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Comparison of cell disruption methods for a recombinant *Escherichia coli* strain

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Abstract

Different methods of cell disruption were investigated in order to release the inulase II from the cells of a recombinant *Escherichia coli* strain. For small volumes, the mixer mill and the ultrasounds device are appropriate methods, in each case, the optimal working parameters, as the disruption time, loading of the vial, the cell suspension concentration, etc, were examined. Analyzing and comparing the results, the yield of released protein was found to be similar independent of the used method. In some cases, the results were compared with respect to the enzyme activity.

Keywords: cell disruption, grinding with mixer mill, ultrasonication.

Introduction

In the last years, applied microbiology has become a major growth area since the recombinant DNA technology offers the possibility to take advantage of the cellular synthetic capacities. Whereas up till then random mutation and selection had been used to increase the level of preexisting activity in a microbial cell, recombinant DNA technology is now being used to confer to the cells an entirely new synthetic capacity, such as, for instance, the synthesis of human hormones by *Escherichia coli*. Also the enzyme employed in this work, inulase II, was recently produced as recombinant protein in genetically modified *E. coli*. For this, the gene for inulase II was transferred from the natural producer, a strain of *Arthrobacter* spec., to *Escherichia coli* and so the *E. coli* XL-1 blue/pMS*ift*OptWT strain was obtained [1]. The inulase II was further used to obatin di-D-fructofuranose 1,2´:2,3´ dianhydride (DFA III) from inulin as a row material. DFA III is expected to be utilized in various applications due to its properties: (i) it has half of the sweetness of sucrose, (ii) it is low caloric since it is not metabolized in the gastro intestinal tract of mammals (iii) it is chemically more stable than fructo-oligosaccharides due to the presence of a dioxane ring, (iiii) it has growth promoting activity for such enteric bacteria as *Bifidobacteria* [2]. Secreted by *Arthrobacter* sp., inulase II is an extracellular protein. When produced in recombinant *E. coli*, the signal peptide responsible for the posttranslational export of the protein out of the cell is missing [3].

The sequence of *Arthrobacter* would not have been recognized by *E.coli* anyway. The inulase II remains in the cell and in order to recover it from the cytoplasm it is necessary to disrupt the cells.

The type of energy input necessary to isolate the proteins that are accumulated within the cells permits the cell disruption classification in: (i) mechanical cell disruption processes and (ii) non-mechanical cell disruption processes, including chemical, biological and physical methods. Each method has specific advantages and disadvantages, depending on the product and its applications. Mechanical disintegration is generally applicable, while the other approaches need specific procedures for each individual case. Disruption of a small volume of cell suspension in a laboratory for analytical purposes is generally performed using

mechanical forces and especially important are the stability of the cell wall and the size of the cell itself. For instance, animal tissue can be damaged by very small shear forces but the Gram-positive bacteria require a larger energy input [4, 5]. The release of Inulase II from *E. coli* cells was performed using mechanical disruption methods for small volumes, the grinding with mixer mill and ultrasonication.

Mixer- mill: cells are disrupted by shear forces generated during vibration and enforced motion of glass beads in the mixer mill [6]. The protein release for the disruption in the mill is a first-order rate process. The most important parameters to influence the efficiency of cell disruption are: the size of the beads, cell concentration, ratio of cell suspension to the amount of glass beads and the total volume of suspension and beads [7]. To establish the optimal conditions for the cell disruption using the mixer mill, experiments were performed using the cells from an overnight LB medium culture which were harvested and resuspended in phosphate buffer + Mg^{2+}. The magnesium ions are cofactors for the Benzonase, the enzyme used to reduce the viscosity of the suspension due to nucleic acids. For every g of cell suspension 0.2 µl Benzonase (Merck, Darmstadt) were used. The necessity of Benzonase is a critical step for the use of this method for large-scale disruption considering the price of the enzyme. The disruption evaluation was made by quantifying the total protein.

Ultrasonication: the energy developed by ultrasound depends on resonance frequency of the device (15-25 kHz). Due to acoustic waves created by the probe vibration, air cavities are formed in the cell suspension. When these cavities are breaking down, the mechanical energy dissipated in the cell suspension is bigger than the cell elasticity and so the cell wall is destroyed [8]. As for all mechanical procedures, the disruption kinetics is a first-order one [9]. An important problem that has to be overcome is the temperature increase in the cell suspension during disruption.

Materials and Methods

The genotype of *Escherichia coli* XL-1 blue is: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI* ^{q}*Z ΔM15* Tn*10 (*Tet^{r}*)*].

