Electronic Journal of Biotechnology 56 (2022) 12–21

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage:

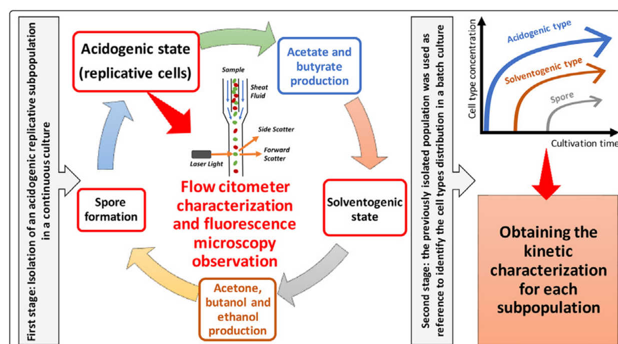
Research Article

Kinetics of ABE fermentation considering the different phenotypes present in a batch culture of *Clostridium beijerinckii* NCIMB-8052

Iván Paredes *, Julián Quintero [1], Karlo Guerrero, Roberto Gallardo [2], Silvia Mau, Raúl Conejeros, Juan Carlos Gentina, German Aroca

Escuela de Ingeniería Bioquímica, Pontificia Universidad Católica de Valparaíso, Avenida Brasil 2085, Valparaíso, Chile

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 26 July 2021
Accepted 17 December 2021
Available online 23 December 2021

Keywords:
ABE fermentation
Acidogenic
Batch cultures
Butanol production
Clostridium
Clostridium beijerinckii
Fermentation kinetics
Flow cytometry
Fluorescence microscopy

ABSTRACT

Background: In solvent-producing *Clostridium* species, several physiological conditions and morphological forms can be observed during batch ABE fermentation. Currently, the contribution made by each condition or form to the production of acids and solvents is not clear. Flow cytometry and fluorescence microscopy have been used to analyze the presence of different morphological and physiological conditions for *Clostridium* species; however, there are few studies to quantify their contribution to fermentation kinetics.

Results: Morphological changes of the batch cultures of *Clostridium beijerinckii* NCIMB-8052 were followed by flow cytometry and epifluorescence microscopy. Three cell types were identified, and the percentage of each one was estimated along the culture, showing that they are present throughout the culture and its population distribution was in agreement with the kinetics of growth and acids and solvents production. Yields for the global population and for each subpopulation were estimated. The values of the yield for butanol and acetone based on the fraction of solventogenic cells were 2.5 times the values of the parameters obtained for the whole biomass, while those for acetic and butyric acids based on the acidogenic cell type was 1.5 times.

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso

* Corresponding author.
E-mail address: ivan.paredes@pucv.cl (I. Paredes).
[1] Present address: Departamento de Ingeniería Química, Universidad de Santiago de Chile, Avenida Libertador Bernardo O'Higgins 3363, Estación Central, Santiago, Chile.
[2] Present address: Departamento de Ingeniería Química, Universidad de Magallanes, Avenida Bulnes 1855, Punta Arenas, Chile.

https://doi.org/10.1016/j.ejbt.2021.12.002
0717-3458/© 2022 Pontificia Universidad Católica de Valparaíso. Production and hosting by Elsevier B.V.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Solventogenic
Solvents production

Conclusions: Quantitative analysis of the subpopulations by flow cytometry allows the calculation of the yields related to specific cell types, isolating the contribution of each subpopulation to the production of acids and solvents.

How to cite: Paredes I, Quintero J, Guerrero K, et al. Kinetics of ABE fermentation considering the different phenotypes present in a batch culture of *Clostridium beijerinckii* NCIMB-8052. Electron J Biotechnol 2022;56. https://doi.org/10.1016/j.ejbt.2021.12.002

© 2022 Pontificia Universidad Católica de Valparaíso. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Because of the growing concern about the depletion of fossil fuels and the environmental problems associated with their use, in recent years, attention has been paid to the development of technologies that allow obtaining renewable energies such as biofuels. Butanol is a biofuel that has numerous advantages over others, including its high energy content, less corrosive nature, lower freezing point, high octane rating, high hydrophobicity, and the fact that it can be used in a mixture with gasoline. Despite these advantages, currently obtaining butanol through fermentation using bacteria of the genus *Clostridium* has been hampered mainly by the costs associated with the substrate and the recovery of the final product [1,2,3,4].

Butanol is naturally produced by several strains of the genus *Clostridium*. In the metabolism of *Clostridium beijerinckii* and other solvent-producing bacteria, two metabolic phases are described: an acidogenic phase associated with a replicative state, where organic acids, hydrogen, and CO_2 are the main products, and a solventogenic phase associated with a non-replicative state, in which acetone, butanol, and ethanol are produced from the organic acids in a process known as ABE fermentation because these compounds are the main products [5,6]. In a batch culture, the acidogenic phase occurs during the initial part of the exponential growth, while the solventogenic phase occurs by the end of the exponential growth and stationary phase.

It has been shown that the transition from the acidogenic phase to the solventogenic phase is the result of a shift in gene expression triggered by a decrease of the pH in the medium due to the accumulation of the acids produced, causing the joint metabolization of the main carbon source and accumulated organic acids to produce solvents [7]; both acids and solvents influence the physiological state of the cells, and it is thought that acids contribute to the initiation of sporulation [8]. Although the sequence of these phases have been described for *Clostridium acetobutylicum* [9], they are not clearly identifiable for *Clostridium beijerinckii* [10]. In addition, the relationship between signal pathways of solventogenesis and sporulation is not yet fully clear [8,11,12]. Despite numerous studies, it has been shown that although there is a similarity in the metabolic pathways and in the cell cycle, gene regulation and expression varies from one solventogenic strain to another [13].

In solvent-producing *Clostridium* species, up to five morphological forms could be observed by contrast-phase microscopy during batch ABE fermentation: motile rod-shaped cells, swollen non-motile cells, forespore, endospore, and free spores. A relationship between the morphological forms and the production of solvents has been proposed [14,15]. In this regard, flow cytometry together with fluorescent markers and direct microscope observation have been successfully used to characterize the role of different morphological forms found in ABE fermentation.

The first study showing a detailed characterization of the morphological forms of *C. acetobutylicum* in batch cultures was conducted by Jones et al. in 2008 [11] by means of transcriptional analysis and fluorescence microscopy by a double staining method using Syto-9 and propidium iodide (PI). These stains are usually used to distinguish between viable and non-viable cells. However, it was found that in *C. acetobutylicum*, they did not describe properly the viability of the cells, and instead, they could be used as an identification methodology for morphological subtypes. Using these fluorescent dyes, it was possible to describe several physiological conditions of *C. acetobutylicum*: vegetative cells colored red indicating a higher uptake of PI than Syto-9, an intermediate condition (named clostridial) that stains almost equally with both dyes generating an orange color, and sporulated forms (forespores, endospores and free spores) colored mostly green indicating a higher uptake of Syto-9 than PI [16].

Several attempts have been made to identify cell types in *Clostridium* culture. In 2014, Patakova et al. [17] using PI, carboxyfluorescein diacetate (CDFA) and bis-(1,3-dibutylbarbituric acid) trimethineoxonol (BOX) together with flow cytometry, identified four cell types in batch cultures of *C. beijerinckii* CCM6218 and *C. tetanomaphum* DSM 4473. In 2015, González-Peñas et al. [18] identified four physiological states in a batch culture of *C. acetobutylicum* ATCC824 by using light diffraction analysis (LS) and PI staining. In 2016 Kolek et al. [8] using flow cytometry and combinations of PI/CFDA, PI/Syto9 and BOX determined the variation in the viability and the appearance of spores in batch cultures of *C. beijerinckii* NCIMB-8052 and *C. pasteurianum* NRRL-B-598. In 2018, Branska et al. [19] identified four cell types in batch cultures of *C. beijerinckii* NRRL-B-598 by means of flow cytometry and PI/CFDA stains. More recently, Karstens et al. [20] developed a mathematical model to predict the distribution of the physiological states in a cascade of reactors operating in continuous mode, using for this purpose the concentrations of different metabolic products.

