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Isolation of *Bacillus subtilis* spores by Tangential Flow Filtration using the Bionet M1 system

ABSTRACT

In downstream processing (DSP), Tangential Flow Filtration (TFF) is one of the best options to separate different molecules and/or biomass according to particle size. *Bacillus sp* and related products are one of the bacteria most produced by fermentation technology since it has several applications in the biotechnology industry. The main goal in DSP operations with *Bacillus sp* processes is the biomass or spores concentration or removal, depending on which is the target product. In this work, several filtrations using the Bionet M1 TFF system have been performed to concentrate or isolate spores using ceramic membranes of different pore sizes, and Bacillus broth produced by batch and fed-batch modes.

KEYWORDS

Bacillus subtilis, Spores, Tangential-Flow Filtration, Microfiltration, Ceramic membrane

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1 INTRODUCTION

In Tangential or Cross Flow Filtration (TFF/CFF) systems, the fluid flows tangentially to the membrane surface instead of frontally, making it possible to sweep away the material build-up on the membrane surface, reducing or delaying fouling. As a consequence, two different streams are generated, the retentate or concentrate, which contains the biomolecules that cannot go through the membrane pores, and the permeate, a cleaner stream with the smaller particles that are not retained (see Figure 1).

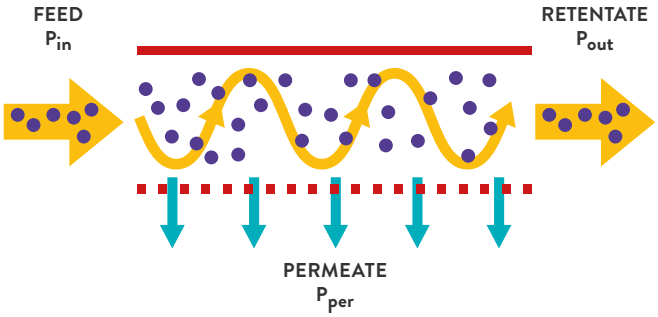

Fig. 1: Tangential/Cross Flow Filtration (TFF/CFF) Concept.

Four different types of filtrations can be established depending on the pore size and the applied pressure: Microfiltration (MF), Ultrafiltration (UF), Nanofiltration (NF), and Reverse Osmosis (RO). MF and UF separate components by means of a physical screening, small particles cross the membrane and large particles are retained, while NF and RO are also selective to the passage of ions, so they are influenced not only by the particle size but also by the osmotic pressure of the feed stream.

MF and UF are the most commonly used technologies in the biotechnology industry for downstream purposes, like biomass clarification or biomolecule concentration and/or purification (Michaels et al., 2020). Membranes are commercially available and integrated into different modules, with the tubular (polymeric and ceramic version), hollow fibre and cassettes being the most commonly used in biotech.

Filtration yield and membrane module selection are highly affected by the physical and chemical characteristics of the feeding product (https://bionet.com/expert-references/a-guide-on-membrane-selection). For example, a high cell density (DCW, dry cell weight) broth could cause premature fouling and aggravate filtration performance, leading to lower permeate fluxes (higher operation time) and even harder cleaning conditions. Thus, the feeding product (i.e. DCW, pH, viscosity, shear, and temperature sensitivity) must be characterized before starting any filtration process (Van Reis et al., 1997).

Bacterium *Bacillus subtilis* is widely used in the biotechnology industry for enzymes, vitamins, and antibiotics production, or as plant biostimulants (Kaspar et al., 2019; Kovács, 2019). These bacteria usually are produced by submerged fermentation at an industrial scale. Most bioprocesses have well-defined upstream parts. However, downstream operations, like separation or purification stages, are usually neither well-defined nor selected with scalable technologies in mind.

In the case of processes used in certain sectors to produce *B. subtilis*, it is necessary to concentrate the cells or even eliminate the spores depending on the application.

The most implemented and classical technology for biomass separation in bioprocesses is centrifugation, but despite being a robust, scalable, and well-characterized technology, centrifugation needs further downstream steps to remove and clear remaining small particles like spores, with limitations for the separation of cell debris (Pieracci et al., 2018). TFF is also a robust technology but it provides more selective results in terms of particle size screening while at the same time providing better temperature control.

