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A microscale bacterial cell disruption technique as first step for automated and miniaturized process development

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ABSTRACT

Cell disruption is crucial during recovery of biopharmaceuticals overexpressed in *E. coli*, which tend to be produced intracellularly as insoluble inclusion bodies. Miniaturized high-throughput systems can accelerate the laborious downstream protocol for such biopharmaceuticals and enable integrated process-development. A fast and robust cell disruption method reflecting the protein and impurity profile of homogenates obtained by large-scale methods is required for such an approach. We established a miniaturized bead mill for parallel mechanical cell disruption at the microscale. Its total protein and impurity release, protein pattern, and particle size distribution were compared to results from microscale enzymatic digestion and referred to laboratory-scale high-pressure homogenization. Bead mill disruption led to equivalent protein and impurity release as well as to the same particle size profile as the large-scale reference. In contrast, lysates obtained by enzymatic digestion contained only 30–47% of overall protein, 17% of dsDNA, and 7–10% of endotoxin compared to those obtained by high-pressure homogenization; also larger debris was present in lysates after enzymatic digestion. The established method is fast, efficient, robust and comparable to current large-scale standards, allowing for parallelization of experiments. Thus, it is the method of choice for rapid integrated process development at the microscale.

1. Introduction

Intracellular expression in *E. coli* offers a variety of options for high-yield product formation. The product may be 1) soluble, expressed in the cytosol; 2) soluble, expressed in the periplasm; 3) insoluble, deposited in inclusion bodies (IBs) within the cell; or 4) associated with the cell membrane. In all cases, a cell disruption step to release the expressed products is required [1,2]. Clearly, focusing on soluble production as a favorable format represents a severe restriction for protein overexpression in *E. coli*. IBs can indeed represent an advantage or even be the only possibility of expressing specific proteins. Systems enhancing IB formation have enabled overexpression to a high product concentration up to 12 g/L of high purity with the N^{pro} fusion system [3,4], suppressed degradation of products [5], and even supported the production of cell toxic molecules [4,6,7]. Admittedly, protein recovery from IBs with a high yield is usually an empirically driven, laborious, and time- and material-consuming process.

An automated miniaturized platform for integrated process development at the microscale is of increasing interest for biopharmaceutical production from IBs to hasten process development while reducing material and time consumption. A high-throughput screening method for IB solubilization and its scalability has been published recently [8,9]. Microscale methods for screening of refolding conditions [10,11], approaches for online monitoring of such processes [12] and a number of publications on small-scale chromatography and related scalability are available [13–16].

The technology chosen for the initial product recovery is critical because it will determine the quantity of product and impurities released as well as the physical properties of the homogenate. The first crucial step for the recovery of intracellular products after cell harvesting is cell disintegration. For *E. coli* cells, the two major options are mechanical or non-mechanical methods. The first option can be further divided into methods applying hydrodynamic (high-pressure homogenization, HPH; ultrasonic) or solid shear stress (e.g., bead mill, X-press), while the second can be grouped into 1) chemical (detergents, organic solvents, etc.), 2) enzymatic (lytic enzymes, autolysis, phage lysis), and 3) physical treatments (osmotic shock, heat, freezing) [17,18].

Obviously, the ratio of product to impurity content significantly influences the subsequent purification process, including filtration/centrifugation and chromatography [18–24]. Clearly, this influence has

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to be addressed by a process-development platform. Host cell–derived impurities to be considered throughout the downstream process of *E. coli*–derived biopharmaceuticals are proteins and cell debris, DNA, and endotoxin. All of these impurities need to be depleted in a reliable and verifiable manner for the production of biopharmaceuticals.

Even though mechanical disruption by HPH does not selectively release the product, it is the standard method for large-scale industrial applications because it is efficient, is based on a well-controlled and understood technology, and allows for high flow rates [25–30]. As a consequence, alternative cell disruption methods are usually compared to HPH as a reference [25,31–35]. Hence, a miniaturized cell disruption method enabling integrated process development for intracellular products has to deliver homogenates of equivalent properties to those of the large-scale HPH, namely concerning the quantity and quality of released product and impurities and their physical properties.

Methods applicable for this purpose that have been compared to HPH are enzymatic digestion with lysozymes [32], mechanical breakage with a bead mill [34] or French press [35] and ultrasonication [14,21,22,31,33,36,37]. Enzymatic digestion of *E. coli* is a common method used for analytical sample preparation to characterize fermentation performance which is either carried out in free solution or with lysozyme immobilized on carriers [38]. Van Hee et al. investigated this method and its applicability for IB release compared to HPH. In that study, the enzymatic digestion prevailed over the mechanical method at the laboratory scale and showed good separation properties during subsequent centrifugation [32], but this outcome has not been verified for microscale experiments. Comparing cell disruption with a bead mill to HPH and a microfluidizer, Agerkvist and Enfors showed equivalent protein and enzyme release among these three methods. However, the homogenates obtained showed differences in their physical properties, which had an impact on the subsequent filtration and centrifugation steps [34]. These investigations were carried out at the laboratory scale only. Parallelized approaches of cell disruption by bead mill were established by Hummel et al. [39] for up to eight samples with working volumes of 0.2–2 mL, for more than 60 samples by Ramanan et al. [40] and for high-throughput in 96 well plates by Allender et al. [41]. The latter one was established for DNA extraction only. Adaptive focused acoustics, an ultrasonication approach, has been used as an ultra-scale–down cell disruption method to study the interaction of homogenization with downstream processing for yeast and *E. coli* [21,22]. This ultrasonic device released soluble products and impurity levels equivalent to the laboratory-scale HPH but showed limited performance for the release of products deposited as IBs [33]. As a limitation AFA can be performed for minimal sample volumes of 1.5 mL but only in serial operation mode [33]. Ho et al. established a bead mill method for bacterial cell disruption on the microliter scale and discussed the comparison to other disruptive methods [42–44]. The comparison of French pressing, sonication, freezing–thawing, and bead vortexing at the milliliter scale revealed that bead vortexing is preferable regarding protein quality by maintaining enzyme activity. Benov also showed that this method is simple, applicable for multi-parallel experimentation, and suitable for periplasmic extraction [35]. While most miniaturized methods were shown to reach protein release to similar extend as other small scale techniques [40,45] the comparison to large scale method such as HPH is rarely available.