The above-mentioned studies provide valuable qualitative information; however, for quantitative characterization, a methodology allowing the direct isolation and quantification of each subpopulation needs to be established. Flow cytometry distinguishes the subpopulations on the basis of size and complexity, allowing to establish the relative amount of each one of them. Ideally, each subpopulation should be identified separately, but this is not an easy task to perform in the case of a *Clostridium* culture. In this regard, the use of continuous culture operated at a dilution rate close to its critical value would allow to isolate at least a population of vegetative cells in an acidogenic state, because in that condition, the other cell types would not have the replicative capacity and they would be washed out from the culture [9,21]. Combining flow cytometry with staining and microscopy techniques allows a more detailed description and analysis of the culture and easier identification of the contribution of each cell type in the production of acids or solvents, resulting in a deeper understanding of the kinetics of ABE fermentation. This work aims to identify and characterize the cell types in a batch culture of *Clostridium beijerinckii* NCMB-8052 by means of flow cytometry and to relate the identified phenotypes to the kinetic behavior of the cell population

for determining the contribution of each cell type to the production of organic acids and solvents.

2. Materials and methods

2.1. Microorganism and culture medium

Clostridium beijerinckii NCIMB-8052 obtained from the American Type Culture Collection (ATCC) was used in all the experiments. The microorganism was maintained under anaerobic conditions as a spore suspension at 4°C in a P2 culture medium, which is traditionally used for bacteria of the genus *Clostidium* [22] and has the following composition: glucose (60 g·L^{-1}), ammonium acetate (2.2 g·L^{-1}), buffer phosphate (KH_2PO_4 0.5 g·L^{-1}, K_2HPO_4 0.5 g·L^{-1}), mineral salts ($MgSO_4 \cdot 7H_2O$ 0.2 g·L^{-1}, $MnSO_4 \cdot H_2O$ 0.01 g·L^{-1}, $FeSO_4 \cdot 7H_2O$ 0.01 g·L^{-1}, NaCl 0.01 g·L^{-1}), vitamins (aminobenzoic acid 1 mg·L^{-1}, thiamin 1 mg·L^{-1}, biotin 0.01 mg·L^{-1}), and L-cysteine hydrochloride (0.5 g·L^{-1}). Spores as inoculum were obtained through heat shock of a suspension of freshly propagated cells in a water bath at 80°C for 10 min. All cultures were performed in a 500 mL flask sealed with butyl septa and aluminium crimps; nitrogen was sparged to displace oxygen before autoclaving them at 121°C. The vitamins were filter-sterilized through a 0.22 µm PVDF sterile membrane and added just before inoculation.

2.2. Batch culture of Clostridium beijerinckii NCIMB-8052

The kinetic characterization studies of *Clostridium beijerinckii* NCIMB-8052 and ABE production were performed in batch cultures in a 2.5 L laboratory-scale bioreactor (Ez-Control, Applikon Biotechnology, Delft, The Netherlands), using a working volume of 1.6 L. All cultures were performed in duplicate. Ammonium acetate was replaced by ammonium sulfate (3.3 g·L^{-1}) in the culture medium to avoid the use of acetate as a carbon source. The culture medium was sparged with nitrogen before autoclaving at 121°C to eliminate oxygen. The vitamins solution was sterilized by filtering through a 0.22 µm PVDF sterile microporous membrane and added just before inoculation. To initiate each batch culture, the bioreactor was inoculated with 10% v/v (160 mL) of an active culture at exponential growth with a cell concentration of 1.5 g·L^{-1}. During the experiment, the pH was maintained at 6.0 using NaOH 3 N and H_2SO_4 2 N and the temperature and agitation were maintained at 37°C and 200 rpm, respectively. To ensure anaerobic conditions, sterile nitrogen was bubbled during the startup.

2.3. Sample treatment

Batch cultures were monitored for 90 h, samples of 5 mL of culture broth were taken every 4 h; 1 mL of each sample was analyzed in a spectrophotometer at 600 nm (6705 UV–Vis, Jenway, Bibby Scientific, Staffordshire, UK), and the resulting absorbance was correlated with the dry weight of cells to determine the cell concentration; and 3 mL were centrifuged at 9000 g for 5 min (Prism C2500 centrifuge, Labnet International, Edison, NJ, USA), and the supernatant was filtered using syringe filters with 0.22 µm PDVF membrane to determine the concentration of glucose, organic acids, and solvents by chromatography. The remaining 1 mL was centrifuged at 9000 g for 5 min; the pellet was washed twice and then preserved in a sterile solution of 1% NaCl for flow cytometry and staining analysis.

2.4. Determination of the culture parameters

The maximum specific growth rate (μ_m) for the total population was determined using a modified logistic equation (Equation (1)), which has been reported to describe successfully the growth kinetics of *Clostridium* species [23]. Experimental data were fitted using the least squares method.

$$x = \frac{x_0 \hat{A} \cdot x_{max\hat{A}\cdot} e^{(\mu_m \hat{A}\cdot t)}}{x_{max} - x_{t_o} + x_{t_0} \hat{A} \cdot e^{(\mu_m \hat{A}\cdot t)}} \tag{1}$$

The glucose to total biomass yield ($Y_{x/s}$); the specific yield of products ($Y_{P/x}$) for acetone, butanol, ethanol, acetic acid, and butyric acid; the specific yield of solvents (acetone, butanol, and ethanol) for the solventogenic subpopulation ($Y_{P_{sol}/x_{sol}}$); and the specific yield of acids (acetic and butyric acid) for the acidogenic subpopulation ($Y_{P_{ac}/x_{ac}}$) were determined using the following expressions:

$$Y_{x/s} = \frac{x_{max} - x_{t_0}}{G_{t_0} - G_{t_f}} \tag{2}$$

$$Y_{P/x} = \frac{p_{max} - p_{t_0}}{x_{p_{max}} - x_{t_0}} \tag{3}$$

$$Y_{P_{sol}/x_{sol}} = \frac{p_{sol\,max} - p_{sol_{t_0}}}{x_{sol} - x_{sol_{t_0}}} \tag{4}$$

$$Y_{P_{ac}/x_{ac}} = \frac{p_{ac\,max} - p_{ac_{t_0}}}{x_{ac} - x_{ac_{t_0}}} \tag{5}$$

All these yields were calculated from the experimental data using the values obtained at the cultivation time at which the highest concentration of the product was reached, in agreement with the criterion proposed by Branska *et al.* for *Clostridium beijerinckii* NCIMB-8052 [19].

Table 1 shows the list of variables used as well as their definitions and units.

2.5. Continuous culture of Clostridium beijerinckii NCIMB-8052

In previous cultures (data not shown), a maximum specific growth rate of 0.23 h^{-1} was obtained in batch cultures. In order to avoid culture washout, 90% of the maximum growth rate was selected as the dilution rate in continuous culture (D = 0.2 h^{-1}). The bioreactor was filled with sterile medium with a working volume of 1.6 L, the pH was manintained at 6.0, the temperature was maintained at 37°C, and the agitation was maintained at 200 rpm; these growth conditions have been previously reported for *Clostridiums* species [5,24].