In this study, the objective was to validate TFF as a feasible separation technology of spores from *B. subtilis* broth.

2 MATERIALS AND METHODS

2.1 *Bacillus sp* broth

Two types of *B. subtilis* fermentation broths were generated following two fermentation modes, Batch and Fed-Batch. Each mode is derived in a final CFU spore's concentration of 1.10^9 and 1.10^{10}, respectively.

2.2 Tangential Flow Filtration system

2.2.1 — Main Equipment

The Tangential Flow Filtration trials with *B. subtilis* broths were carried out in the BIONET's tangential flow filtration system, M1. Its main characteristics are:

- Supports work with any membrane technology from any vendor (ceramic, hollow fibre, cassettes, and flat sheets).
- Gentle and scalable pump technology. Possibility of working with shear-sensitive products.
- Real-time view of filtration parameters (retentate flow, pump speed, feed/retentate/permeate pressure, TMP, temperature and permeate weight) and data registration via ROSITA software.

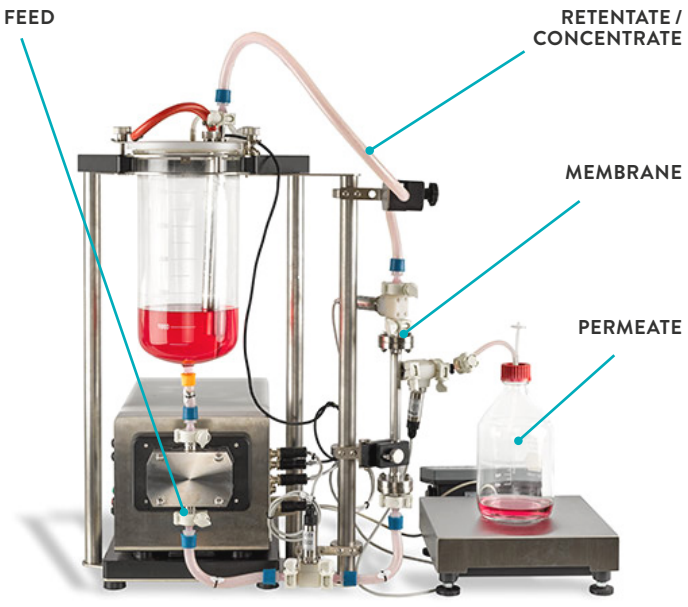

Fig. 2: Bionet M1 Tangential-Flow Filtration system.

2.2.2 — Membranes

Multi-tubular membranes based on ceramic material and different pore sizes were selected to perform the trials. Their main characteristics are shown in Table 1.

Table 1: Main characteristics of the membranes used for the trials.

MATERIAL	PORE SIZE	HYDRAULIC CHANNEL DIAMETER	FILTRATION AREA
$TiO_2 + ZrO_2$	0.45 µm	2 mm	0.0132 m²
$TiO_2 + ZrO_2$	0.20 µm	2 mm	0.0132 m²

2.3 Filtration

Figure 3 summarises the process flow diagram followed for the TFF trials.

