Development of a versatile and continuously operating cell disruption device

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Abstract

Cell disruption is a recurrent unit operation in biotechnology. Interesting biotechnological products like proteins, lipids or biopolymers are synthesized intracellularly and are often not secreted. Furthermore, cell-free biotechnology uses defined fractions of the cytoplasm for in vitro protein synthesis. Bacteria, yeast, algae and filamentous fungi are surrounded by rigid cell walls that have to be disrupted by physical, chemical or mechanical methods in order to retain the valuable cell content. High pressure homogenization is a widely used procedure to disrupt cells and it has been applied to bacteria, algae and yeast. However, the mode of cell disruption has not been fully elucidated and performance is not predictable, thus time consuming iterative cycles are always necessary to define the best parameters for each microorganism, chemical environment and the corresponding product. Therefore, physical parameters of different biological systems were analyzed and boundary conditions defined in order to construct an adjustable disruption device to allow economical efficient, predictable and adjustable cell disruption processes.

Keywords: cell disruption, S30 lysate, cell free protein expression, high pressure homogenizer

1. Introduction

Bacteria, yeast, algae and filamentous fungi are surrounded by rigid cell walls that have to be disrupted by physical, chemical or mechanical methods in order to retain the valuable cell content. Thus, cell disruption is a recurrent unit operation in biotechnology as interesting biotechnological products like proteins, lipids or biopolymers are synthesized intracellular and are often not secreted [1]. Furthermore, cell-free biotechnology uses defined fractions of the cytoplasm for in vitro or so called cell-free protein synthesis (CFPS). The CFPS represents a new biotechnological production system that offers significant advantages compared to conventional in vivo protein production [2]. This system is especially interesting for proteins which are difficult to express using conventional systems including toxins, recombinant antibodies as well as membrane proteins and has been used in small and large scale up to 100 l [3].

A wide range of principles and disruption systems exists for different application. Well known is the operating principle of the French press where cells are exposed to high pressure which is released through a valve. The disruption is caused by the high pressure difference, cavitation and shear stress [4]. All devices that operate in a similar mode but different set ups are summarized as high pressure homogenization. Using a ball mill, cells are grounded by balls of appropriate size. The size depends on the cells that have to be disrupted. Ball diameter and the ratio of balls to cells have to be adapted. Both systems have to be cooled to minimize the thermal effects on proteins due to the heat production during the processes.

However, the mode of cell disruption in all systems has not been fully elucidated and performance is not
predictable, thus time consuming iterative cycles are always necessary to define the best parameters for each microorganism, chemical environment and the corresponding product. The new production system for proteins, the CFPS requires a special cell extract. Therefore, physical parameters of a model biological system were analyzed and boundary conditions defined in order to design an adjustable disruption device to allow economical efficient, predictable and adjustable cell disruption processes.

2. Materials and Methods

*E. coli* cell were cultivated in LB medium at 37°C. S30 extract was prepared from *E. coli* BL21 Star™ and BL21 (Life Technologies GmbH) as described in EMBL protocols (http://www.embl.de/pepcore/pepcore_services/protein_expression/ecoli/lysate/). For homogenization a French press (SLM Instruments) and “Avestin” EmulsiFlex-B15 high pressure homogenizer (Avestin Europe GmbH, Germany) were employed.

Batch cell free expression reactions were also performed as described in EMBL protocols employing pIX 3.0 GFPSII vector (S. Kubick Fraunhofer IBMT) as template DNA and monitored using the RT-PCR device (TOptical Thermocycler, Analytic Jena). Aliquots of the cell free expression reaction and cell lysis samples were separated by vertical polyacrylamide gel electrophoresis (10% gel, SDS-PAGE) according to Schägger and von Jagow [5]. The gels were run at 120 V for 2-3 hours. Simulation was carried out using ANSYS Workbench, CFX Version 14.0 was applied. The cell suspension was considered as isothermal transient at 4°C with a total simulation time of 50 * 10^{-3} s. Boundary conditions were a velocity of 0 m * s^{-1} and static pressure of 0.12 MPa in front of the inlet valve relative to the atmospheric pressure behind outlet valve.

3. Results and Discussion

3.1. Comparison of different disruption methods for CFPS lysate preparation

The CFPS can be performed in a prokaryotic or eukaryotic system. The prokaryotic system usually uses a cell extract from *Escherichia coli*, known as S30 lysate. All significant factors for protein expression such as ribosomes, transcription factors, elongation factors and tRNA have to be isolated during S30 lysate preparation (Fig. 1). Cell disruption and cell content purification must aim to isolate the machinery of protein synthesis and all necessary factors that are summarized in Figure 1.

The components of a S30 lysate range from 15 kDa up to 1.6 MDa. Furthermore, ribosomes are large complexes composed of proteins and RNA. Those complexes must not be destroyed by the disruption method. Thus, first boundary conditions are the size range of proteins and the integrity of protein complexes that must be obtained. Therefore, using a high pressure homogenizer, a ball mill as well as an enzymatic cell disruption the size range of proteins obtained was evaluated (Fig. 2).