The bioreactor was fed from a 20 L autoclavable bottle (Nalgene, USA) containing sterile medium. The anaerobic condition was maintained by bubbling nitrogen into the broth through a membrane filter (0.22 µm). To avoid gas exchange, Tygon (Masterflex, USA) was used in all tubing and connections, a material that was selected for its low oxygen permeability. Feed was controlled by a level sensor within the bioreactor, while the discharge was made by using an external peristaltic pump (Masterflex EasyPump, USA) that took culture broth from the bottom of the bioreactor. The culture was monitored through measuring biomass, glucose, organic acids, and solvent concentrations until a steady state was reached; at that point, samples of biomass were collected for microscopy and flow cytometry analysis.

Since the steady state was obtained at a dilution rate close to the critical, only the cells with replication capacity remained in the culture, thus isolating a predominantly acidogenic population.

Table 1
Variables used for the determination of the culture parameters as well as their definition and units.

Variable	Definition	Units
x_{max}	Highest achieved concentration of total biomass	$g{\cdot}L^{-1}$
x_{t_0}	Initial concentration of total biomass	$g{\cdot}L^{-1}$
μ_m	Maximum specific growth rate	h^{-1}
t	Cultivation time	h
$Y_{x/s}$	Glucose to total biomass yield	$g{\cdot}g^{-1}$
G_{t_0}	Initial concentration of glucose	$g{\cdot}L^{-1}$
G_{t_f}	Concentration of glucose when the maximum biomass was reached	$g{\cdot}L^{-1}$
$Y_{P/x}$	Specific product yield for total biomass	$g{\cdot}g^{-1}$
p_{max}	Highest achieved concentration of products	$g{\cdot}L^{-1}$
p_{t_0}	Initial concentration of products	$g{\cdot}L^{-1}$
$x_{p_{max}}$	Concentration of total biomass when the maximum products were obtained	$g{\cdot}L^{-1}$
$Y_{P_{sol}/x_{sol}}$	Specific solvent yield of solventogenic population	$g{\cdot}g^{-1}$
p_{solmax}	Highest achieved concentration of solventogenic products	$g{\cdot}L^{-1}$
$p_{sol_{t_0}}$	Initial concentration of solventogenic products	$g{\cdot}L^{-1}$
x_{sol}	Concentration of solventogenic biomass when the maximum solventogenic products were obtained	$g{\cdot}L^{--1}$
$x_{sol_{t_0}}$	Initial concentration of solventogenic biomass	$g{\cdot}L^{-1}$
$Y_{P_{ac}/x_{ac}}$	Specific solvent yield of acidogenic population	$g{\cdot}g^{-1}$
p_{acmax}	Highest achieved concentration of acidogenic products	$g{\cdot}L^{-1}$
$p_{ace_{t_0}}$	Initial concentration of acidogenic products	$g{\cdot}L^{-1}$
x_{ac}	Concentration of acidogenic biomass when the maximum acidogenic products were obtained	$g{\cdot}L^{-1}$
$x_{ace_{t_0}}$	Initial concentration of acidogenic biomass	$g{\cdot}L^{-1}$

By applying a light scattering (LS) flow cytometry analysis, it is possible to characterize this isolated population in terms of size (FS: forward scatter) and complexity (SS: side scatter), further correlating their metabolic state with the products generated and finally verifying the results by qualitative inspection using staining and microscopy. This information applied later to a batch culture would allow to isolate the contribution of a vegetative population to the generation of products and then identify the other populations present in the culture and their location in the flow cytometry graphs in terms of SS and FS.

2.6. Cell staining for microscopy and flow cytometry analysis

The staining protocol was adapted from method reported by Tracy et al. [16]. Cells preserved in sterile solution of 1% NaCl were diluted until an OD of 0.5 ± 0.1 (measured at 600 nm, path length 1 cm) was reached; after this, 100 μL of this cell suspension were mixed with 1 μL of Syto9 (1.67 mM) and 1 μL of PI (10 mM) (Sigma-Aldrich, St. Louis, Missouri, USA) and then incubated in the dark for 15 min at room temperature. With these samples, a visual verification was carried out in an epifluorescence microscope equipped with a mercury lamp (Eclipse 50i, Nikon Instruments Inc, Amsterdam, Netherlands). For flow cytometric analyses, 10 μL of the cell suspension was diluted in 990 μL of 1% sterile saline solution. The data acquisition was carried out using the MXP software of the same equipment (Cytomics FC 500, Beckman Coulter Inc, CA United States). For each sample, 35,000 events were captured, which correspond to cells that pass individually through the hydrodynamic focus point. The populations were differentiated through light scattering analysis, which were correlated with the observations of the microscopy analysis of the culture. The percentage of cells at each gate during the evolution of the fermentation was calculated from the areas defined in the flow cytometry analysis and the number of events were recorded in each of the defined gates. With this information, the concentrations and proportions of acidogenic, solventogenic, and sporulated cell types over time were estimated.

2.7. Analytical determinations

Concentrations of organic acids and glucose were determined by HPLC (Agilent 1260 infinity, Agilent Technologies, Waldbronn, Germany) using an Aminex HPX-87H column (Bio-Rad, USA) and an IR-UV detector. The injection volume was 20 μL, and the mobile phase was a 5 mM H_2SO_4 solution at a flow rate of 0.7 $mL{\cdot}min^{-1}$. The column and IR detector temperature was 55°C, and the UV detection wavelength was 210 nm. Concentrations of ABE products were determined by gas chromatography (Clarus 500, Perkin Elmer, Massachusetts, USA) using an Equity-1 column (Supelco, Bellefonte, PA, USA). The injection volume was 0.5 μL, and the carrier gas (helium) flow rate was 6.5 $mL{\cdot}min^{-1}$. Methanol was used as the internal standard. The temperature of the oven, injector, and FID detector was 200°C.

3. Results and discussion

3.1. Isolation of a vegetative cell population in a continuous culture of Clostridium beijerinckii NCIMB-8052

The continuous operation mode was started after 14 h of a batch culture, when the culture was in the exponential growth phase and the cell concentration was 2.98 $g{\cdot}L^{-1}$. The biomass and glucose concentrations oscillated for almost 60 h before reaching a steady state and then stabilized for 45 h, which corresponds to 9 residence times. During the steady state, cell and glucose concentrations were kept at an average value of 1.56 ± 0.09 and 24.72 ± 0.40 $g{\cdot}L^{-1}$, respectively (Fig. 1A). Fig. 1B shows the concentrations of acetic and butyric acids and its variations during the operation; their average values at the steady state were 1.01 ± 0.03 $g{\cdot}L^{-1}$ for acetic acid and 1.71 ± 0.06 $g{\cdot}L^{-1}$ for butyric acid. During steady state, butanol, ethanol, and acetone were not detected.

In the genus *Clostridium*, during continuous culture, cells continue to undergo changes between acidogenesis and solventogenesis, the cell population is composed of a mixture of actively dividing cells (acidogenic), non-dividing cells (solventogenic), sporulated cells, and dead cells, generating oscillations that prevents the achievement of a true steady state. Clarke et al. [25] and Ezeji et al. [26] reported this oscillatory behavior of a continuous culture of *C. beijerinckii* BA101 at a dilution rate of 0.03 h^{-1}. Similarly, Gallazzi et al. [27], during the continuous culture of *C. pasteurianum* DSM 525 for butanol production, did not achieve a steady state due to the oscillatory behavior working at various low dilution rates (0.07 h^{-1}, 0.05 h^{-1}, and 0.01 h^{-1}). However, it has been reported that in a continuous culture of *C. acetobutylicum* ATCC 824 run at a high dilution rate, the fermentation produced mainly acids and a true steady state was reached [28]. At high dilution rates, such as those used in this work, the culture is dominated by replicative cells in the acidogenic phase, with acetic and butyric acids being the main products.

