applied sciences

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Efficient Disruption of *Escherichia coli* for Plasmid DNA Recovery in a Bead Mill

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Received: 5 November 2017; Accepted: 22 December 2017; Published: 26 December 2017

Featured Application: In general, the alkaline lysis is more acceptable than the mechanical lysis for pDNA recovery due to the risk of product degradation when the latter is used. In this work, the experimental design allowed us to explore mechanical disruption conditions under which such an effect was minimized. The application of this technique is important for novel pDNA-vaccine process development, since bead milling is one of the most preferable mechanical cell lysis methods on an industrial scale due to its scalability, ease of operation, controllability, and ability to load concentrated cell slurry.

Abstract: The release kinetics of pDNA in a bead mill was studied. Samples taken during the process were analyzed to determine total pDNA (pDNA(t)) and supercoiled pDNA (pDNA(sc)) concentration. In order to identify important variables of the process and to develop an empirical model for optimal pDNA(t) and pDNA(sc) release, a two level 2^3 factorial design was used with variables: mill frequency, cell concentration, and bead size. The results were analyzed by response surface methodology. The optimized conditions for pDNA(t) yield 13.26 mg/g dcw (93.41% recovery), with a mill frequency of 30 Hz, a bead size of 0.10–0.25 mm, and a cell concentration of 20 g wcw/L. However, the optimized conditions for pDNA(sc) yield 7.65 mg/g dcw (92.05% recovery), with a mill frequency of 15 Hz, a bead size of 0.10–0.25 mm, and a cell concentration of 10 g wcw/L. Cell disruption in a bead mill was proved efficient for the release of pDNA(t) and pDNA(sc) compared to the alkaline treatment. The results obtained suggest a compromise between pDNA(sc) purity and recuperation in the process development.

Keywords: mechanical cell lysis; bead mill; plasmid DNA recovery; release kinetics of pDNA

1. Introduction

During the last decade, interest in the use of pDNA vaccines has strongly increased [1]. The pDNA vaccines are believed to be potentially useful in a wide range of applications including preventive and therapeutic vaccines for viral, bacterial, and parasitic diseases as well as cancer [2,3]. The production of pDNA follow the steps of fermentation, primary and intermediate recovery, and purification. After the fermentation step, a sequence of operations is followed to recover pDNA molecules from the host cells and to remove impurities and contaminants from the lysate, until the desired level of purity and other specifications are obtained [4]. Cell disruption is the first and the most critical step in the bioprocess since this step influences the yield and quality of the product [5,6]. The cell disruption method for

plasmid isolation must be chosen such that minimal damage is caused on the pDNA product, and in most cases, it is also desired to avoid shearing of the host cell genomic DNA (gDNA) into smaller fragments that are more difficult to separate from pDNA [7].

Typically, the disintegration of bacterial cells for pDNA production is performed by alkaline treatment described by Birnboim and Doly [8]. The method successfully removes most of the cell walls, organelles, proteins, and gDNA leaving RNA as the main contaminant of the target plasmid together with some proteins [9]. During this procedure, cells are subjected to an alkaline solution such as NaOH containing a detergent as SDS. Under this condition, cell wall structures are solubilized, thereby releasing the plasmid and other cell-related compounds, resulting in a highly viscous solution. While gDNA and proteins are denatured at the resulting pH-value, pDNA stays intact if homogeneous mixing is provided to avoid local pH-extremes that would lead to an irreversible denaturalization of the pDNA and the formation of undesired isoforms and pDNA loss trapped in flocs [7].

Several works aimed to improve the alkaline lysis method have been reported. Lezin et al. [10], developing a one-step approach for isolate pDNA, found that non-ionic detergent (NID) plasmid DNA performs better than alkaline treatment in many downstream applications. Bag et al. [11] developed an improved method for the extraction of community DNA from different environmental and human origin samples by a combination of physical, chemical, and mechanical lysis methods for proper lysis of microbial DNA. A novel approach to lyse cells on-chip through the application of electric discharges can be used in plasmid recovery [12]. The combined action of enzymes, detergents, and high temperatures constitutes an interesting alternative for the large-scale disruption of *E. coli* cells and plasmid release [13]. Although the method works well at the laboratory scale, the implementation of the high-temperature batch incubation at a process scale is clearly problematic [5]. The heat lysis is possibly the most commonly used method after alkaline lysis [14]. Recently, Thatcher et al. [15] described various methods and devices aimed at performing alkaline lysis at a large scale.