The suspension derived from cell disruption was centrifuged to remove cell debris and the supernatant was applied to SDS-PAGE, where proteins are denatured and distributed according to their size. The resolution of the protein gel ranges from 25 kDa to 300 kDa, larger protein complexes cannot migrate and concentrate at the beginning of a lane. It can be clearly seen that using the ball mill and the lysozyme approach proteins within the range of 25 and 280 kDa are present, but larger proteins and protein complexes are not present. Only when a high pressure homogenization is used, larger proteins and complexes are present. Thus, cell disruption for S30 lysate preparation should be performed using high pressure homogenization.

<table>
<thead>
<tr>
<th>cell disruption</th>
<th>French press</th>
<th>Ball mill</th>
<th>Lysozyme</th>
</tr>
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<tbody>
<tr>
<td>pressure in MPa</td>
<td>400</td>
<td>1000</td>
<td>0.5 2.0</td>
</tr>
<tr>
<td>balls g</td>
<td>0.5 1.0</td>
<td>0.5 2.0</td>
<td></td>
</tr>
<tr>
<td>lysozyme in mg</td>
<td>280</td>
<td>140 100</td>
<td>35 25</td>
</tr>
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Fig. 2. Derived size distribution of proteins using French Press (FP), Ball mill ULTRA-TURRAX® Tube Drive and enzymatic disruption with lysozyme and ultra-sonication

Fig. 1. Required components for the CFPS adapted from [6]
3.2. Optimization of a high pressure homogenization cell disruption method for S30 lysate preparation

High pressure homogenization is a widely used procedure to disrupt cells and it has been applied to bacteria, algae and yeast [7]. However, the mode of cell disruption has not been fully elucidated and performance is not always predictable. Parameters that have an influence on cell disruption and that can be adapted are the pressure that is applied, the geometry of the seat, valve and impact ring. Furthermore, the velocity of cell suspension release and the number of passages can be modified.

Geometry in standard devices is fixed but pressure and number of passages can be changed. Two different available devices were used; a standard French press and a commercially available device called “Avestin” (see 2. Materials and Methods). Cell disruption was followed by S30 lysate preparation for cell free protein expression. The quality of the S30 lysate was evaluated by comparing the amount of expressed green fluorescence protein (GFP) in the cell free system. The amount of active protein can be quantified by measuring the fluorescence intensity during and after cell free protein expression.

Using the Avestin system, first 100 MPa disruption pressure was chosen and one to four passages were carried out. Cell disruption efficiency can be evaluated by protein concentration of the supernatant. As depicted in Fig. 3 the protein yield in the supernatant in relation to the total protein content does not change significantly after the first passage. Thus, one passage of cells is sufficient for cell disruption and no further shear stress has to be applied.

In order to reduce the shear stress to the isolated proteins, the pressure was reduced from 114.5 MPa to 41.25 MPa. Here the efficiency of disruption and impact on the S30 lysate quality was tested by using the corresponding S30 lysate in a cell free expression system expressing the green fluorescence protein. The highest expression activity can be obtained using 41.25 MPa disruption pressure. An increase of pressure results in a decrease of expression efficiency (Fig. 4). Using the obtained parameters, one passage and 40.0 MPa disruption pressure, cells were disrupted employing French press and Avestin. Size distribution of released proteins was assayed and the amount of active GFP was determined. Surprisingly, although protein size distribution and concentration does not differ significantly, the amount of active GFP derived from Avestin is considerably higher than from the French press S30 lysate (Fig. 5 a and b).

Consequently, the total amount of expressed GFP was determined to differentiate between total and active protein. Protein folding and maturation have an influence on the activity of proteins [8]. For GFP it is known that folding factors so called chaperones, oxygen and a certain time of maturation is needed for a fully active protein [9].

The protein quantification by SDS-PAGE and Bradford unambiguously depicts (Fig. 6) that the activity assay using GFP as a reporter protein does not reflect total GFP synthesis during CFPS. Thus, the total amount of expressed GFP from French press S30 lysate is higher than from Avestin S30 lysate. This can be due to
differently isolated maturation factors or other factors that influence the chemical environment. Future work will elucidate the reasons for this difference in order to further optimize cell lysis for S30 lysate preparation.

Fig. 6. (a) Quantitative analysis of expressed GFP from S30 lysates French press (FP 1-3) and Avestin (A 1-3) of biological triplicates after 2h/37°C; (b) Corresponding protein gel that depicts the actual GFP amount derived within the CFPS (NK - control)

3.3. Development of a versatile disruption device

As described within the previous two chapters high pressure homogenization is the method of choice for S30 lysate preparation but when using different devices results are not consistent. Reasons for this inconsistency have not been clarified yet.