After reaching steady state, the cell samples were analyzed by flow cytometry with regard to their of size (FS) and complexity (SS). Fig. 2 shows the flow cytometry graphs obtained at three different moments of the steady state of the continuous culture. In these graphs, a compact and clearly defined cloud of events is observed, which correlates with cells of similar morphological characteristics. Similarly, fluorescence microscopy inspection showed that these cells were predominantly stained with PI, corresponding to the cells in an acidogenic vegetative state, as reported previously for *Clostridium* strains [11,16]. Also, according to the products distribution throughout the culture (Fig. 1B), the cell population only produced acetic and butyric acids, confirming the predominance of acidogenic vegetative cells at the steady state.

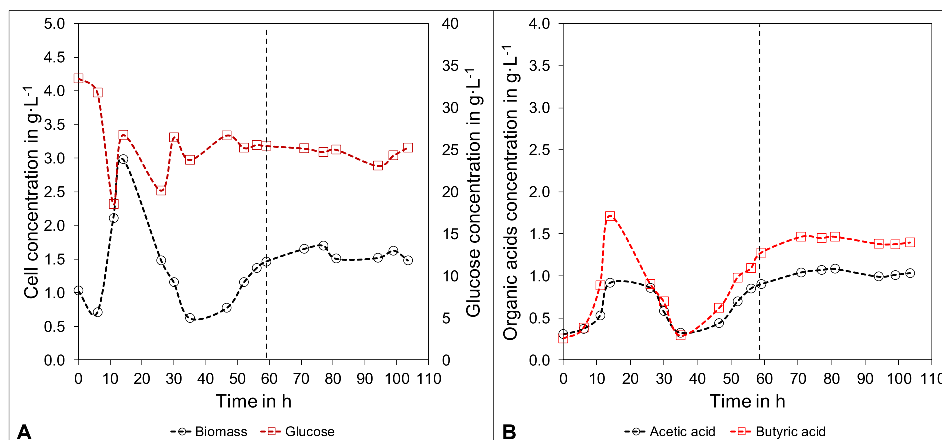


Fig. 1. Continuous culture of *Clostridium beijerinckii* NCIMB-8052, at dilution rate 0.2 h^{-1}, pH 6.0, temperature 37°C, and agitation 200 rpm. (A) Variations in the cell and glucose concentrations. (B) Variations in the organic acid concentrations. The dotted line shows the time from which the steady state is considered.

3.2. Quantification of phenotypes in a batch culture of Clostridium beijerinckii NCIMB-8052

The location in the cytograms of vegetative cells in terms of FS and SS obtained from the continuous culture allowed to establish a gate for this type of cells in the flow cytometry analysis. Therefore, every cell with values of FS and SS within that gate can be considered as vegetative. It was further applied to the flow cytometry analysis of samples taken from a batch culture at different times to determine the variation of the vegetative population over time in the batch operation. Fig. 3 shows the cytograms and microscopy fluorescence images during the batch culture.

At 16 h (Fig. 3A), the individual cells exhibited a regularity of size, shape, and thickness, and the cells are mainly stained with red, which corresponds to the entry of PI. A compact and clearly defined cloud is observed in cytometry graphs, corresponding to cells with similar morphological characteristics; this distribution is in accordance with the identification criterion obtained in continuous culture. Therefore, the event cloud is located inside the limits of the defined gate, confirming a population in a vegetative state.

At 20 h (Fig. 3B), the cells stained with Syto9 were observed, probably due to a change in the cell membrane permeability allowing the entry of the dye, which may be associated with a change in the physiological state [29,30]. The modifications of the membrane permeability are a consequence of the changes in the environmental conditions (presence of solvents and other metabolites) [7]. At this time, clostridial cells with granular accumulation began to appear, exhibiting a characteristic swollen and cigar-shaped morphology. Under fluorescence microscopy, they were stained with both dyes, acquiring an orange color. This clostridial form is generally assumed to be the solvent-producing *Clostridium* cells [9]. Studies carried out with *C. pasteurianum* have reported that there are changes in the composition of membrane lipids (difference in the proportion of saturated to unsaturated fatty acids) due to the stress caused by the presence of butanol [31]. Also, flow cytometry analysis showed slight displacement of the event cloud in terms of size and complexity toward the upper right quadrant, which may be related to the appearance of clostridial forms. At the same time, the number of events at the gate that defines the vegetative cells decreased, which could be due to the transformation of vegetative cells into clostridial cells.

At 28 h (Fig. 3C), the response to Syto9 increased and another dense cloud of events was observed in the flow cytometric analysis, its location accounting for a population of smaller homogeneous forms. These smaller forms were associated with the presence of sporulating cells, which were observed by light microscopy (Fig. 3C). From this cloud of events, a second gate was defined, which allowed the quantification of sporulating forms. In addition, another gate that encompasses the intermediate region was defined considering physiological conditions between the vegetative cells and spores (mainly solventogenic clostridial forms). In this way, by applying a light scattering flow cytometry analysis, it was possible to characterize the three populations in terms of size (FS) and complexity (SS) without the use of fluorescent markers or additional techniques. The cell types identified are: solventogenic cells (gate 2), spores (gate 3), and acidogenic cells (gate 4). Tracy et al. [16] and Jones et al. [11] achieved a more detailed differentiation of cell types using a similar analytical technique supported by a transcriptional analysis and a sorting procedure in a flow cytometry protocol. For the purpose and aims of this work, discrimination in three cell types was enough, since these were related to the production of acids (acidogenic cells), solvents (solventogenic cells), and the final state of spores. The additional cell types reported in the mentioned studies correspond to early and late acidogenic cells and solventogenic cells in their clostridial, forespore, and endospore states.

At 44 h (Fig. 3D), the cells were predominantly stained green due to the action of Syto9 and orange due to the combined action of both dyes. The flow cytometry analysis showed a displacement of the event cloud from the acidogenic gate to the solventogenic gate, and at the same time increased the sporulating cells. This trend continued, and according to the defined gates, it indicates a change of phase from acidogenic to solventogenic, as be seen at 56 h (Fig. 3E). Only toward the end of the culture (Fig. 3F) could a loss of the staining intensity be observed, probably due to the damage of the membrane caused by the accumulation of butanol and butyric acid.

3.3. Kinetic characterization of a batch culture of Clostridium beijerinckii NCIMB-8052

Fig. 4 shows the concentrations and proportions of acidogenic, solventogenic, and sporulated cell types over time. The percentages of cells of each cell type were calculated from the areas defined in the flow cytometry analysis and the number of events were recorded in each of the defined gates.

