Flow cytometric method for cell viability evaluation of *Gordonia alkanivorans* strain 1B in fossil fuels biodesulfurization processes

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Abstract

This work reports the development of a rapid flow cytometric method for the viability assessment of *Gordonia alkanivorans* strain 1B, a bacterium used in the biodesulfurization process. To demonstrate that it is possible to monitor by flow cytometric analysis changes in this bacterium physiological state, positive controls using the 5(6)-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) staining mixture were set. The loss of viability of *G. alkanivorans* resting cells in the presence of different concentrations of 2-hydroxybiphenyl, a very toxic end product of the dibenzotiophene desulfurization process, was assessed over a period of time. The results here reported demonstrate the potential of this technique for the biodesulfurization process monitoring and consequent enhancement.

Introduction

One of the major problems related with fossil fuels is the air pollution caused by the gaseous emissions resultant of the fuel combustion, such as nitrogen oxides, volatile organic compounds and sulfur dioxide. The decreased availability of crude oils with low sulfur content increases the process cost due to the necessity of removing larger amounts of sulfur to comply with an increasingly stricter legislation that requires the reduction of sulfur levels in fossil fuels. The most commonly method used for sulfur removal from fossil fuels is hydrodesulfurization, a physico-chemical process at very high temperatures and pressures. An alternative to this process is biodedesulfurization (BDS), a microbiological process that works at atmospheric pressure and temperature making it easier to work with and less expensive. It also as the advantage of easily desulfurizing recalcitrant sulfur compounds which are hard to remove by hydrodesulfurization [1]. Several bacteria species, such as *Gordonia alkanivorans* strain1B [2], are able to desulfurize dibenzothiophene (DBT), a model compound used commonly in BDS studies, to 2-hydroxybiphenyl (2-HBP) via the 4S pathway without destroying the carbon structure [3], therefore maintaining the fuel potential energy. BDS limitations are related with process parameters and with the cost of maintaining bacterial cultures so to enhance the BDS process, it is necessary to monitor how changes in the experimental system affect the microbial cells viability and consequently the process

efficiency. An alternative method to conventional microbial techniques to determine cell viability is flow cytometry. This method provides a fast and accurate quantitative method for measurement of thousands of individual cells, based on scattered light and fluorescence emitted by specific dyes. Propidium iodide (PI) is a nucleic acid dye that only enters into cells with a compromised cytoplasmatic membrane, and 5(6)-carboxyfluorescein diacetate (CFDA) is a membrane permeant non-fluorescent substrate that is an indicator of cell viability as a function of enzymatic activity. The goal of this study was to develop a rapid method for viability assessment of *G. alkanivorans* cells using flow cytometry for further application to monitor and optimize BDS processes.

Experimental

G. alkanivorans strain 1B was cultured in sulfur-free mineral medium and maintained in shake flasks at 30ºC and 150 rpm. To obtain resting cells, the bacterium was harvested during the exponential growth phase, washed and ressuspended in phosphate buffer at a concentration of approximately 10 g l^{-1} of dry cell weight. *G. alkanivorans* cells at different physiological states were used to establish controls with positive staining for PI and CFDA. For the staining procedure the bacterium cells were incubated with CFDA for 30 minutes at 37ºC in the dark. The cells were then washed, PI was added and the flow cytometric analysis was performed immediately.

Results and Discussion

Controls with positive staining for PI (PI^+) and CFDA ($CFDA^+$) were established for cells of *G. alkanivorans* grown on culture media with DBT as the only sulfur source. Samples from exponential growing cells with all nutrients in excess, stressed cells submitted to a starvation period and ethanol treated cells, were stained with the mixture CFDA and PI and analyzed by flow cytometry for the assessment of their viability.

Fig. 1 shows the results of flow cytometry analysis of the samples of microbial cells at different physiological states (control tests). These qualitative results allowed the identification of three distinct subpopulations: viable cells (A), stressed or injured cells (B) and dead or enzymatically inactive cells (C). Exponential growing cells (i) were stained mainly with CFDA ($CFDA^+$ cells) and some stained with PI or with both dyes. In the starved culture (ii) the proportion of PI^+ cells ($CFDA^-$ and $CFDA^+$) was much higher compared to the exponential growing cells density plot (i), indicating as supposed a more compromised culture. Ethanol-killed cells (iii) only showed fluorescence emission for PI (PI^+ cells).