The intention of the present studies was to establish a microscale *E. coli* cell disruption method for integrated process development. This method should be able to mimic cell breakage by laboratory-scale HPH regarding product and impurity release, as well as the physical properties of the homogenate, and allow parallelization of experiments. Such a method has to be 1) simple, fast, and economical, 2) easily automated, 3) robust and reproducible, and 4) consistently applicable.

A mechanical disruption method simulating a bead mill at the microscale by moving glass beads in linear motion with high frequency in microtiter plates was chosen and optimized by adjustment of the following: 1) the diameter of the glass beads, 2) shaking frequency and duration, 3) ratio of biomass to beads, and 4) biomass concentration. Enzymatic digestion does not require any additional instrumentation and is very efficient at the laboratory scale [32], so this method was included as an alternative microscale disruption method. Both methods were compared to HPH at the laboratory scale, which was accepted as the reference method. All homogenates were analyzed for 1) total protein, 2) product, 3) endotoxin, and 4) dsDNA release as well as for particle size distribution and protein pattern. The optimization is shown for disruption of *E. coli* overexpressing a single chain antibody fragment scFv-BIWA4 as IBs and was then verified with green fluorescent protein (GFP) as the model protein for soluble expression.

2. Material and methods

2.1. Equipment

For the HPH, a laboratory homogenizer APV 1000 (SPX FLOW Inc., Charlotte, NC, USA) was used including a cooling unit (Frigomix 2000, Sartorius Stedim Biotech, Goettingen, Germany). Resuspension of the wet cell paste prior to homogenization and the harvested pellet was carried out with an Ultra-Turrax® (T25 digital, S 50 N – G 45 G attachment, IKA®-Werke, Staufen, Germany). Centrifugation at the laboratory scale was done in a tubular centrifuge (Type: GLE, CEPA, Lahr, Germany). A Geno/Grinder® 2010 (SPEX® SamplePrep LLC, Metuchen, NJ, USA) was used for small-scale cell disruption experiments. Quantification of total protein and dsDNA release was carried out on a Freedom Evo™ 200 (Tecan Group Ltd., Männedorf, Switzerland) with an integrated centrifuge (Hettich Rotanta 460 R, Hettich GmbH & Co. KG, Kirchlengern, Germany), a microplate reader Safire^2 (Tecan, Männedorf, Switzerland), and a Te-Shake orbital shaker (Tecan, Männedorf, Switzerland). A bench-top Mini Spin Plus centrifuge (F45-12-11, Eppendorf, Hamburg, Germany) was used for small-scale centrifugation. Particle size distribution was measured with a laser diffraction particle size analyzer (LS 13 320, Beckman Coulter Ltd., High Wycombe, UK).

2.2. Materials

Urea, Tris-base, glycin, NaCl, KCl, Na_2HPO_4, KH_2PO_4, NaH_2PO_4*$2H_2O$, L-arginine, and acetic acid were purchased from Merck (Darmstadt, Germany). Tris-HCl was bought from Amresco Inc. (Solon, OH, USA) and DTT from Mallinckrodt Baker (Mulhuddart, Ireland), Triton X-100 from Dow Chemical (Midland, MI, USA), GuHCl from American International Chemical Inc. (Framingham, MA, USA), and cysteine and bovine serum albumin from Sigma Aldrich (St. Louis, MO, USA). All reagents were of analytical grade. All materials used for the labeling and first dimension of 2D DIGE except for regular chemicals were purchased from GE Healthcare Life Sciences (Wauwatosa, WI, USA). Gels and the marker used for the second dimension were purchased from Invitrogen (Paisley, UK). Wet cell pastes of *E. coli* after overexpression scFv-BIWA4-LCHC as the IB or GFP in soluble form was kindly provided by Boehringer-Ingelheim RCV (Vienna, Austria).

2.3. Methods

2.3.1. High pressure homogenization

The fresh wet cell pastes were resuspended in cell disruption buffer containing 25 mM Tris-HCl, 1 M urea, pH 8.0, in a ratio of 1:5 (w/w) and dispersed with an Ultra-Turrax® at 10,000 rpm on ice. This suspension was homogenized with a high-pressure homogenizer at 700 bar for three passages. The homogenates were cooled to 4 °C at the homogenizer outlet and transferred into 1.5 mL reaction tubes and centrifuged for 15 min at 13,400 rpm. The supernatants were stored at −20 °C prior to analysis of total soluble protein, dsDNA, endotoxin, and protein pattern.