Fig. 5 shows the variation in the concentration of biomass, glucose, acids, and solvents in a batch culture of *Clostridium beijerinckii* NCIMB-8052. After an initial phase of approximately 16 h, the con-

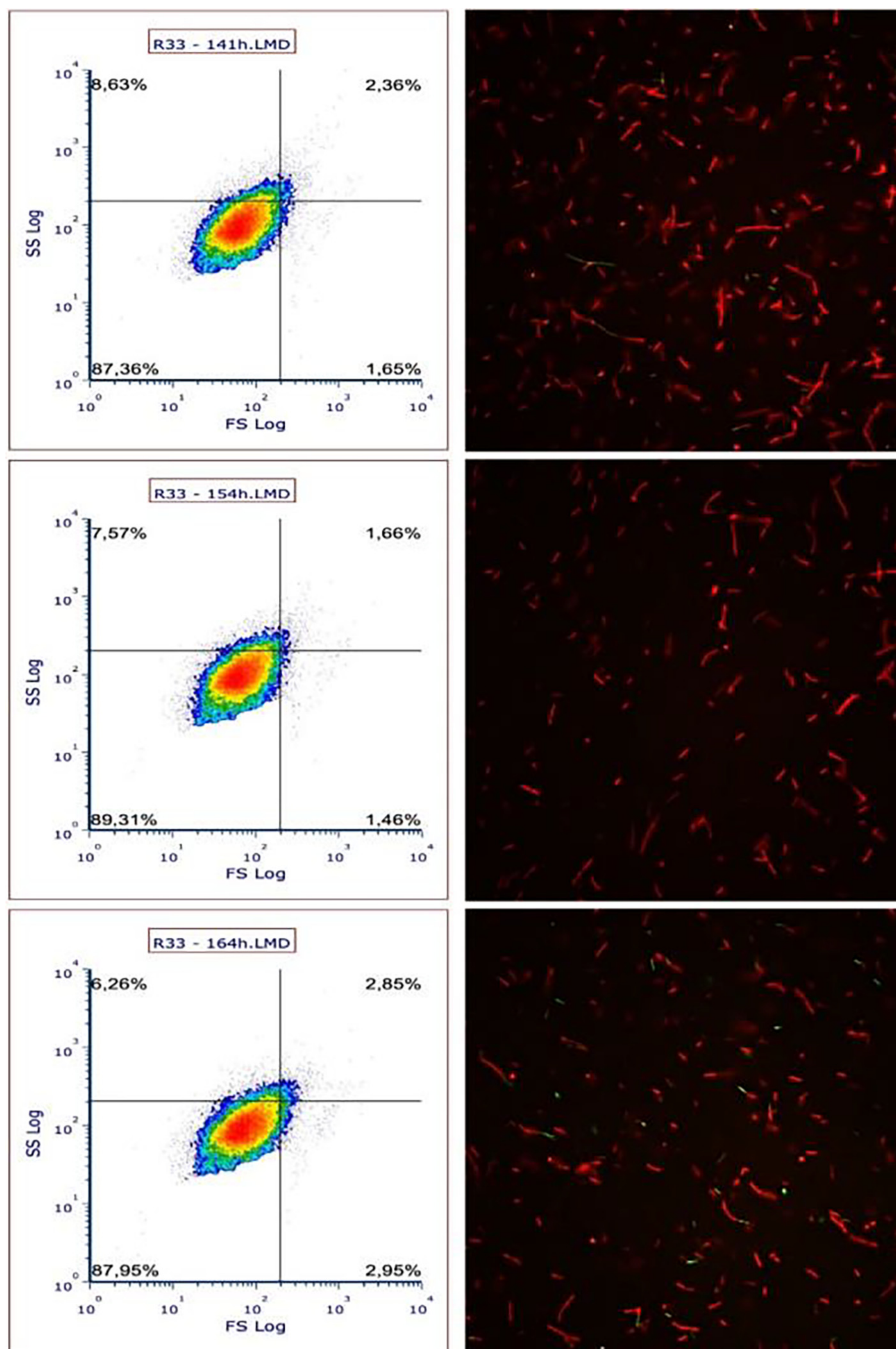
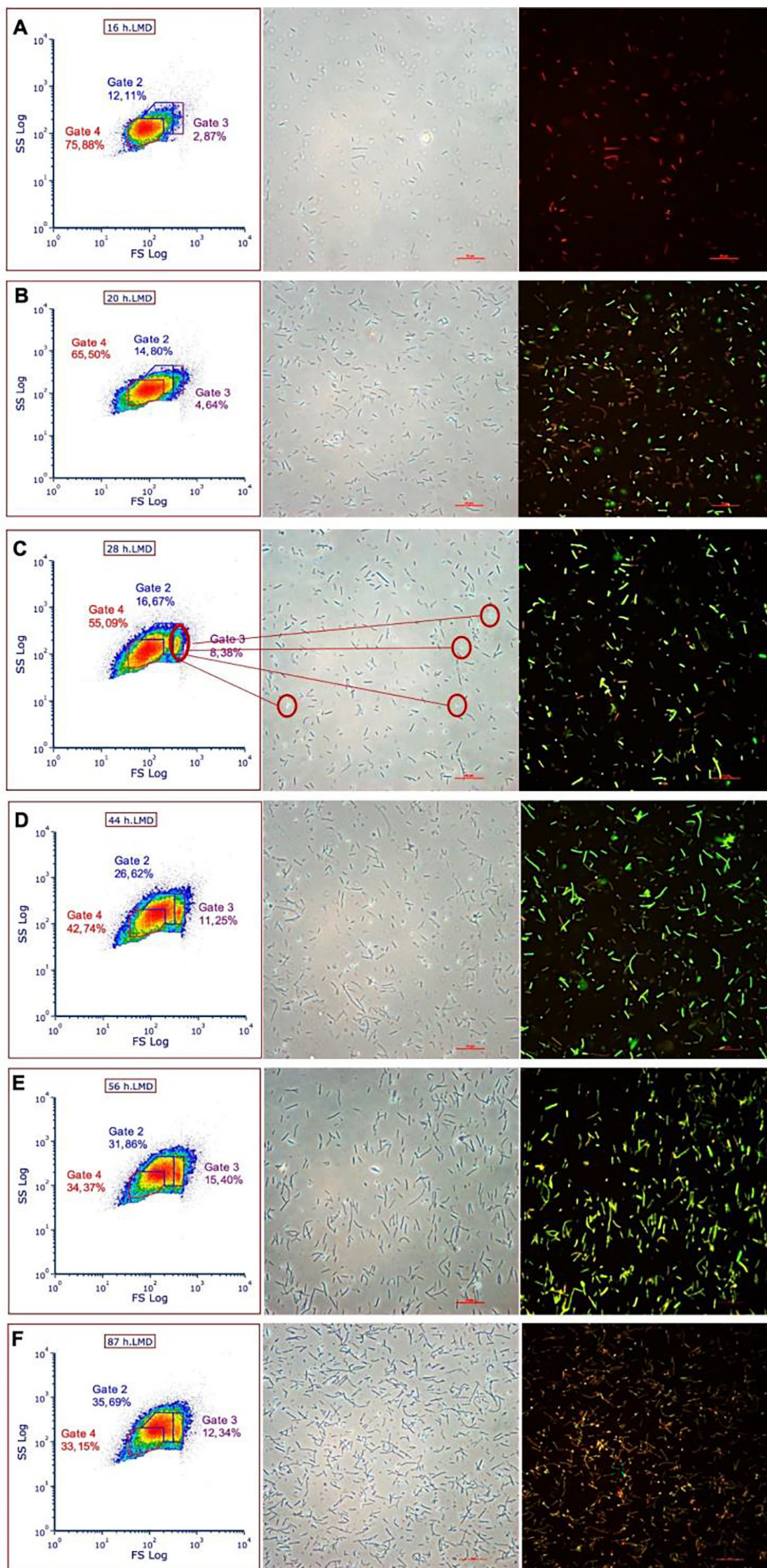


Fig. 2. Characterization of a vegetative population by flow cytometry and epifluorescence microscopy at different times of a continuous culture of *Clostridium beijerinckii* NCIMB-8052 running at steady state.

centration of biomass increased until reaching a maximum concentration of 5.5 g·L^{-1} at 32 h of cultivation (Fig. 5A). At that time, 47.3% of the initial glucose concentration had been consumed, with a $Y_{x/s}$ of 0.21 g·g^{-1}, while the butanol concentration was 5.9 g·L^{-1}, 60% of the total produced (Fig. 5B). The exponential growth phase lasted 24 h with a maximum specific growth rate of 0.23 h^{-1} and a correlation coefficient (r^2) of 0.989 with respect to the logistic model used [23]. Toward the end of the culture (after 60 h), a decrease in cell concentration was observed, which may be due to an increase in the sporulating cells and a decrease in the cell viability. This was confirmed by microscopy; loss of intensity of fluorochromes labeling was observed, which is an evidence of cell membrane damage.