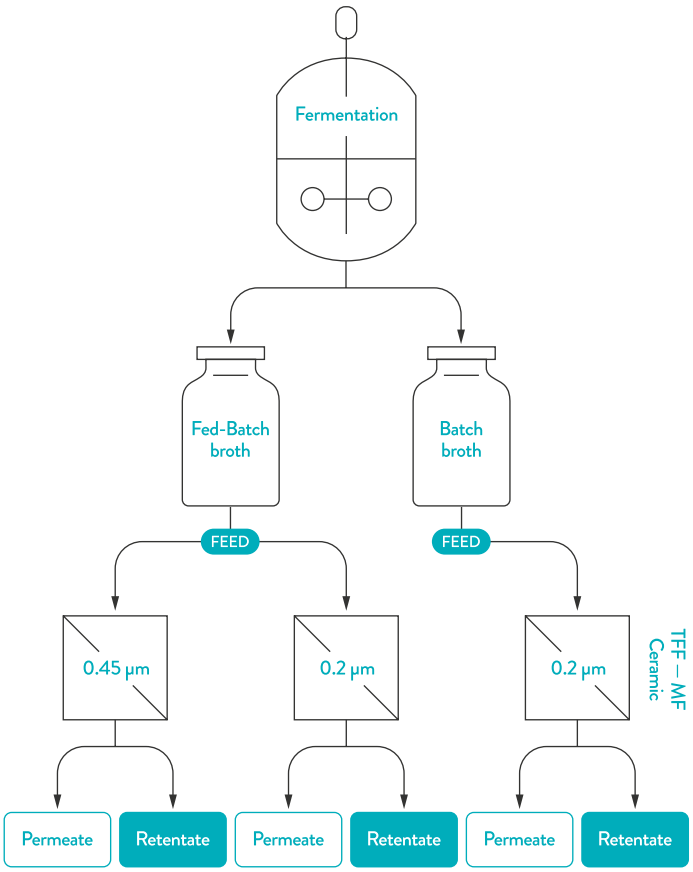

Fig. 3: Process flow diagram followed for the TFF experiments.

In each experiment, three streams were generated and characterised: feed (*Bacillus sp* broth), permeate (low spores' concentration), and retentate (high spores' concentration). Filtration trials were carried out according to the conditions represented in Table 2.

Table 2: Filtration conditions at each experiment

	BIOMASS/SPORES CONCENTRATION	SPORES REMOVAL
TMP (barg)	0.7	1.4
CFV (m/s)	3.5	5
Temperature (°C)	27	22–26

2.4 Key Performance Indicators

TFF is characterized by a number of Key Performance Indicators, KPI, where the following stand out:

- ***Transmembrane pressure,*** or TMP, is defined as the average feed pressure minus the permeate pressure, and it is calculated according to the following formula. It is an indicator of the force needed to promote filtration across the membrane layer. All the terms in the equation must be introduced in the same pressure units.

$$TMP = \frac{P_{feed} + P_{retentate}}{2} - P_{permeate}$$

- ***Permeate flux,*** refers to the permeate flow obtained during filtration per membrane area. Its units are normally referred to as "lmh". It is calculated according to the following formula.

$$Flux\left(\frac{L}{h \cdot m^2}\right) = \frac{Permeate\ flow\left(\frac{L}{h}\right)}{Membrane\ area\ (m^2)}$$

- ***Cross Flow Velocity,*** or CFV, refers to the linear velocity of the flow tangential to the membrane surface. It is a key parameter to control the thickness of the fouling layer over the membrane and delay the permeate flux decay. In the case of tubular membranes, it can be determined by the following equation.

$$CFV\left(\frac{m}{s}\right) = \frac{4 \cdot Feed\ flow\left(\frac{m^3}{h}\right)}{3600\left(\frac{s}{h}\right) \cdot \pi \cdot (channel_{diameter})^2(m^2) \cdot number_{channels} \cdot number_{parallel\ membranes}}$$

2.5 Analytics

The Colony Forming Units (CFUs) method was applied in all of the streams generated to analyze the yield of each filtration trial in terms of spores' recovery/isolation. This method gives an estimated number of viable cells.

3 RESULTS

3.1 Biomass / Spores concentration

In this set of experiments, filtration performance was compared starting from two broths with different cell densities, one produced in a batch mode fermentation (low cell density) and the other in a fed-batch (high cell density).

Figure 4 shows the evolution of permeate fluxes where a significant difference in permeability was observed due to the difference in cell concentration. As expected, the fluxes obtained from filtering the broth generated in the batch mode were between 2 to 4 times higher than with the high-concentration samples obtained in the fed-batch mode. In the end, the higher the cell density the higher the fouling layer set over the membrane, making it more difficult to permeate generation.