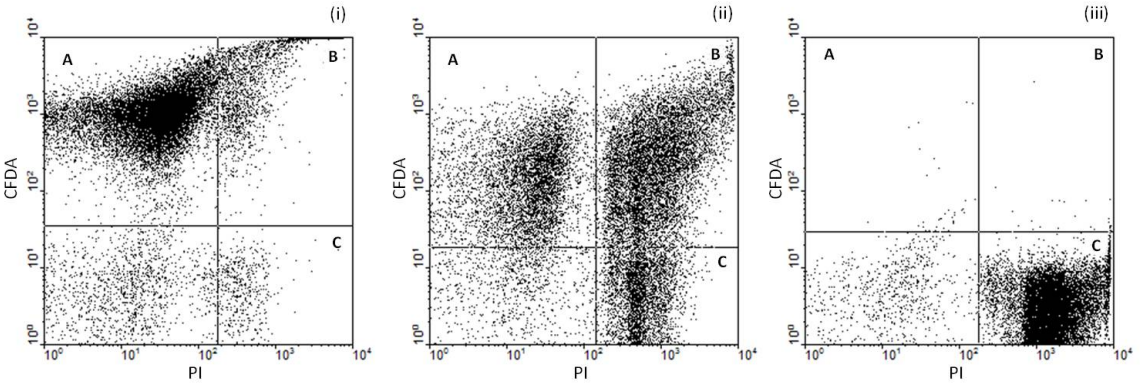

Fig. 1. Flow cytometric analyses of *G. alkanivorans* stained with CFDA and PI. Analyzed cells were at different physiological states: healthy exponential growing cells (i), cells submitted to starvation (ii) and dead cells (iii). Three subpopulations can be differentiated: (A) viable cells, (B) stressed or injured cells and (C) dead or enzymatically inactive cells.

Based in the obtained controls, physiological studies of 2-HBP toxicity, a very toxic end product of the desulfurization process, were perform on *G. alkanivorans* resting cells. Five different concentrations of 2-HBP (0.25, 0.45, 1, 5 and 10 mM) were tested and the percentages of viable, stressed or injured cells and dead or inactive cells for each concentration of toxicant over time were obtained by analysis of the flow cytometry density plots (Fig. 2). A control with resting cells without adding 2-HBP was also performed.

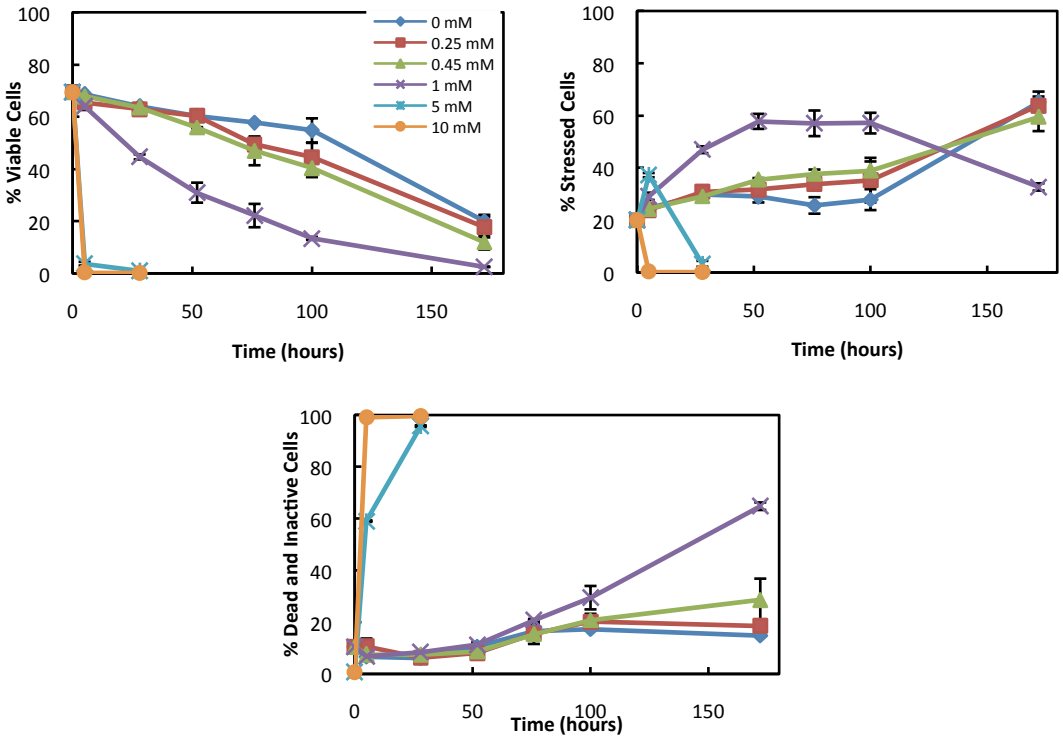

Fig. 2. Time-course profile of *G. alkanivorans* resting cells viability in the presence of 0.25, 0.45, 1, 5 and 10 mM of 2-HBP. Percentages of viable cells, stressed or injured cells and dead or inactive cells over time are presented for each toxicant concentration tested.

The lower concentrations of 2-HBP (0.25 and 0.45 mM) did not seemed to affect significantly the viability of the resting cells, however a slightly faster decrease of viable cells can be observed. When added 1 mM of 2-HBP to the resting cells the decrease of viable cells was much faster, having approximately 50% less viable cells than the control culture without 2-HBP after 52h. However, this loss of viable cells was not reflected in an increase of dead or inactive cells but instead in an increase of the percentage of stressed or injured cells. This is an important indicator for the desulfurization process because even though the culture is compromised it does not mean that it cannot be recovered, since the cells still retain some enzymatic activity. The higher concentrations of 2-HBP (5 and 10 mM) showed to be very toxic to the resting cells, being the percentage of dead or inactive cells above 90% after 28h with 5 mM and practically 100% with 10 mM after just 5h.

Conclusion

Considering the obtained results, the use of this technique seems to be a promising tool to monitor the viability of microbial desulfurizing cells during BDS processes, since the possibility of performing frequent evaluations of how cells viability vary in response to parameters changes could enable a faster optimization and a better control of the process.

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