of $\times 1000$ and $\times 400$ led to a dramatic photobleaching of the fluorescent probe, a final magnification of $\times 100$ was chosen. Using this magnification, a 25 ms exposure time was used to capture images.

Optimization of the BCECF-AM staining technique. The protocol for pH_i measurement was first optimized by testing the effect of various dye concentrations and staining times on the measured fluorescence of *S. pristinaespiralis*. Cell samples were submitted to increasing concentrations of BCECF-AM, from 1 to $60 \mu\text{M}$, for 30 min at 28°C . The measured mean $R_{527/600}$ values of the samples are reported in Fig. 1.

For fluorochrome titres below $20 \mu\text{M}$, the fluorescence ratio varies with the dye concentration, whereas at higher concentrations it remains rather constant. Because titres over $20 \mu\text{M}$ gave an increasing SD and an

Fig. 3. $R_{527/600}$ of stained pellets (20 μM BCECF-AM, 30 min) of *S. pristinaespiralis* suspended in pH 7.0 high $[\text{K}^+]$ buffer as a function of nigericin concentration. The cells were incubated for 10 min with the ionophore.

important stain release, 20 μM BCECF-AM was retained as a compromise concentration for further experiments. The optimized staining time with the fluorescent dye was determined using the same criteria (Fig. 2): 30 min staining was chosen for further experiments.

In vivo calibration. The effect of nigericin concentration was first assessed on stained samples suspended in high $[\text{K}^+]$ buffer (pH 7.0) (Fig. 3). The optimal nigericin concentration was found to be 20–30 μM , since concentrations above or below reduced the $R_{527/600}$ values. Concentrations higher than 40 μM led to cell lysis. However, the $R_{527/600}$ SD values found for different nigericin titres remained rather high. In addition, cell suspensions in high $[\text{K}^+]$ buffers at different pH values (ranging from 6.5 to 8.5) using only nigericin did not show a linear relationship between $R_{527/600}$ and external pH.

Thus, the effect of simultaneous addition of 20 μM nigericin and valinomycin concentrations ranging from 0 to 10 μM on the $R_{527/600}$ values of the cells was assessed. It appeared that valinomycin at low concentrations had no significant influence on ratio values, but the heterogeneity of pellet $R_{527/600}$ values was diminished considerably, i.e. at 1 μM valinomycin the SD of the mean ratio was reduced fivefold (1.41 ± 0.1) compared to the assay without valinomycin (1.43 ± 0.5). When higher valinomycin concentrations were used, dye was released, which considerably decreased the precision of the measurements. An investigation into the sequence of ionophore addition revealed that the best combination was a simultaneous addition of nigericin and valinomycin (data not shown). Thus, simultaneous addition of 20 μM nigericin and 1 μM valinomycin was chosen for further experiments.

To optimize the ionophore incubation time, suspensions of *S. pristinaespiralis* were stained with BCECF-AM and incubated with 20 μM nigericin/1 μM valinomycin at 28 °C in high $[\text{K}^+]$ buffer (pH 7.0) for times varying from 10 to 30 min. $R_{527/600}$ was constant (1.46 ± 0.05) whatever

Fig. 4. Calibration curve of *S. pristinaespiralis* ($R_{527/600}$ vs pH_i). The calibration curve was determined in triplicate using three different cultures of growing cells sampled after 24 h.

Fig. 5. Calibration curves for pellets having a mean 600 nm grey level ranging between 20 and 25 ($\square$), and 25 and 30 ($\circ$).

incubation time was used. On this basis, a 10 min incubation time was chosen for further experiments.

For the calibration of the technique, cells were re-suspended in high $[\text{K}^+]$ buffers at different pH values ranging from 6.5 to 8.5. The osmolality of the buffers was adjusted to that of the culture medium by means of xylose to avoid osmotic shock. Nigericin and valinomycin were added to the samples to equilibrate the pH_i of the stained cells to the pH of the surrounding buffer.

Calibration curves – green/red fluorescence ratios vs pH – were established for three samples of growing cells sampled after 24 h culture (Fig. 4). After being captured by the monochrome camera, image analysis showed that the equation of the calibration curve was slightly different for pellets with grey level images between 20 and 25, and between 25 and 30, at 600 nm. Thus, two calibration curves were used. One curve was drawn for pellets having mean grey levels between 20 and 25 at 600 nm and another was determined for mean grey levels between 25 and 30 (Fig. 5). For pellets with grey

Fig. 6. $R_{527/600}$ profile along the straight line of a calibration pH image. (a) Line drawn on a pellet pH image. (b) $R_{527/600}$ values as a function of the distance in pixels along the straight line.

levels at 25 at 600 nm, pH_i values were calculated using both equations.

