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Use of fluorescent staining and flow cytometry for monitoring physiological changes in solventogenic clostridia

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ABSTRACT

Physiological changes in populations of *Clostridium beijerinckii* and *Clostridium tetanomorphum* were monitored by fluorescence staining and flow cytometry. To estimate the number of metabolically active cells in exponential growth, a combination of the dyes propidium iodide and carboxy fluorescein diacetate appeared to be a good choice for both species. During stationary phase, these stains did not reflect physiological changes sufficiently and therefore additional labeling with bis-(1,3-dibutylbarbituric acid) trimethineoxonol was applied. Results of fluorescence staining in solventogenic batch fermentations were compared with substrate-use data, the concentration of key metabolites and growth curves. We demonstrate that measurements by all methods were mutually compatible.

1. Introduction

The anaerobic genus *Clostridium* is phylogenetically diverse and species can be found in soil, sea sediments, plants, animal and human digestive tracts, and wounds. Sporulation of pathogenic species such as *Clostridium botulinum, Clostridium tetani, Clostridium perfringens* and *Clostridium difficile* is often coupled with toxin formation, and sporulation of solventogenic species may be associated with solvent production [1]. Currently, the most well-known sporogenic *Clostridium,* usually with negative connotations, is *C. difficile*, which can cause serious nosocomial infections in hospitals [2]. Although clostridia are bacteria of undisputed importance within their own right, they are not so often investigated in comparison with other spore-formers, such as the genus *Bacillus*. Thus, the development of new methods for monitoring changes in their physiology is desirable and flow cytometry (FC) may be a useful and unique tool for this purpose because it enables single cell analysis within bacterial populations.

The most frequent application of FC in bacteriology is in estimating the number of viable cells in a population. For this task, many fluorescent probes, employing different principles of cell labeling (membrane integrity, esterase activity, transmembrane potential) can be used, however the actual labeling of cells must be evaluated carefully with the aid of a fluorescent microscope because the direction and extent of cellular responses to non-specific staining can differ from expectations. As a result, staining protocols must be tailored for particular bacterial species and FC results cannot be generalized. The use of FC for solventogenic clostridia, although not widespread, is summarized in review articles [3,4]. Flow cytometry is also used for the detection of pathogenic or toxinogenic clostridia [5,6]. The goal of this study is to investigate the staining of two solventogenic clostridial populations with the common vital fluorescent probes (BOX, PI, CFDA), under sporulation conditions, and to determine whether morphologically distinct cell types might be distinguishable with the aid of specific stains. With respect to *Clostridium beijerinckii*, the work expands our previous research dealing with viability estimation of the vegetative cells by single-stain fluorescent labeling and flow cytometry [10]. To the best of our knowledge, this is also the first documented application of fluorescent labeling and FC on *Clostridium tetanomorphum* population.

2. Material and methods

2.1. Strains and culture conditions

The solvent-producing strains *C. beijerinckii* CCM 6218 and *C. tetanomorphum* DSM 4473, maintained as spore suspensions, were

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used throughout this study. *C. beijerinckii* was grown in modified TYA medium, containing 40 g/l glucose [7] and *C. tetanomorphum* was grown in cultivation medium according to [8] with the addition of 40 g/l glucose. The bacteria were cultivated in a 5 l laboratory BIOSTAT B bioreactor (B. Braun Biotech Int., Germany) with 3 l of appropriate medium, 200 rpm agitation, without pH control, at 37 °C. The bioreactor was inoculated with a 10% v/v 24-h culture grown from spores.

2.2. Growth measurement

Cell concentration was measured spectrophotometrically at 600 nm (A_{600}), using a Cary 50 Bio UV–Visible spectrophotometer (Varian, Spain).

2.3. Substrate and product analyses

The concentration of glucose, butyric acid and butanol were measured by HPLC (Agilent Series 1200 HPLC; Agilent, Spain) using a Polymer IEX H+ column (Watrex, Czech Republic) at 60 °C, with a mobile phase of 5 mM H_2SO_4 (flow rate 0.5 ml/min) and refractive index detection (for more details see Ref. [7]).

2.4. Staining protocols

Samples from bioreactor cultivation were centrifuged (6000× *g*, 3 min), washed twice in sterile 0.8% (w/v) NaCl and diluted in the same solution to an A_{600} of 0.20 ± 0.01. Fluorescent probes, bis-(1,3-dibutylbarbituric acid) trimethineoxonol (BOX), propidium iodide (PI) and carboxy fluorescein diacetate (CFDA) were added to the cell suspension, to a final concentration of 10.0 μg/ml. The cultures were incubated in the dark, at room temperature, for 10 min and subsequently analyzed.