Butyric acid showed a first production peak at 24 h, reaching a concentration of 1 g·L^{-1}, then it decreased due to its reassimilation, and after 40 h increased again until reaching 2 g·L^{-1} (Fig. 5B). Production of organic acids continued as expected since acidogenic cells populations never dropped below 40% (Fig. 4). The acetic acid concentration increased until 1.5 g·L^{-1} at the end of the exponential phase (30 h); its production continued at a lower rate, up to a concentration of 1.9 g·L^{-1} (Fig. 5B). The final butanol concentration reached 9.7 g·L^{-1} (Fig. 5B). Therefore, in Fig. 5, three zones can be identified in the culture: a predominantly acidogenic zone that extends from the beginning of the culture until approximately 24 h (at this point, the accumulation of acids would trigger the onset of solventoge-


A
16 h.LMD
Gate 2
12,11%
Gate 3
2,87%
Gate 4
75,88%
SS Log
FS Log
B
20 h.LMD
Gate 4
65,50%
Gate 2
14,80%
Gate 3
4,64%
SS Log
FS Log
C
28 h.LMD
Gate 2
16,67%
Gate 4
55,09%
Gate 3
8,38%
SS Log
FS Log
D
44 h.LMD
Gate 2
26,62%
Gate 3
11,25%
Gate 4
42,74%
SS Log
FS Log
E
56 h.LMD
Gate 2
31,86%
Gate 3
15,40%
Gate 4
34,37%
SS Log
FS Log
F
87 h.LMD
Gate 2
35,69%
Gate 3
12,34%
Gate 4
33,15%
SS Log
FS Log

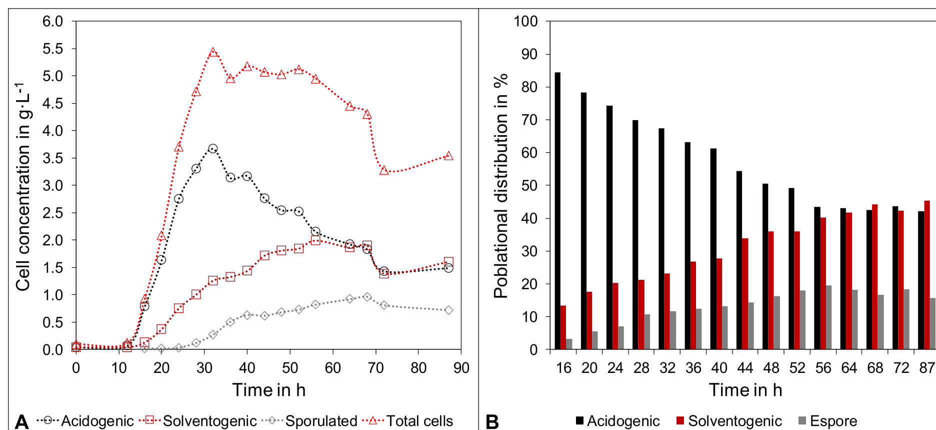


Fig. 4. Subpopulation distribution of the phenotypic states present in a batch culture of *Clostridium beijerinckii* NCIMB-8052. (A) Cell-type concentration time evolution. (B) Cell-type percentage time evolution.

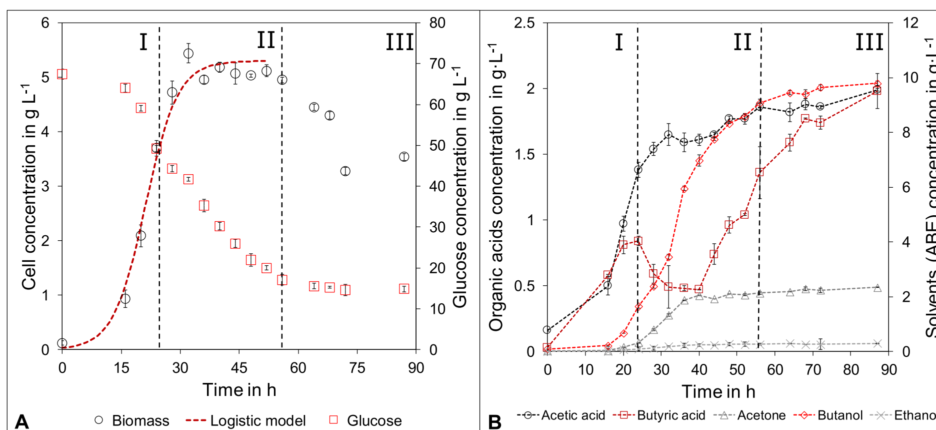


Fig. 5. Batch culture of *Clostridium beijerinckii* NCIMB-8052 at pH 6.0, temperature 37°C, and agitation of 200 rpm. (A) Cell growth and glucose concentration time evolution. (B) Organic acid and solvent concentration time evolution.

sis); a predominantly solventogenic zone that extends from 24 to 68 h; and finally, a third zone of biomass viability loss where the cell concentration decreased due to sporulation and cell lysis after reaching the highest concentrations of organic acids and solvents.

Considering the variations in the acidogenic, solventogenic, and sporulating populations obtained through flow cytometry analysis (Fig. 4), it is possible to analyze the behavior for each cell type: at the beginning of the batch culture, at 16 h, the population distribution was 85% acidogenic, 13% solventogenic, and 2% spores. The concentration of acidogenic cells increased to 3.67 $g{\cdot}L^{-1}$ at 32 h, which accounts for 67.4% of the population, then decreased to a concentration of 1.43 $g{\cdot}L^{-1}$, which corresponds to 42.1% of the population. On the other hand, the solventogenic cells increased constantly until 56 h, reaching a concentration of 2 $g{\cdot}L^{-1}$, which accounts for 40.3% of the population. A gradual increase in the spore population could be observed until 52 h, then it remained around 18% of the total population.

Most of the butanol production occurred between 32 and 56 h (23% of the population were solventogenic cells), registering a slowdown in its production starting from the 45 h (34% of the population were solventogenic cells). Since most of the butanol is generated from the beginning of the deceleration of the cell growth rate, it could be considered that clostridial cells would be responsible for the production of butanol. The coexistence of populations is in agreement with the reported by Zhang et al. [32], who observed that biomass growth in *Clostridium beijerinckii* batch cultures continued during solvents production.

The concentration of butyric acid increased during the exponential growth phase to later decrease as a result of the re-assimilation due to the production of butanol. Later, around 40 h, it began to increase again, which coincided with the slowdown of butanol production. The high concentration of butanol added to that of butyric acid in the media could affect the metabolic activity of the cells in the acidogenic state (which never dropped below 40% of the population), preventing or hindering the cells' transition to the solventogenic state [33]. The activation of solventogenesis and sporulation as a response mechanism for cell survival can be mainly attributed to the butyric acid concentration, close to 1 $g{\cdot}L^{-1}$ and a pH between 4.5 and 4.8, but there are no reports on any protection mechanism against the high concentrations of butanol. Cell damage observed at the end of the culture may be a consequence

Fig. 3. Characterization of the physiological states by flow cytometry and epifluorescence microscopy at different times of a batch culture of *Clostridium beijerinckii* NCIMB-8052: gate 4 for acidogenic cells, gate 3 for spores, and gate 2 for solventogenic cells.