Interestingly, calibration curves established for bacteria sampled at different culture times showed the same relationship between $R_{527/600}$ and pH_i , indicating that the age of the *S. pristinaespiralis* culture has no influence on the *in vivo* calibration.

To investigate a possible effect of the three-dimensional size and shape of the pellets on axial resolution (depth of field), topographic sections across pH pellet images were taken (Fig. 6a). If there was an effect, a central pixel displaying a pH_i similar to another at the periphery of the pellet would have a higher $R_{527/600}$. In fact, as shown in Fig. 6(b), the mean $R_{527/600}$ values of the peripheral pixels (at the termini of the straight line) were identical to the values of the pixels from the centre of the pellet (middle of the line), indicating that the surface fluorescence is not affected by the depth of the pellet. Pellets presented a homogeneous pH_i over a large part of their surface and the SD of mean $R_{527/600}$ values for pixels of a given pH_i remained very low over the whole surface of the pellets.

Using these experimental conditions, pH_i was determined with satisfactory precision, since the error did not exceed 0.3 pH units for pH_i values between 6.5 and 8.5.

Bioreactor batch culture of pristinamycin-producing *S. pristinaespiralis*

The protocol presented for pH_i determination was applied to the pristinamycin-producing *S. pristinaespiralis* grown under stable pH conditions (external

Fig. 7. Properties of *S. pristinaespiralis* cultivated in batch mode in a 2 l bioreactor. (a) Dry weight biomass ($\diamond$), glucose concentration ($\square$) and pristinamycin profiles ($\circ$). (b) pH_i ($\times$) and ΔpH ($\triangle$) profiles. Error bars represent the SD of mean pH_i values. Thirty pellet images were recorded at each wavelength.

pH 6.8). To evaluate growth, glucose consumption, pristinamycin production and pH_i of *S. pristinaespiralis*, a bioreactor was operated in batch mode over a 40 h period (Fig. 7a, b). The pH_i of the pellets of each culture sample was calculated with the calibration curve corresponding to the mean grey level at 600 nm. The dry weight biomass level, initially 0.4 g l^{-1} , increased to reach a maximum of 8.0 g l^{-1} within 40 h (Fig. 7a). Growth stopped independently of carbon limitation as the glucose concentration was still around 20 g l^{-1} at that time.

Contrary to the external pH, pH_i showed important variations during the course of fermentation (Fig. 7b). Starting from an initial value of 7.2, pH_i quickly increased to values around 8.2 during the first 10 h. Then it dramatically decreased, to reach 6.2 after 17 h culture. This value remained stable for a 10 h period and finally came back to near its initial value during the last period of the process. Further pH_i determinations during the stationary phase were not possible due to significant probe leakage. Because the extracellular pH was quite constant around 6.8, the pH_i and ΔpH profiles were similar during the batch process. As seen, the cells were capable of maintaining a positive ΔpH throughout the culture period, except during the 10 h period preceding the excretion phase.

This culture was performed in triplicate (data not shown) and identical pH_i profiles were observed, though minor differences regarding the pH_i values were obtained during the three processes (± 0.4 pH units).

DISCUSSION

The first aim of this study was to develop a new method for the measurement of pH_i in filamentous streptomycetes. Because flow cytometry cannot be used for filamentous bacteria, and NMR or radiolabelled weak acid/base distribution analyses are even more difficult and tedious techniques requiring high cell densities and specific materials, an image-analysis-based fluorometric method was developed.

The fluorochrome BCECF-AM, with a pK_a of 7.0, was found to be efficient for pH_i determination of neutrophilic bacteria, such as *S. pristinaespiralis*, grown in a bioreactor: the fluorescence was proportional to the pH_i of the cells between pH values of 6.5 and 8.5, and the fluorescence of the stained cells was stable for at least 30 min.

During this study only pellets of *S. pristinaespiralis* were taken into account because the bacteria mainly grow in this form during the pristinomycin-producing process. Another reason for this choice is that the origin of dispersed filaments present in the culture medium is not always clear. They can be present as a consequence of the alteration of fluffy pellets caused by shearing forces produced by the increased agitation necessary for the regulation of dissolved oxygen. Moreover, simultaneous analysis of all morphologies present in the culture has been reported to be impossible since a $\times 100$ oil immersion objective ($\times 1000$ final magnification) generally requires the use of coverslips for filament analysis which can disturb the physiology of the cells in the pellets (Nielsen *et al.*, 1995; Cox *et al.*, 1998).

