# New physical cell lysis process for sensitive biopharmaceuticals: Comparison with the reference method.

Christian Rhême<sup>a</sup>, Simon Crelier<sup>b</sup>, Loredana Alibrando<sup>b</sup>, Stéphanie Chédel<sup>b</sup>, Sacha Stadelmann<sup>b</sup>

<sup>a</sup> Frewitt SA, Rte du Coteau 7, 1763 Granges-Paccot, Switzerland c.rheme@frewitt.com

<sup>b</sup> HES-SO Valais-Wallis, Institut ITV, Rte du Rawyl 47, 1950 Sion, Switzerland simon.crelier@hevs.ch

# INTRODUCTION

Cell lysis is a process which is applied to release intracellular molecules by breaking the plasma membrane. More specifically, in biopharmaceutical applications cell lysis releases proteins produced by organisms such as yeasts or bacteria.

The proteins are generally very sensitive, especially in terms of temperature, and the objective is to recover a maximum of the target molecule without losing biological activity.

The numerous methods available are divided into two classes: chemical lysis and physical lysis. Chemical lysis is used regularly. However, it can create contamination and thus requires complex additional stages of purification. It can also be complicated and costly to scale up industrially.

## **High-pressure homogenisation**

Physical lysis is usually carried out by means of high-pressure homogenisation (HPH), a process which takes place over several cycles. It is considered the physical method of reference in spite of the significant temperature rises generated by the increase in pressure and the presence of uncontrollable phenomena such as cavitation.

In the case of heat-sensitive proteins, the pressure used is crucial since the temperature increase is directly linked to it (adiabatic heating of about +1 °C for 4.2 MPa pressure rise). Lyses are therefore often produced under low pressure, which reduces the rate of proteins released and necessitates many cycles through the homogenizer.

# **NEW FREWITT PROCEDURE**

The suggested equipment is a mill intended mainly for the production of active pharmaceutical particles between 100 and 500 nanometres in size.

Based on bead mill technology, this equipment has been developed and optimised for pharmaceutical applications. Compared with its competitors, it stands out with its GMP design, high energy efficiency allowing a process at almost constant temperature, and a drastic reduction in product contamination [1].

The mill functions mainly as an agitator; a grinding media (usually zirconia beads) is used to transmit by means of shock the energy required to rupture the cell membranes. Thanks to the unique Frewitt technology, the energy can be dosed very precisely according to the type of cells treated – bacteria require more energy than yeasts, for example.

The technology described in this article is protected by an international patent.



Figure 1: NanoWitt-LAB equipment of Frewitt SA

#### **METHOD**

Two cellular systems were selected for the different tests:

- Suspension at 38.2 g/l of S.cerevisiae yeasts, whose mitochondria and cytoplasm contain the enzyme fumarase
- Suspension at 37.6 g/l of E.coli bacteria producing recombinant GFP (green fluorescent protein).

For both systems the medium is physiological water, i.e. a solution of NaCl 9 g/l.

## Lysis equipment

High-pressure homogenisation lysis is performed on Stansted SPCH-10 type equipment, the pressure is set at 1000 bar and a volume of 50 ml is treated at each cycle. The temperature is measured before and after each cycle, and the suspension is cooled between each cycle.

The new process is performed on Frewitt NanoWitt-LAB type equipment (Fig. 1) in recirculation mode (Fig. 2), which allows a 250 ml volume of suspension to be processed and samples to be taken at any time. Different stirring speeds were used to adjust the energy according to the work to be carried out. The beads chosen are made of zirconia and their diameter is between 400 and 600 micrometres. The temperature of the product is measured continuously at the outlet of the chamber.

Figure 2 shows the NanoWitt-LAB installation during E. coli grinding. The green-yellow colour of the suspension is due to the presence of GFP.



Figure 2: Lysis of E.coli bacteria in recirculation mode

## **Analytical equipment**

All samples are kept on ice and centrifuged for 10 minutes at 10,000 g at a temperature of 4 °C. The supernatant is then recovered and stored on ice until it is analysed.

The Bradford method is used for the quantification of total proteins, and the measurement is made after staining at a wavelength of 595 nm. To perform a calibration curve, a stock 1 mg/ml BSA solution is prepared as well as a dilution range up to 0.1 mg/ml. A blank is also measured by means of demineralised water.

Fumarase activity [2,3] was measured with a 50 mM L-malic acid solution (substrate) in a 100 mM phosphate buffer at pH 7.6. The kinetics measurement is carried out immediately after the substrate is added, and the absorbance evolution is measured at a wavelength of 240 nm for 10 minutes.

The fluorescence measurement of GFP [4] is performed by excitation at 475 nm, and the emission is measured at 509 nm. A calibration curve is measured with fluorescein solutions (reference substrate) with concentrations ranging from 0.1 to 8.0 µmol/l.

#### **RESULTS AND DISCUSSION**

## High-pressure homogenisation (HPH)

For the suspension of S.cerevisiae yeasts, three cycles were carried out. The maximum temperature of the suspension reached 39 °C, the total protein concentration increased linearly with the number of cycles to reach 3 mg/ml, but no plateau was reached.

Likewise, the fumarase activity increased linearly up to 4.5 U/ml but did not reach a maximum.

The cells were therefore not fully lysed and more cycles should be considered. Protein concentration increases even more after 10 cycles according to the literature [5].