Table 2
Yields of acids and solvents in a batch culture of *Clostridium beijerinckii* NCIMB-8052 for total, acidogenic, and solventogenic cells.

	Global population			Acidogenic subpopulation	Solventogenic subpopulation
	Concentration [g·L^{-1}]	$Y_{P/S}$[g·g^{-1}]	$Y_{P/X}$[g·g^{-1}]	$Y_{P_{ac}/X_{ac}}$[g·g^{-1}]	$Y_{P_{sol}/X_{sol}}$[g·g^{-1}]
Butanol	9.39 ± 0.16	0.18	2.22		4.74
Acetone	2.28 ± 0.064	0.04	0.54		1.15
Ethanol	0.26 ± 0.041	0.005	0.06		0.13
Acetic acid	1.88 ± 0.042	0.005	0.41	0.02	
Butyric acid	1.77 ± 0.042	0.005	0.42	0.96	

of the synergistic action of butanol and butyric acid, which finally caused the lysis of a number of cells [33,34].

Toward the end of the batch culture, a decrease in cell concentration was observed. When contrasted with fluorescence microscopy images, membrane damage and cell lysis were observed in part of the population, together with a change in the intensity of the marking with fluorochromes in the cells. This damage may be the result of the joint effect caused by the high concentration of butyric acid and butanol, as it is known that the presence of carboxylic acids increases the toxic effect of butanol [30], causing damage at the metabolic and membrane levels [33,35]. Unlike what happens with acids, the clostridial cell do not have a response mechanism against a high concentration of solvents. In these batch cultures, the concentrations were over 9.5 g·L^{-1} and 1.5 g·L^{-1} for butanol and butyric acid, respectively, which may be the cause of cellular damage. While the butanol concentration tolerance range has been widely reported in *Clostridium* species, the exact mechanism of tolerance and its relationship to metabolism has not been fully understood [33].

Kinetic parameters were calculated for total biomass, as well as, for the acidogenic and solventogenic subpopulations, $Y_{P/X}$ was determined independently for the acidogenic ($Y_{P_{ac}/X_{ac}}$) and solventogenic subpopulations($Y_{P_{sol}/X_{sol}}$) in terms of organic acids or solvents, respectively. The coherence of the values obtained can be verified by comparing the $Y_{P/X}$ calculated from continuous culture using vegetative cells growing at a dilution rate of 0.2 h^{-1} (in the steady state equal to μ) and those calculated for the acidogenic population in full phase of exponential growth, being 1.09 and 0.97 g·g^{-1} for butyric acid and 0.65 and 0.61 g·g^{-1} for acetic acid, respectively. Table 2 summarizes the results obtained in batch culture.

As shown in Table 2, the yield values by subpopulation are significantly different with respect to those obtained when considering the population as a whole. The values of the$Y_{P_{sol}/X_{sol}}$ for the solventogenic cell type are 2.5 times the values of $Y_{P/X}$ obtained considering all the biomass. Also, the estimated values for acetic and butyric acids for the acidogenic cells type are 1.5 times their values when considering all the biomass. Literature reports regarding ABE fermentation are usually globally estimated, i.e.: considering the total biomass; however, as shown in this work, it is possible to estimate the parameters for each population group, allowing the determination of each group's contribution to the global performance of the fermentation.

4. Conclusions

The morphological and physiological changes of *Clostridium beijerinckii* NCIMB-8052 observed in a batch culture were not synchronized during the fermentation. A heterogeneous distribution of cellular types could be found through the culture. The quantification of the distribution of these phenotypic states by means of the data obtained from the flow cytometry allowed the calculation of the yields considering the different cell types coexisting in the culture, isolating the contribution of each one to the production of acids and solvents. The values of the$Y_{P_{sol}/X_{sol}}$, which consider just the fraction of solventogenic cells were 2.5 times the $Y_{P/X}$ of the culture, which consider all the biomass. Also, the values estimated for acetic and butyric acids by the acidogenic cells type were 1.5 times the values when considering all the biomass. Yields estimated for a specific subpopulation allows the quantification of the effects of changes in growing conditions that might otherwise be masked by using the total population. In this way, it would be possible to observe the effect of changes in culture conditions or culture medium formulation directly on each subpopulation, which would help make better decisions in order to design an improved culture strategy and to obtain deeper knowledge regarding ABE fermentation.

Financial support

This work was financed by the National Research and Development Agency (ANID, former CONICYT) through Projects FONDECYT 21151422, and FONDECYT 3140232, Project SMIBIO ERANET-LAC ELAC2014/BEE0249, and the Pontificia Universidad Católica de Valparaíso.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

The authors would like to thank the Environmental Biotechnology Group (BioGroup) of the Institute of Technological Research at the Universidad de Santiago de Compostela, especially Prof. Juan Lema and PhD Thelmo Lu.

References

[1] Charubin K, Bennett RK, Fast AG, et al. Engineering *Clostridium* organisms as microbial cell-factories: challenges & opportunities. Metab Eng 2018;50:173–91. https://doi.org/10.1016/j.ymben.2018.07.012 PMid: 30055325.

[2] Magalhães BL, Grassi MCB, Pereira GAG, et al. Improved *n*-butanol production from lignocellulosic hydrolysate by *Clostridium* strain screening and culture-medium optimization. Biomass Bioenergy 2018;108:157–66. https://doi.org/10.1016/j.biombioe.2017.10.044.

[3] Moon HG, Jang YS, Cho C, et al. One hundred years of clostridial butanol fermentation. FEMS Microbiol Lett 2016;363(3):fnw001. https://doi.org/10.1093/femsle/fnw001 PMid: 26738754.

[4] Yoo M, Nguyen NPT, Soucaille P. Trends in systems biology for the analysis and engineering of *Clostridium acetobutylicum* metabolism. Trends Microbiol 2020;28(2):118–40. https://doi.org/10.1016/j.tim.2019.09.003 PMid: 31627989.

[5] Veza I, Muhamad Said MF, Latiff ZA. Recent advances in butanol production by acetone-butanol-ethanol (ABE) fermentation. Biomass Bioenergy 2021;144:105919. https://doi.org/10.1016/j.biombioe.2020.105919.

[6] Xue C, Zhao J, Chen L, et al. Recent advances and state-of-the-art strategies in strain and process engineering for biobutanol production by *Clostridium acetobutylicum*. Biotechnol Adv 2017;35(2):310–22. https://doi.org/10.1016/j.biotechadv.2017.01.007 PMid: 28163194.

[7] Bharathiraja B, Jayamuthunagai J, Sudharsanaa T, et al. Biobutanol – An impending biofuel for future: A review on upstream and downstream

processing tecniques. Renew Sustain Energy Rev 2017;68(2):788–807. https://doi.org/10.1016/j.rser.2016.10.017.

[8] Kolek J, Branska B, Drahokoupil M, et al. Evaluation of viability, metabolic activity and spore quantity in clostridial cultures during ABE fermentation. FEMS Microbiol Lett 2016;363(6):fnw031. https://doi.org/10.1093/femsle/fnw031 PMid: 26862145.

[9] Jones DT, Van Der Westhuizen A, Long S, et al. Solvent production and morphological changes in *Clostridium acetobutylicum*. Appl Environ Microbiol 1982;43(6):1434–9. https://doi.org/10.1128/aem.43.6.1434-1439.1982 PMid: 16346038.