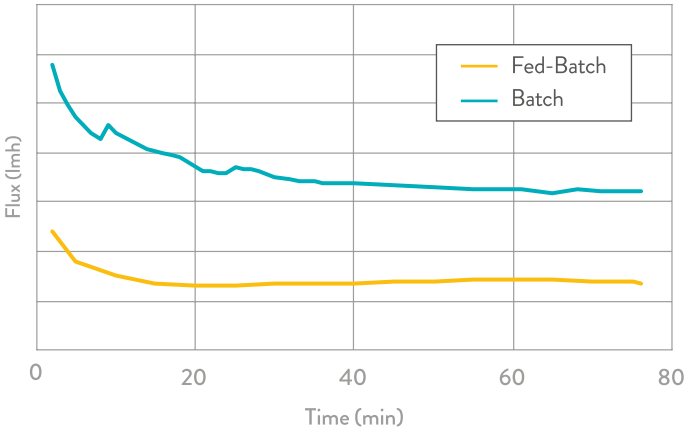

Fig. 4: Evolution of permeate flux during the microfiltration of *Bacillus sp* broths with different cell densities.

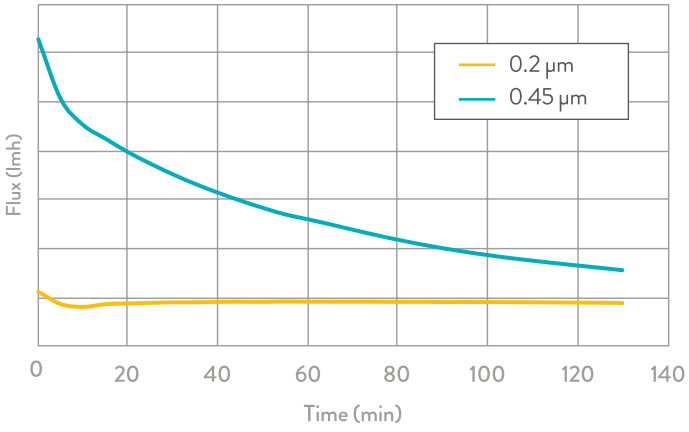

Fig. 5: Evolution of permeates flux during the microfiltration of *Bacillus sp* broths with different pore sizes.

Despite the difference in cell density, it was possible to concentrate both samples 10 times (10X) (see Table 4) with scalable permeate fluxes e.g. with the batch mode broth the average permeate flux was 196 L/h/m² while with the broth from the fed-batch fermentation the average permeate flux was 70 L/h/m².

Table 4: CFUs analysis after the filtration process of batch and fed-batch broths

FERMENTATION MODE	FEED (CFUs/mL)	PERMEATE (CFUs/mL)
Batch	$3{,}40 \cdot 10^{9}$	$2{,}35 \cdot 10^{10}$
Fed–Batch	$6{,}90 \cdot 10^{10}$	$3{,}07 \cdot 10^{11}$

3.2 Spores removal

The second group of experiments consists of comparing not only the permeate flux but also the spore removal effectiveness depending on the membrane pore size used (0.2 and 0.45 µm).

In some processes, the objective is to separate as many spores as possible from the product of interest, in case it is in the supernatant, mainly due to regulatory aspects. As expected, the permeate flux obtained with a 0.45 µm membrane was significantly higher than that of 0.2 µm but, despite being lower, it was very stable from the beginning (see Figure 5).

Membranes used in TFF do not achieve an absolute filtration grade. A subsequent depth filtration of the permeate stream is required to guarantee complete particle removal. This becomes particularly important when considering the use of the 0.45 µm membrane, as the spore passage is significantly higher than with the other (see Table 5).

Table 5: CFUs spores analysis after filtration with 0.45 µm and 0.2 µm

MEMBRANE PORE SIZE	FEED (CFUs/mL)	PERMEATE (CFUs/mL)
0.45 µm	$6{,}9 \cdot 10^{10}$	$1 \cdot 10^{3}$
0.2 µm	$6{,}9 \cdot 10^{10}$	$1 \cdot 10^{1}$

The combination of permeate flux stability with the decreased particle passage makes the 0.2 µm ceramic membrane the most recommended for the task, even in terms of process scalability.

4 CONCLUSIONS

The Bionet's M1 system has validated TFF as a feasible technology for managing processes that involve high cell concentration B. subtilis broths (approx. 10^{10} logs). Specifically, ceramic membranes with 0.2 µm pore sizes have resulted in a good alternative (1) to concentrate the spores or (2) to remove the spores from a supernatant where other biomolecules of interest are found.

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