In order to have an influence on geometry and to overcome the limitation of a batch wise disruption of small volumes of cell suspension, a continuously operating system with interchangeable geometry was constructed. This will assist in elucidating more parameters and boundary conditions for a disruption method for highly active S30 lysate and serve as a device for up-scaling S30 lysate preparation.

The developed device is composed of two subassemblies, which could be separated from each other. Therefore it is possible to autoclave the relevant parts separately. The construction consists of a hydraulic power unit, to exert a pressure \( p_1 \) on a hydraulic piston. This piston passes the force \( F \) to a second piston with a minor diameter \( D \). The second piston transfers the pressure \( p_2 \) on the fluid with the cells which have to be disrupted. A schematic overview is given in Fig. 7.

Furthermore a specially shaped shaft to trigger the pistons is a component of the system. This shaft works as a valve. In a defined position the shaft links the pressure pipe from the hydraulic power unit with the piston. The shaft enables a disconnection between the pressure pipe of the hydraulic power unit and the piston. The shaft is continuously driven by an electric motor and pivoted by two ball bearings. Thus the rotational speed \( n \) defines the cycle time \( t_c \). A pump carries the fluid and the cells into a cavity under the piston arrangement. The cavity will be closed by an inlet and an outlet valve, which are pivoted inside. In addition the valves will close if the shaft couples the hydraulic power unit and the piston. Over this depicted process step the piston puts pressure \( p \) on the fluid. The outlet valve includes a spring, which assures the opening process of the outlet valve in the cycle time with normal pressure \( p_0 \). Because of the inrushing fluid the inlet valve will open without a spring. The design requirement for the pressurization chamber of the high pressure homogenizer were that the incoming fluid, containing the non-disrupted \( E. coli \) cells, does not pass the outlet duct until it has been pressurized.

This ensures that no cells leave the high pressure homogenizer without being disrupted. In order to assure this and to analyse the flow in the pressurization chamber Computational Fluid Dynamics (CFD) was employed. For this purpose the suspension has to be defined as a fluid in the CFD-Software. The viscosity is a characteristic property of the fluid where phenomena such as friction stall and eddy development can be deduced from. Therefore, viscosities have been obtained at 4 °C for suspension containing non-disrupted cells as well as for suspension containing disrupted cells of \( E. coli \). The obtained measurement data served as input values for a transient flow analysis, based on the \( k-omega \) Turbulence Model, of the pressurization chamber.
flow by means of the ANSYS Workbench, CFX Version 14.0 software. The flow is visualized by the use of particle tracks which describe the path of the cells through the pressurization chamber (Fig. 8).

![Simulated particle track](image)

Fig. 8. Simulated particle respectively E. coli cells track as a result of the CFD analysis

As a result of the flow analysis an optimized pressurization chamber, valve and clap geometry and regulation has been designed for E. coli disruption. Due to the modular set up a substitute of the clap arrangement in the pressure cabin through another selectable valve setup is enabled. A fixed force press is described for the cell disruption. In consequence of this concept different filling levels are tolerated with a constant pressure inside the cabin. The transmission ratio of the pressure $p$ is default by the arrangement of the pistons. Nevertheless the pressure within the chamber is adjustable by a relief valve. The maximum working pressure $p_w$ is limited by the maximum pump pressure $p_p$ and the ratio of the piston diameters. The developed system is a modest setup and enables a continuous and on different cells and application adaptable cell disruption device.

4. Summary

High pressure homogenization is currently the best method to produce an E. coli cell lysate where a highly active S30 lysate for CFPS can be prepared from. First results demonstrate that for this purpose a lower homogenization pressure than normally used for cell disruption is needed. For the two device tested Avestin and French press, 40 MPa was an optimal homogenization pressure. A higher passage number could not increase the total yield of E. coli cell protein content, thus one passage was sufficient for lysate production. Remarkably, using these parameters on both devices, starting with the same E. coli cell culture the prepared S30 lysates did not show the same protein expression activity. The S30 lysates did show similar protein distribution and concentration but using the extract in the CFPS the Avestin S30 lysate produced more active protein, whereas the total protein amount was higher using the French press S30 lysate. Reasons for this are unclear at the present state of research. Current investigations are focusing on biochemical and mechanical influence on cell disruption. As described in the previous chapters, geometry cannot be changed in standard devices. Thus, for the mechanical characterization, a homogenizer has been designed that can work continuously. Furthermore, claps and valves are interchangeable and the influence of geometry can be analyzed in future work.

Acknowledgements

We thank Sophie Skarlatou and Stefanie Weber for technical assistance. We are very grateful to Jennifer Langbein and Wolfram Volkwein for advice, correction of the English language, and particularly for part of the disruption studies This work was supported by the German Federal Ministry of Education and Research for support through grant FKZ 0312037 and FKZ 0315942.