2.5. Fluorescence microscopy and flow cytometry

Cell fluorescence was monitored by epifluorescence microscopy as described previously [9].

Stained suspensions were analyzed with an Accuri C6 cytometer (BD Accuri Cytometer Inc., USA) equipped with an argon laser (488 nm). FSC and SSC signals, and green (FL1; 515–565 nm) and red (FL3; >605 nm) fluorescence were measured. The sample flow rate was 10 μl/min, in all experiments, standard 1 and 2.5 μm beads (Align Flow, KRD) were used to check for operational consistency.

3. Theory

The work focused on two species, *C. beijerinckii* and *C. tetanomorphum*. *C. beijerinckii* is a common solventogenic *Clostridium* that ferments carbon sources via a biphasic acetone–butanol–ethanol (ABE) process. *C. tetanomorphum* also belongs to solventogenic clostridia but displays some special characteristics; it does not produce acetone but forms an equimolar mixture of butanol and ethanol, and differs from most other solventogenic clostridia in its sporulation in that it does not accumulate granulose before sporulation and forms round spores. Morphological changes during the cell cycle are shown for both species (Fig. 1).

In solventogenic clostridia, the definition of cell viability is complicated by sporulation because spores, and sometimes sporulating cells, tend to label as if they were non-viable cells. This work extends our previously published data [10] obtained under conditions of limited sporulation and pursues ideas published in Ref. [11].

4. Results and discussion

For investigating changes in the physiological state of two clostridial populations throughout batch fermentation, single staining with BOX and double staining with PI and CFDA were used (see Fig. 2). Figs. 3 and 4 show photomicrographs of *C. beijerinckii* and *C. tetanomorphum* cells double stained with PI and CFDA, displaying red and green fluorescence, respectively. Propidium iodide, as a membrane integrity probe, should stain nucleic acids in cells with permeabilized membranes and non-fluorescent CFDA, which is used for estimation of esterase activity in cells, should be enzymatically converted (by cellular esterases) into a fluorescent product. Theoretically, all cells in a population, whether active or non-active, should be labeled and the sum of labeled cells should approach 100% (see dashed lines in Fig. 2A, C). During both *C. beijerinckii* and *C. tetanomorphum* batch fermentations, the number of cells labeled with PI increased, while those labeled with CFDA decreased, but their sums remained almost constant until stationary growth phase was reached after 30 h of fermentation (see Fig. 2A, B). For stationary phase however, PI and CFDA staining did not yield consistent results, whereas labeling with BOX, which reacts to changes in transmembrane potential, appeared to better reflects changing population physiology.

Our results show that double staining (PI + CFDA) is a convenient method for estimating the number of vegetative, rod shaped, non-sporulating cells during exponential growth. A similar combination of dyes (PI + Syto 9), was used for monitoring the cell cycle in cultures of *Clostridium acetobutylicum* [11] and for estimations of

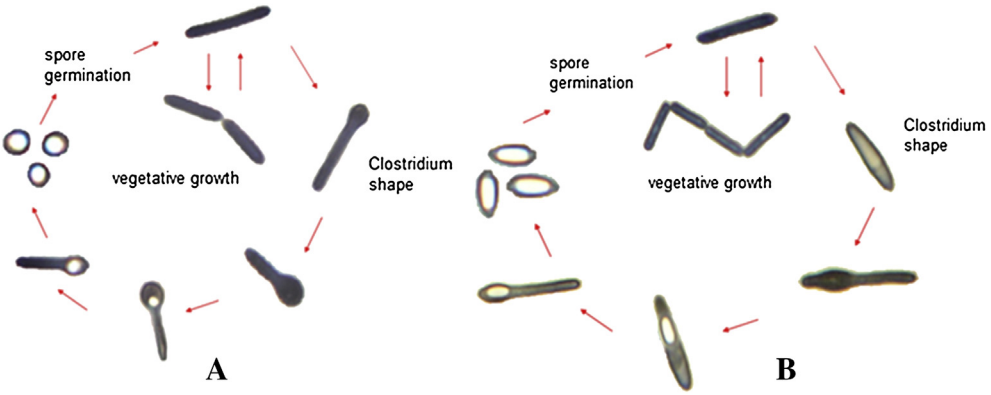

Fig. 1. Cell cycles of *C. tetanomorphum* (A) and *C. beijerinckii* (B).