The optimal cultivation parameters for this strain are: 37°C, 170 rpm, 16 hours.

Bacterial cells were harvested by centrifugation in a refrigerated centrifuge and suspended in buffer (0.04 M Sörensen phosphate buffer containing 2mM Mg^{2+}, pH 7, see below) to obtain different concentrations (% of wet cell weight –WCW-) of cell suspension. Various amounts (g) of this suspension, and an appropriate volume (µl) of Benzonase (Merck KGaA, Darmstadt, Germany) (0.2 µl per g of wet cells) were placed in each stainless grinding chamber (chamber volume 30 ml). Glass beads were added (diameter 0.3 mm) (employing different ratio between wet cell suspension and glass beads). After 30 minutes treatment in the mixer-mill (Retsch MM 2000, Windaus Labortechnik, Clausthal-Zellerfeld, Germany) at 100% intensity, the chambers were emptied using a spatula and washed with buffer (0.04 M Sörensen phosphate buffer, pH 7). Both disruption mixture and washing buffer were collected in the same tube (SS 34 vials), which was further centrifuged to remove the cell debris for 10 minutes at 27,000 g in the refrigerated centrifuge (Sorvall, RC-5B Refrigerated Superspeed Centrifuge, Fa. DuPont Instruments, Bad Homburg, SS 34 rotor). The clear supernatant was used for enzyme analysis. The glass beads were repeatedly used after sterilization, washing and drying at 50°C over night.

Sörensen phosphate buffer + Mg^{2+} (0.04 M, pH 7): 4.360 g/L $Na2HPO_4$; 2.110 g/L KH_2PO_4; 0.493 g/L $MgSO_4 \times 7H_2O$.

Ultrasonication: the following standard procedure was used for cell disruption. 0,5 ml from the culture broth were centrifuged in a 10 ml glass test tube (∅=13mm) using a Jouan

Centrifuge (BR.4, Jouan Centrifuge, France) (rotor S40, 2,100 g, 10°C for 10 minutes). After centrifugation, the supernatant was removed by inverting the test tubes until all medium had been drained. Pellets were suspended in 5 ml of cold NaCl 0,9% (from ice) using a vortex device (dilution 1:10 regarding the native culture broth).

The cell suspension was then assayed for disruption, using an ultrasounds device (Sonopuls Homogeniser, Bandelin *electronic* GmbH & CoKG, Berlin, Germany) with the following parameters: (i) probe: KE76, (ii) cycle: 5×10%, (iii) power: 60% (iv) time: 2 minutes. The probe was not more than 2 cm immersed in the cell suspension, with no contact to the walls of the test tube. To prevent overheating, the test tubes were placed on ice during cell disruption. At the end, 2 ml from the disrupted cell suspension were further centrifuged in 2 ml Eppendorf cups for 5 minutes (Jouan centrifuge, rotor AB 2.14, 9,400 g, 4°C) and the supernatant was assayed for enzyme analysis.

The protein concentration was determined following Bradford [10]. This procedure is based on the Coomassie-Brilliant-Blue colorant binding to proteins. 20 µl of sample were mixed with 1 ml Bradford reagent and incubated 5 minutes at room temperature. The extinction was measured at 584 nm using a Spectrophotometer (Shimadzu Spectrophotometer UV-120-02), against Bradford reagent + 20 µl dist. water. The calibration was made with BSA (Bovine Serum Albumin).

Bradford reagent:

Serva Blau G-250	70 mg
Ethanol 96% (v/v)	50 ml
H_3PO_4 85% (v/v)	100 ml
H_2O dist.	to 1000 ml

The colorant was dissolved in ethanol, phosphoric acid was added and then filled up with distilled water to 1000 ml. The reagent was filtered and kept at 4°C protected from light. LB (Luria Bertani) medium [11]: 5 g/L yeast-extract; 10 g/L peptone from casein (pancreatic digest); 10 g/L NaCl; pH adjusted to 7 with NaOH 1 M.

The medium was sterilized at 121°C for 20 minutes (Varioklav, Fa. H+P Labortechnik GmbH, Oberschleissheim). After sterilization, the vessel containing the medium was temperated at 50°C for one hour using a water-bath. Sterile ampicillin solution (with a concentration of 60 mg/ml) was added for strain selection (*E.coli* XL-1 blue/pMS*ift*OptWT is carrying an ampicillin resistance gene) in a ratio of 1:1000.Aqueous ampicillin solution is sterile filtered using 0,22 µm filters (Millex-GS) and stored at –20°C.