Unfortunately, the commonly used cell lysis methods (non-mechanical: chemical, physical, or enzymatic) prove efficient only at the laboratory scale [16,17] but fail to address the needs of large-scale cell disruption processes [18]. Mechanical methods, on the other hand, are promising as these can be scaled up with minimum or no pre-treatment/chemical additives and can be used in batch or continuous modes. Bead milling is one of the most preferable mechanical cell lysis methods at the industrial scale due to its ease of operation, controllability, and ability to load concentrated cell slurry [17,19]. This method has been a subject of continuing research in batch and continuous modes [19,20]. The common principle of a bead mill is that the cells are subjected to high stress produced by abrasion during rapid agitation with glass beads, resulting in the breaking of the membrane and cell wall, releasing all intracellular components [21].

Response surface methodology (RSM) is apt for optimization studies where the process variables interact with each other and need to be varied simultaneously to estimate the interaction effects between any two selected variables at one point in time [22]. Based on the experimental design, the results obtained are used to develop a best-fit mathematical equation, considering ANOVA. The equation is used further to validate the results at different levels of the process variables [18]. RSM has become the leading methodology for most laboratories and industries working on bioconversions of biological products [23,24].

To develop an economically viable downstream bioprocess, an easy-to-scale cellular disruption method must be studied to ensure low operating costs and high pDNA recovery. Here, we studied the influence of parameters such as frequency, cell concentration, and bead size on the disruption of *E. coli* to release pDNA in a small-scale batch-mode bead mill.

2. Materials and Methods

2.1. Fermentation

The *E. coli* DH5α hosting the plasmid pVAX1-NH36 (Center for Research and Advanced Studies of the National Polytechnic Institute, Mexico City, Mexico) was propagated in a fermentation TB enriched medium (glycerol 13 g/L, vitamins, yeast extract 24 g/L, tryptone 12 g/L, KH_2PO_4 2.31 g/L, K_2HPO_4 12.54 g/L). To prepare seed cultures of fermentation, vials of *E. coli* DH5α were inoculated in shake flasks containing 200 mL of TB-enriched medium with 50 µg/mL of kanamycin and grown overnight with vigorous shaking (350 rpm) at 37 °C. The cell biomass was harvested in a centrifuge Biofuge Stratos (Thermo Fisher Scientific, Waltham, MA, USA) at 14,300× *g* and 4 °C for 20 min.

2.2. Primary Recovery

2.2.1. Mechanical Lysis

The harvested biomass was resuspended to prepare cells suspensions of 10 and 20 wcw/L using the TE buffer (25 mM Tris-HCL and 10 mM EDTA, pH 8.0). A small-scale bead mill MM400 (Retsch, Haan, Germany) operating in batch mode was used with a 1:2 volume ratio of cell suspension and glass beads with two 35 mL steal chambers [25]. Glass beads of 0.10–0.25 mm and 0.25–0.50 mm diameter and mill frequencies of 15 and 30 Hz were used. During cell disruption, 300 µL samples were taken at 30 s intervals for 4 min to perform kinetic studies of total pDNA (pDNA(t)) and supercoiled pDNA (pDNA(sc)) release. The sample volume was replaced with TE buffer. Each experiment was conducted using two replicates. The lysate samples were clarified by centrifugation at 14,000× *g* and 4 °C for 20 min [25]. The final lysate obtained was vacuum filtered using a 0.45 µm filter to perform electrophoretic and chromatographic determinations.

2.2.2. Alkaline Treatment

As a control, cellular disruption was performed by alkaline treatment as reported by Diogo et al. [26]. Briefly, a sample of 1.0 g wcw was resuspended in 8 mL of 50 mM glucose, 25 mM Tris–HCl and 10 mM EDTA, pH 8.0. Then, an equal volume of a solution of 200 mM NaOH and 1% (*w*/*v*) sodium dodecyl sulfate solution was added. Cellular debris, gDNA, and proteins were precipitated by adding an equal volume of pre-chilled (on ice) 3 M potassium acetate, pH 5.0. The obtained lysate was clarified by centrifugation at 13,000× *g* for 30 min at 4 °C. The final lysate obtained was vacuum filtered using a 0.45 µm filter to perform electrophoretic and chromatographic determinations.