In the case of E.coli bacteria, five cycles were executed. The temperature rise during each cycle was approx. 10 °C. The protein concentration increased during the first two cycles, then stabilised at the value of 10.8 mg/ml.

The concentration of GFP required four cycles to reach a stable value of  $0.6 \ \text{mg/ml}.$ 

## **New Frewitt procedure**

Two operating conditions were tested for the lysis of S.cerevisiae yeasts, namely a rotation speed of 1000 and 1500 rpm.

At 1000 rpm, the temperature remained stable throughout the process ( $\Delta T$ =0.2 °C) and a maximum of 7.6 mg/ml was reached for protein concentration after 10 minutes.

At 1500 rpm, the temperature rise was limited to  $1 \,^{\circ}$ C (maximum temperature 15.4  $^{\circ}$ C) and the protein concentration reached 10.2 mg/ml after only 5 minutes.

The fumarase activity followed the same logic for the two rotation speeds tested. A maximum was reached after 10 minutes of lysis and the values were 2.6 U/min at 1000 rpm and 3.3 U/min at 1500 rpm.

The lysis of E.coli bacteria was also performed at two different speeds: 1500 and 2000 rpm.

The temperature increase was measured at 1.1 °C at a speed of 1500 rpm and at 2.6 °C at 2000 rpm; the maximum temperature reached was 15.7 °C.

The maximum protein concentration was reached in 20 minutes, confirming that E.coli bacteria require more lysis energy than S.cerevisiae yeasts. The values were 6.2 mg/ml at 2000 rpm and 12.2 mg/ml at 1500 rpm.

Times of 15 to 20 minutes were also necessary to reach a maximum concentration for GFP. At 1500 rpm, 0.37 mg/ml was obtained; at 2000 rpm, 0.1 mg/ml was obtained.

It should be noted that the 1500 rpm test proved to be more effective than the 2000 rpm test, which makes it important to be able to precisely dose the energy fed into the suspension which is to undergo lysis.

### Comparison of results

Figure 3 compares the performance of the two technologies for S. cerevisiae yeast in terms of total protein (located mainly in the cytoplasm) and fumarase (present in the mitochondria).

Figure 4 offers a similar comparison but for *E. coli*. This time, however, GFP is the target molecule.

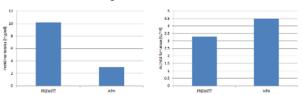


Figure 3: S.cerevisiae, total protein (g.) and fumarase (d.)

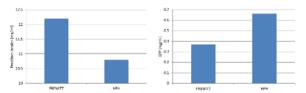


Figure 4: E.coli, total protein (g.) and GFP (d.)

Figure 5 shows the gradual disappearance of GFP in E. coli centrifuge pellets during lysis. This shows that the target molecule was successfully extracted during the liquid phase (eliminated after centrifugation).

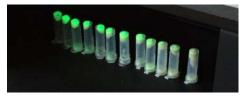


Figure 5: Evolution of the GFP rate in the pellets after centrifugation in terms of lysis time per process Frewitt (t=1 min to t=15 min from left to right)

#### CONCLUSION

Developed to perform nanomilling operations on active ingredients under GMP conditions, NanoWitt equipment demonstrates very interesting qualities in this cell lysis application.

Under optimal parameters, the process can be carried out without any rise in temperature or with a controlled rise of 2 to 3 °C maximum, while allowing a higher total protein recovery rate for E.coli bacteria and an even higher rate for S.cerevisiae yeasts compared to the high-pressure homogenisation process considered the "gold standard" in the field.

The high-pressure homogenisation process retains a slight advantage in terms of fumarase activity and GFP concentration. However, if temperatures of the order of 30 degrees generated by high pressure prove unacceptable for the molecules to be released, then milder conditions should be applied [5] (multiple cycles at low pressure), resulting in a decrease in profitability and, probably, in repeatability.

The new process suggested in this study therefore presents interesting lysis efficiency and surpasses competing technologies in terms of respect for the integrity of thermosensitive active ingredients.

It allows a continuous, reproducible process, and the suspension temperature can be maintained. In addition, the process can be monitored and controlled by various process analytical technology (PAT) sensors.

The build of the equipment is specially designed for GMP use, the materials meet FDA requirements, and aseptic process use in an isolator with a sterilisation system is available.

The excellent results that are obtained during scale-up validations carried out for nanomilling of active ingredient applications will certainly also be confirmed for this cell lysis application.

# **REFERENCES**

- 1. C. Rhême, C. Lefebvre and X. Gao. Investigating a novel approach to drastically minimize zirconium contamination during the production of drug nano-suspensions by wet-stirred media milling. Frewitt.
- 2. O. Pines, S. Even-Ram, N. Elnathan, E. Battat, O. Aharonov, D. Gibson and I. Goldberg. (1996). The cytosolic pathway of L-malic acid synthesis in Saccharomyces cerevisiae: The role of fumarase. Applied Microbiology and Biotechnology 46, (4), pp. 393-399.
- 3. Fumarase Activity Colorimetric Assay Kit MAK206. Sigma-Aldrich.
- 4. T. C. V. Penna, M. Ishii, O. Cholewa and L. C. D. Souza. Thermal characteristics of recombinant green fluorescent protein (GFPuv) extracted from Escherichia coli. Letters in Applied Microbiology, 38, (2), pp. 135–130
- 5. High-pressure cellular grinding homogeniser. Microfluidics Inc.