The enzymatic activity of the enzyme is determined from the amount of DFAIII produced. 1U of enzyme responds to 1µmol DFAIII per minute. A standard assay for enzyme activity was carried out as follows.

Enzyme activity test: 100 µl enzyme solution and 900 µl Inulin solution (100 g/L, see below) were incubated in 2 ml Eppendorf cups at 50°C for 30 minutes. The enzymatic reaction was stopped boiling the samples at 100°C for 5 minutes. 10 µl Novozym SP 230 were added (to hydrolyze the remaining inulin to fructose and glucose) and samples were incubated at 60°C for another 30 minutes. For desalting, ca. 150 mg ion-exchanger were introduced in every cup, and then were shaken at room temperature for 30 minutes (Edmund Buhler Swipe KS10). Samples were filtered through a 0.22 µl filter and measured by HPLC. The enzyme solutions had to be diluted with water for a final concentration of DFAIII in the reaction tube of around 3 g/L.

Inulin solution: 100 g inulin/L were dissolved in 0.04 M phosphate buffer pH 5.25 by heating the solution at 90-100°C under stirring. When the inulin was completely dissolved the solution was cooled down to 50-60°C using an ice bath. This solution was freshly prepared before use and will be further referred to as Inulin solution

Phosphate buffer 0.04 M: 0.14 g/L $Na_2HPO_4 \times 2H_2O$; 5.33 g/L KH_2PO_4 pH 5.25
Serva Blue G was obtained from Serva, Heidelberg, Novozym from Novo Nordisk, Mainz and all the other chemicals were supplied by Merck, Darmstadt.

Results and discussions:

1. Mixer mill

1.1. Ratio of cell suspension to glass beads

Experiments were performed varying the cell concentration of the tested suspension. Different amounts of beads (0.3 mm diameter) were added to a constant volume of cell suspension to find an optimum ratio of cell suspension and beads. The size of the beads was chosen according to the studies of Schütte and Kula [12], which found that for bacteria suspension the appropriate bead size varies in the range of 0.2-0.5 mm and for yeasts in the range of 0.4-0.7 mm. The disintegration was carried out at room temperature with precooled samples. Considering that Inulase II is stable up to 60°C, the temperature increase during disruption had no influence on the enzyme activity. The highest temperature recorded after proceeding the disruption for 30 minutes was 29°C. As control parameter for the cell disruption efficiency, the protein concentration was determined according to Bradford [10].
The ratio glass beads: cell suspension was varied from 0.5:1 to 3:1 and all samples were treated for 30 minutes at maximum amplitude of the mixer-mill. The results presented in table 3.1 were obtained for a 5% cell suspension and a 40% degree of loading.

Table 1. Influence of the ratio of beads: cell suspension ratio on protein release

Ratio Beads: cell suspension	Protein concentration µg/ml)
0.5:1	1,500
1:1	2,400
1.5:1	3,000
2:1	3,800
3:1	3,850

The degree of cell disruption increases with the amount of glass beads as demonstrated by the release of protein. For further experiments a ratio of 2:1 of glass beads and cell suspension was considered as appropriate. Since there is no significant difference between the results obtained for 2:1 ratio and 3:1 ratio, the use of a higher amount of beads is not necessary.

1.2. Cell suspension concentration

Suspensions containing different cell concentrations were tested and the results are presented in figure 1. The cells were suspended in phosphate buffer, so that the final suspension concentration was varying in the range of 2 to 30% wet cell weight (WCW).

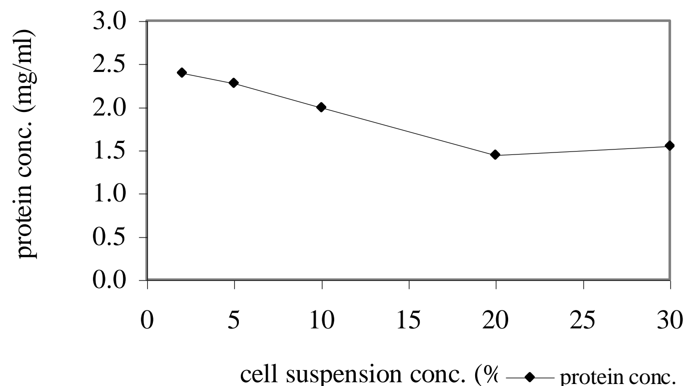

Figure.1. Release of protein during disruption for different cell concentrations

The cell disruptions were performed under identical conditions for all samples (ratio beads: suspension- 2:1, 40 % loading degree and 30 minutes). The highest protein concentration was obtained for 2% cell concentration, which means that the protein release using grinding with glass beads is less efficient with the increase of the cell suspension concentration as is known from the literature. For further experiments involving the mixer-mill cell disruption a suspension concentration of 2% WCW was employed.