The pH_i of *S. pristinaespiralis* cells only from the surface of the pellets (projected area) was considered. In fact, surface cells within the pellets can be considered as the most active cells because pellets have been described to have hollows in their centre (Thomas, 1992). These hollows form due to cell necrosis as a result of limitations in oxygen and nutrients.

In vivo calibration is essential to avoid response variability due to differences in microscope focusing between BCECF solutions (*in vitro* calibration) and BCECF-AM-stained pellets.

Satisfactory correlation coefficients and linearity were found with the proposed nigericin-valinomycin protocol over the pH 6.5–8.5 range. Contrary to the results of Boyarsky *et al.* (1996a, b) using eukaryotes, enough K^+ was able to enter the pellets to equilibrate extracellular and pH_i , even at basic pH values. Addition of xylose, resulting in increased osmolality of high $[\text{K}^+]$ buffers, could be used to avoid K^+ efflux in response to hypotonic shock. Such inward or outward cationic fluxes have been described in response to medium osmolality changes (Guillouet, 1996). One explanation for the efficient combination of valinomycin and nigericin at these concentrations could be their overwhelming action on mechanisms of cell regulation, allowing equal distribution of K^+ at intracellular and extracellular levels,

without any residual K^+ gradient, contrary to the results of Boyarsky *et al.* (1996a, b).

Slight differences in 600 nm pixel intensity for images of pellets resuspended in buffer with the same pH could generate slightly different $R_{527/600}$ ratios, as seen during this study. Differences in staining have been reported for cells having different esterase activities in the culture (Franck *et al.*, 1996). However, the difference in 527 nm fluorescence should have been of the same order. One explanation could be a different sensitivity of the camera, which is described as more efficient around 527 nm, at the two wavelengths. Another reason is that 600/40 nm fluorescence is not really an isosbestic point (Franck *et al.*, 1996). Actually, this artefact could be corrected using two different calibration curves, according to the level of fluorescence at 600 nm.

The advantage of the image analysis algorithm developed here is that it does not modify any pixel level. This results in the collection of unmodified data and it gives a more accurate estimation of actual pH_i . Since fluorescence was pellet-area-independent, contrary to other studies dealing with morphology by image analysis (Sieracki *et al.*, 1989; Durant *et al.*, 1994a, b), no delimitation problems were encountered by using an automated entropy threshold. The simplicity and rapidity (less than 1 min for calculation) of this procedure are favourable to on-line routine analysis during fermentation processes.

This paper presents for the first time a method for the determination of pH_i of filamentous bacteria by means of epifluorescence microscopy and image analysis using a pH-sensitive fluorescent probe. Although a positive control was not employed during this study (use of a bacterium with a known pH_i), the different tools and techniques utilized have already been reported to be efficient for the determination of pH_i in bacteria. First, BCECF is a valuable fluorochrome to determine pH_i since it has been shown to perfectly corroborate ^{31}P -NMR spectroscopy data obtained for *Propionibacterium acnes* (Futsaether *et al.*, 1993). Moreover, Slavik (1997) has shown that the BCECF emission ratio technique can be employed for confocal microscopy to determine pH. In addition, pH_i determination of *Listeria innocua* using epifluorescence microscopy and image analysis (Siegumfeldt *et al.*, 1999) confirmed the results obtained by spectrofluorimetry (Breeuwer *et al.*, 1996).

During this study, the pH_i of *S. pristinaespiralis* grown in batch culture was measured by an image analysis-based method using the fluorochrome BCECF-AM at a final concentration of 20 μM at 28 °C and for 30 min. As shown, pH_i could be correlated to pristinomycin excretion and glucose consumption in batch process. In fact, pH_i was found to be a good marker of the physiological state of the cells cultivated under pristinomycin-producing conditions. Furthermore, pH_i assessment can contribute to the understanding of variations in carbon fluxes through the enzymes involved in glucose metabolism, and ΔpH calculation may help in the

establishment of kinetic models for excretion and consumption of metabolites. Finally, the new method presented in this paper for pH_i assessment of *S. pristinaespiralis* could be extended to filamentous bacteria of fungal cells cultivated *in vitro*, provided that the staining protocol was adapted to each cell line.

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