[10] Seo SO, Lu T, Jin YS, et al. A comparative phenotypic and genomic analysis of *Clostridium beijerinckii* mutant with enhanced solvent production. J Biotechnol 2021;329:49–55. https://doi.org/10.1016/j.jbiotec.2021.02.002 PMid: 33556425.

[11] Jones SW, Paredes CJ, Tracy B, et al. The transcriptional program underlying the physiology of clostridial sporulation. Genome Biol 2008;9:R114. https://doi.org/10.1186/gb-2008-9-7-r114 PMid: 18631379.

[12] Talukdar PK, Olguín-Araneda V, Alnoman M, et al. Updates on the sporulation process in *Clostridium* species. Res Microbiol 2015;166(4):225–35. https://doi.org/10.1016/j.resmic.2014.12.001 PMid: 25541348.

[13] Patakova P, Branska B, Sedlar K, et al. Acidogenesis, solventogenesis, metabolic stress response and life cycle changes in Clostridium beijerinckii NRRL B-598 at the transcriptomic level. Sci Rep 2019;9:1371. https://doi.org/10.1038/s41598-018-37679-0 PMid: 30718562.

[14] Patakova P, Linhova M, Rychtera M, et al. Novel and neglected issues of acetone-butanol-ethanol (ABE) fermentation by clostridia: *Clostridium* metabolic diversity, tools for process mapping and continuous fermentation systems. Biotechnol Adv 2013;31(1):58–67. https://doi.org/10.1016/j.biotechadv.2012.01.010 PMid: 22306328.

[15] Yang Y, Nie X, Jiang Y, et al. Metabolic regulation in solventogenic clostridia: regulators, mechanisms and engineering. Biotechnol Adv 2018;36(4):905–14. https://doi.org/10.1016/j.biotechadv.2018.02.012 PMid: 29477757.

[16] Tracy BP, Gaida SM, Papoutsakis ET. Development and application of flow-cytometric techniques for analyzing and sorting endospore-forming clostridia. Appl Environ Microbiol 2008;74(24):7497–506. https://doi.org/10.1128/AEM.01626-08 PMid: 18931289.

[17] Patakova P, Linhova M, Vykydalova P, et al. Use of fluorescent staining and flow cytometry for monitoring physiological changes in solventogenic clostridia. Anaerobe 2014;29:113–7. https://doi.org/10.1016/j.anaerobe.2013.10.006 PMid: 24211310.

[18] González-Peñas H, Lu-Chau TA, Moreira MT, et al. Assessment of morphological changes of *Clostridium acetobutylicum* by flow cytometry during acetone/butanol/ethanol extractive fermentation. Biotechnol Lett 2015;37:577–84. https://doi.org/10.1007/s10529-014-1702-3 PMid: 25351808.

[19] Branska B, Pechacova Z, Kolek J, et al. Flow cytometry analysis of *Clostridium beijerinckii* NRRL B-598 populations exhibiting different phenotypes induced by changes in cultivation conditions. Biotechnol Biofuels 2018;11:99. https://doi.org/10.1186/s13068-018-1096-x PMid: 29632557.

[20] Karstens K, Trippel S, Götz P. Process engineering of the acetone-ethanol-butanol (ABE) fermentation in a linear and feedback loop cascade of continuous stirred tank reactors: experiments. Model Optimization. Fuels 2021;2(2):108–29. https://doi.org/10.3390/fuels2020007.

[21] Lipovsky J, Patakova P, Paulova L, et al. Butanol production by *Clostridium pasteurianum* NRRL B-598 in continuous culture compared to batch and fed-batch systems. Fuel Process Technol 2016;144:139–44. https://doi.org/10.1016/j.fuproc.2015.12.020.

[22] Buakhiaw B, Sanguanchaipaiwong V. Effect of media on acetone-butanol-ethanol fermentation by isolated *Clostridium* spp. Energy Procedia 2017;138:864–9. https://doi.org/10.1016/j.egypro.2017.10.104.

[23] Al-Shorgani NKN, Shukor H, Abdeshahian P, et al. Enhanced butanol production by optimization of medium parameters using *Clostridium acetobutylicum* YM1. Saudi J Biol Sci 2018;25:1758–70. https://doi.org/10.1016/j.sjbs.2016.02.017 PMid: 30505175.

[24] Pinto T, Flores-Alsina X, Gernaey KV, et al. Alone or together? A review on pure and mixed microbial cultures for butanol production. Renew Sustain Energy Rev 2021;147:111244. https://doi.org/10.1016/j.rser.2021.111244.

[25] Clarke KG, Hansford GS, Jones DT. Nature and significance of oscillatory behavior during solvent production by *Clostridium acetobutylicum* in continuous culture. Biotechnol Bioeng 1988;32(4):538–44. https://doi.org/10.1002/bit.260320417 PMid: 18587752.

[26] Ezeji T, Qureshi N, Blaschek HP. Production of acetone-butanol-ethanol (ABE) in a continuous flow bioreactor using degermed corn and *Clostridium beijerinckii*. Process Biochem 2007;42(1):34–9. https://doi.org/10.1016/j.procbio.2006.07.020.

[27] Gallazzi A, Branska B, Marinelli F, et al. Continuous production of n-butanol by *Clostridium pasteurianum* DSM 525 using suspended and surface-immobilized cells. J Biotechnol 2015;216:29–35. https://doi.org/10.1016/j.jbiotec.2015.10.008 PMid: 26471284.

[28] Li SY, Srivastava R, Suib SL, Li Y, et al. Performance of batch, fed-batch, and continuous A-B-E fermentation with pH-control. Bioresour Technol 2011;102(5):4241–50. https://doi.org/10.1016/j.biortech.2010.12.078 PMid: 21227684.

[29] Linhová M, Patáková P, Lipovský J, et al. Development of flow cytometry technique for detection of thinning of peptidoglycan layer as a result of solvent production by *Clostridium pasteurianum*. Folia Microbiol (Praha) 2010;55:340–4. https://doi.org/10.1007/s12223-010-0054-1 PMid: 20680567.

[30] Mukhopadhyay A. Tolerance engineering in bacteria for the production of advanced biofuels and chemicals. Trends Microbiol 2015;23(8):498–508. https://doi.org/10.1016/j.tim.2015.04.008 PMid: 26024777.

[31] Kolek J, Patáková P, Melzoch K, et al. Changes in membrane plasmalogens of *Clostridium pasteurianum* during butanol fermentation as determined by lipidomic analysis. PLoS One 2015;10(3):e0122058. https://doi.org/10.1371/journal.pone.0122058 PMid: 25807381.

[32] Zhang Y, Jiao S, Lv J, et al. Sigma factor regulated cellular response in a non-solvent producing *Clostridium beijerinckii* degenerated strain: A comparative transcriptome analysis. Front Microbiol 2017;8:23. https://doi.org/10.3389/fmicb.2017.00023.

[33] Peabody GL, Kao KC, Sauer M. Recent progress in biobutanol tolerance in microbial systems with an emphasis on *Clostridium*. FEMS Microbiol Lett 2016;363(5):fnw017. https://doi.org/10.1093/femsle/fnw017 PMid: 26818252.

[34] Wang YF, Tian J, Ji ZH, et al. Intracellular metabolic changes of *Clostridium acetobutylicum* and promotion to butanol tolerance during biobutanol fermentation. Int J Biochem Cell Biol 2016;78:297–306. https://doi.org/10.1016/j.biocel.2016.07.031 PMid: 27477314.

[35] Patakova P, Kolek J, Sedlar K, et al. Comparative analysis of high butanol tolerance and production in clostridia. Biotechnol Adv 2018;36(3):721–38. https://doi.org/10.1016/j.biotechadv.2017.12.004 PMid: 29253526.