2.3. Sample Analysis

2.3.1. Agarose Gel Electrophoresis Analysis

Electrophoretic studies were performed to assess the plasmid integrity in all the samples taken. Samples were analyzed by horizontal electrophoresis in 0.8% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) in the presence of 0.5 µg/mL ethidium bromide. The molecular weight marker was a supercoiled DNA ladder from Sigma-Aldrich (Saint Louis, MO, USA) The gels were run at 75 V for 2 h and subsequently analyzed and photographed using the gel documentation software Multi–Doc, Digital Imaging System, Trans UV (BioRad, Hercules, CA, USA).

To estimate the percentage of the released pDNA(sc) in each experiment, ImageJ (freeware http://imagej.nih.gov/) was used for image analysis. This freeware measures the intensity of pDNA bands in electrophoresis images. Using "area" and "label peaks" commands, the percentage of the area of each band can be calculated. This technique has been employed to conduct image analysis in several studies [10,27–29].

2.3.2. High Performance Hydrophobic Interaction Chromatography

The pDNA(t) concentration was determined by HPLC-HIC as described previously [26]. Briefly, a 4.6 × 100 mm HIC Source 15 PHE PE column (phenyl ligand, polystyrene/divinylbenzene matrix) was connected to an Akta Purifier 10 UPC system (GE Healthcare, Chicago, IL, USA) and equilibrated with 1.5 M ammonium sulfate in 10 mM Tris–HCl, pH 8.0. Diluted samples 1:5 in an equilibration buffer of 30 µL were injected to the column. The column was then washed for 1.4 min with an equilibration buffer at 1 mL/min. At the end of this period, an isocratic elution was performed with 10 mM Tris–HCl buffer, pH 8.0, at 1 mL/min. This condition was maintained over the next 0.7 min to elute the bound species. After this period, the column was re-equilibrated with 1.5 M ammonium sulfate in 10 mM Tris-HCl, pH 8.0 for 5.5 min. The pDNA(t) was quantified using a calibration curve constructed with standards of the plasmid purified prepared in the 10–50 µg/mL concentration range. To calculate the concentration of pDNA(t) in each sample, the area of the corresponding blanks was subtracted from the total area of all peaks on the chromatogram. The HPLC purity degree of pDNA(t) was defined as the percentage of the plasmid to total area.

2.4. Response Surface Methodology

Response surface methodology (RSM) was employed with a factorial design to identify the important variables in the process of cell disruption in a bead mill using the release of pDNA(t) and pDNA(sc) as response variables. Mill frequency, cell concentration, and bead size parameters were used. An experimental design 2^3 at two levels (−1, 1) was used in order to build an empirical model based on an optimized output variable related to the process. The resulting 8 experimental runs are shown in Table 1.

The experimental responses were normalized using the maximum value obtained for each response variable and used as percentage to perform the analysis of variance ANOVA with the fd23 function in MATLAB® (MathWorks, Inc., Natick, MA, USA) for 2^3 experimental design [30], with two replicates using a significance value of $\alpha = 0.1$.

Table 1. Experimental design 2^3 used in the disruption of *E. coli* in a bead mill study.

Exp. No.	Treatment	(1) Mill Frequency	(2) Cell Concentration	(3) Bead Size
1	(1)	−	−	−
2	E_1	+	−	−
3	E_2	−	+	−
4	E_{12}	+	+	−
5	E_3	−	−	+
6	E_{13}	+	-	+
7	E_{23}	−	+	+
8	E_{123}	+	+	+
	Level (−)	15 Hz	10 g wcw/L	0.10–0.25 mm
	Level (+)	30 Hz	20 g wcw/L	0.25–0.50 mm

For a factorial design 2^3, it is assumed that each response can be characterized by a polynomial function of the following form:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \quad (1)$$

where y = the response variable of the process, x_n = the coded variable for each factor assuming values of −1 or 1, and b_0 = the average of the response variable.

Using

$$b_n = \frac{E}{2} = \frac{\frac{1}{N/2}\left[\sum Y_{(level+)} - \sum Y_{(level-)}\right]}{2} \quad (2)$$

where E = is the estimate of each treatment, N = the number of treatments, Y = is the experimental response,

$$y = Avg + \left(\frac{E_1}{2}\right)x_1 + \left(\frac{E_2}{2}\right)x_2 + \left(\frac{E_3}{2}\right)x_3 + \left(\frac{E_{12}}{2}\right)x_1x_2 + \left(\frac{E_{13}}{2}\right)x_1x_3 + \left(\frac{E_{23}}{2}\right)x_2x_3 + \left(\frac{E_{123}}{2}\right)x_1x_2x_3 \quad (3)$$

where y from Equation (3) is the pDNA(t) % or DNA(sc) % predicted for the bead mill operation.