1.3. Bead loading

Different degrees of filling the 30 ml disruption vials with beads were tested. Using a 2 % cell suspension, a ratio of 1:2 for the suspension and beads and a disruption time of 30 minutes, the vial loading degree was varied in a range of 30 to 90%. The results are shown in figure 2.

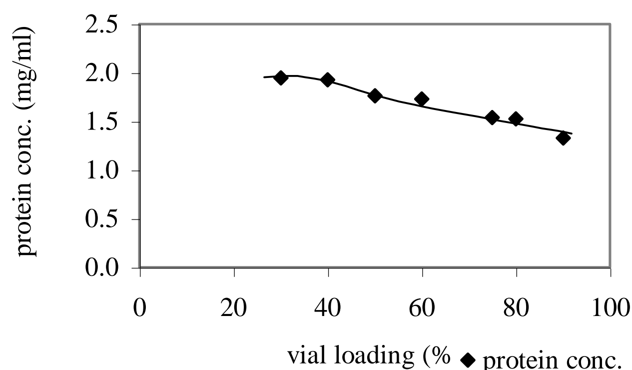

Figure 2. Effect of vial loading on the protein release.

The loading volume influenced the efficiency of disruption as shown in figure 3.2. The protein concentrations obtained are decreasing with the loading volume at a degree of loading higher than 40%, and so for a 90% degree of loading, the protein concentration was 1.3 times lower than for 30%. Therefore, a loading degree of 40% was considered as appropriate for further experiments.

1.4. Disruption time

The disruption time is as well a very important parameter for the efficiency of protein release. The protein concentration was measured after 25, 30, 35 and 40 minutes for two different concentrations of cell suspension (2 and 5%), 40% loading degree and 2:1 ratio of glass beads: suspension. For a better comparison of the 2% and 5% suspensions, the protein concentrations were calculated with reference to a 1% cell suspension (figure 3).

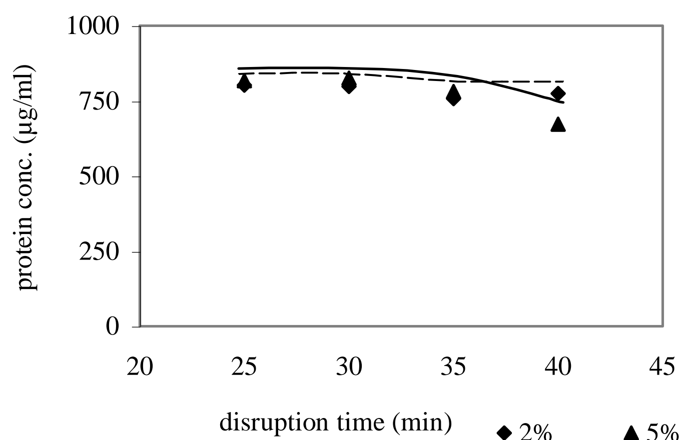

Figure 3. Time course of protein release for 2% and 5% cell suspension

Figure 3 shows that a maximum of 30 minutes is sufficient for a complete disruption and protein release. Once again, in the range of 2% to 5% cell suspension there is no difference, so is not necessary to use a higher concentration than 2% cell suspension.

To summarize, the following parameters were considered appropriate for cell disruption with a mixer-mill: (i) 2% cell suspension, (ii) 2:1 ratio of beads and cell suspension, (iii) 40% vial loading (4 g cell suspension and 8 g beads (g/g)), (iv) disruption time: 30 minutes.

Considering that in 30 minutes only two samples could be disrupted and larger volumes of culture broth are necessary to obtain the 2% cell suspension, for routine analyses of enzyme activity in fermentation experiments, for instance, disruption with ultrasonics is an appropriate method.

2.Ultrasonics

2.1. Time course of enzyme release

0.5 ml from a 2% cell suspension were centrifuged and the pellets were resuspended in 5 ml pre-cooled NaCl solution (0.9%). During cell disruption, which lasted for 10 minutes, the sample was placed in an ice bath in order to prevent the inactivation of the enzyme due to temperature increase. Every minute, 500 µl from the sample were centrifuged and 100 µl from the supernatant were assayed for enzyme activity in order to investigate the time course of enzyme release from the cells. The DFAIII concentration obtained after performing the activity test can be directly correlated with the enzyme concentration and with the efficiency of cell disruption. The results are shown in figure 4.

