**Characterization of a natural isopropanol producer, *Clostridium beijerinckii* DSM 6423, during continuous biofilm fermentation**

Maxime Carrie1, Jean-Christophe Gabelle1\*, Hélène Velly2, Fadhel Ben Chabaane2,

*1 IFP Energie Nouvelles, Rond-point de l’échangeur de Solaize, BP3, 69360 Solaize*

*2 IFP Energie Nouvelles, 1 et 4 avenue de Bois préau 92852 Rueil-Malmaison.*

*\*jean-christophe.gabelle@ifpen.fr*

**Highlights**

* Enzymatic deconstruction is performed to extract immobilized cells of *Clostridium beijerinckii* from their polymerous matrix.
* Viability analysis is achieved by flow cytometry
* Heterogeneous viability repartition within the biofilm is assessed by confocal microscopy.

**1. Introduction**

 Butanol and isopropanol are commonly used chemicals in the industry as solvent. They can also be employed as fuels and are mainly produced from propylene coming from petrochemistry. To a lesser extent, an alternative bioproduction of Isopropanol and Butanol mixture using solventogenic Clostridium is also currently investigated .

However, this fermentation process is not used at an industrial scale because of low productivity and low solvent yield and titer due to butanol toxicity of classical batch or continuous processes. To overcome this issue, continuous fermentation using immobilized cells on a carrier is currently employed at a laboratory scale. This system allows the continuous removal of butanol, can be operated at high dilution rate without causing cell washout, and gives high productivity (4 g.L-1.h-1) 1.

During immobilized cell fermentation, bacteria adhere to the solid and form a structure called biofilm. This biofilm is composed of cells trapped into a gel-like matrix made from a mix of polysaccharides, proteins and extracellular DNA 2. But the scale-up of an immobilized fermentation system is not easy as bacterial adhesion or biofilm growth and evolution are not well known 3. This study focuses on cells viability assessment during IBE immobilized fermentation.

Firstly, confocal microscopy is used to show the cells repartition within the biofilm according to their viability. Then a sequential enzymatic lysis of the biofilm matrix coupled to flow cytometry analysis gave us information about physiological state of both sessile and planktonic cells during continuous fermentation.

**2. Methods**

Four screw scrap bottles, fill with the immobilization carrier, were used as reactors. The system was operated in continuous mode at an useful volume of 80mL. The analysis were performed by scarifying each reactor at different fermentation time.

After staining with a mix of carboxy fluorodesceine diacetate [cFDA] and propidium iodide [PI], the level of viability of the *Clostridium* cells were analysed by flow cytometry (Cyflow Space, Sysmex).

For confocal analysis, the carriers has been removed from the system and gently washed with deionized sterile water to remove planktonic cells attached to the biofilm. Then the carriers were put into staining solution containing propidium iodide and syto 9. After staining the carriers were plonged into PFA solution and then observed under a Zeiss LSM800 confocal laser microscope.

**3. Results and discussion**

Flow cytometry, showed that enzymatic deconstruction using sequentially Dnase and protease are able to extract cells from the biofilm without altering their viability. Using this method, the percentages of viable planktonic and sessile cells follow similar patterns during fermentation. However the amounts of viable sessile cells on the carriers are less important than the planktonic one. Using confocal microscopy, heterogeneity of viability is observed within the biofilm. Those observations also helped us to describe qualitatively the growth of the biofilm inside the foam during continuous fermentation.

**4. Conclusions**

In this study, two methods were validated in order to evaluate the physiological state of *C. beijerinckii* cells during continuous fermentation of immobilized cells. Other parameters of cell viability, such as membrane fluidity or polarization, may complement this analysis in the future. In addition, cell viability analysis using confocal microscopy highlight a high heterogeneity within the biofilm but need some improvement in order to better identified solvent producing cells in the biofilm.

**References**

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