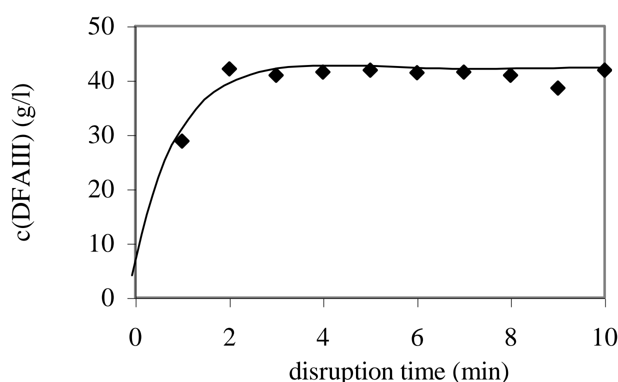

Figure 4. DFAIII concentrations as a function of time.

Figure 3.4 shows that 2 minutes of cell disruption are sufficient for an optimal enzyme release, because no higher DFAIII concentrations could be obtained after longer disruption time.

2.2. Validation of cell disruption method

Due to its advantages (short time, small volume of cell suspension and simple handling procedure), ultrasonication cell disruption method was frequently used for standard analysis. The same tests were performed in order to verify if the disruption method gives reproducible results. Starting from a single 20% cell suspension, 9 identical samples were prepared and assayed for disruption as presented above. The disruption of every sample lasted for 2 minutes. To investigate the released enzyme concentration an activity test was performed for each sample and the DFAIII concentrations obtained are summarized in table 2.

Table 2. Standard deviation for cell disruption by ultrasonics

Sample number	c(DFAIII) (g/l)	Standard deviation (%)
1	4.36	
2	4.31	
3	4.22	
4	4.18	
5	4.01	± 3.9
6	4.67	
7	4.22	
8	4.28	
9	4.45	

There were no significant differences between the DFA III concentrations obtained so the enzyme concentration was similar for all 9 samples. The standard deviation of 3.9% was considered acceptable, meaning that the cell disruption method offers reliable and reproducible results.

2.3. Comparison of ultrasonics and mixer-mill

Cell suspensions of different concentrations were assayed for disruption using both grinding with glass beads and ultrasonication methods. Because each method required different dilutions of cell suspension and the samples were prepared and handled differently, the DFA III concentration only is not sufficient in order to compare the two methods and so

the enzyme activity had to be calculated for 1 liter culture volume. The results are summarized in table 3.

Table 3. Comparison ultrasonication and mixer-mill

Enzyme activity (U/l)		Coefficient
Mixer-mill	Ultrasonication	Ultrasonication/Mixer-mill
180,000	238,000	1.32
180,000	237,000	1.31
150,000	230,000	1.53
40,000	55,000	1.37

The protein release is comparable for both cases. Using the ultrasonication disruption, enzyme activities obtained are 1.4 times higher than those obtained by grinding with glass beads, which means that the disruption by ultrasonication is a slightly more efficient method than the grinding with glass beads.

The cell disruption using the ultrasonication had been shown to be a reliable and easy to perform laboratory procedure, it however cannot be scaled up when larger amounts of enzyme were required. In that case it was necessary to use another technical disruption method, the high–pressure homogenizer, for instance.

Summarizing, grinding with glass beads is a mechanical disruption method that was used for routine enzyme investigations. The efficiency of protein release depends on the disruption time, ratio of beads and cell suspension, cell concentration and the loading volume of the disruption vial. The influence of these parameters has been examined and it was found that an optimal disruption could be obtained after 30 minutes using a 2% cell suspension. These disruption parameters limited the applicability of the method for some experiments, when a large number of samples had to be handled in a short time and the sample volume had to be as small as possible. The necessity of using Benzonase to reduce the viscosity caused by nucleic acids is another disadvantage considering the enzyme price. The temperature increase is not significant, the highest temperature recorded at the end of disruption was 29°C and seemed to be primarily due to the environmental temperature, further increase was found to be in the range of 1°C per 5 minutes [6].

The ultrasonication cell disruption was adopted as a disruption method for routine analysis. As shown in figure 4, a complete release of protein could be obtained after 2 minutes and small only sample volumes are required. The acoustic waves output often reaches values of a few hundred watts. Because of the small volume of the fluid the temperature rises quickly making an intense cooling of the sample necessary. The problem was easily solved placing the sample into an ice bath during disruption. The disruption procedure is easy to perform and gives reliable and reproducible results (see table 2). The yield of released protein was found to be similar comparing the grinding and ultrasonication methods.

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