2.5. Plasmid Release Kinetics

The release of pDNA(t) and pDNA(sc) was described with the first-order kinetics as is shown in Equation (4) [31].

$$V_M \frac{dR}{dt} = k\,(R_m - R)V_M \quad (4)$$

where V_M = is the volume of cellular suspension [mL], R_m = is the maximum concentration of pDNA(t) or pDNA(sc) [mg pDNA/gdcw], R = is the concentration of pDNA(t) or pDNA(sc) at time t [mg pDNA/g dcw], t = is the process time [min], and k = is the specific rate constant for the process [min^{-1}].

The integration of Equation (4) gives

$$R = R_m\left(1 - e^{-kt}\right). \quad (5)$$

The parameters of maximum concentration, R_m, and specific rate constant, k, of pDNA(t) and pDNA(sc), were determined from the experimental data using a MATLAB code with a function *nlinfit*.

3. Results and Discussion

3.1. Agarose Gel Electrophoresis Analysis

Figure 1 shows the electrophoretic analysis of the samples collected at various times from the bead mill of Experiment 1 (Figure 1A) and Experiment 8 (Figure 1B) in Lines 1 to 7. Line 7 is a sample from a nonstop bead mill lysate at the end of 4 min. Line 8 shows the electrophoretic analysis of a lysate sample obtained by alkaline treatment. All the experiments performed (Table 1) had a similar electrophoretic behavior. The first band in Lines 1 to 7 indicates the presence of gDNA, the second band correspond to the open circular isoform, and the third and denser band indicates the presence of pDNA(sc). These results suggest that, as the milling time increases, more pDNA(sc) is released and the quality of the processed solution remains constant. When comparing these results with those of Line 8 of the alkaline treatment, the difference in RNA content is notable. The ribosomes probably remain intact during the bead milling. This assumption is based on one of the methods for the isolation of 70S ribosomes, where the prokaryotic cell begins with cell disruption with a French press at high pressure (16,000 pounds per square inch (psi)). In this methodology, the 70S ribosomes are recovered intact from the crude lysate [32]. In the bead mill, the cells are subjected to high stress produced by abrasion during rapid agitation with glass beads [21]. While in the alkaline treatment, the ribosomes are denatured by adding the lysis solution, and the RNA molecules are released. It is important to notice that, while mechanical lysis does not eliminate genomic DNA (Figure 1), neither proteins nor endotoxins as alkaline lysis does, since there is no precipitation step. This may imply further filtration operations to separate these contaminants.

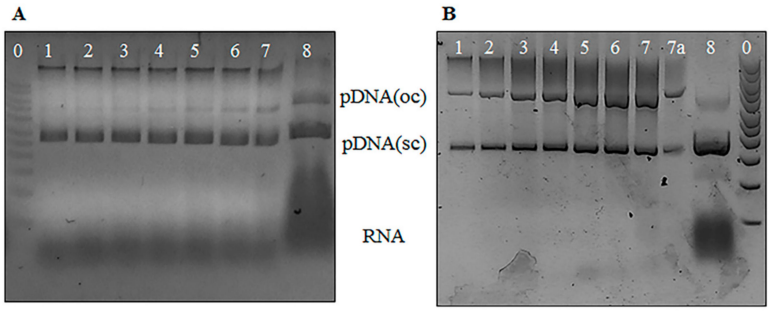

Figure 1. Electrophoretic analysis of samples obtained during *E. coli* disruption in a bead mill: (**A**) Experiment 1 and (**B**) Experiment 8. Line 0: DNA ladder; Line 1: 0.5 min; Line 2: 1.0 min; Line 3: 1.5 min; Line 4: 2.0 min; Line 5: 3.0 min; Line 6: 4.0 min; Line 7: nonstop lysate for 4 min; Line 8: clarificated alkaline lysate. Line 7a: experiment not reported. Open circular plasmid (oc), supercoiled plasmid (sc), and RNA.

In general, the alkaline lysis is more acceptable than mechanical lysis due to the risk of pDNA degradation using the last one. It was noticed that the experimental design allowed for the exploration of disruption conditions where this effect was minimized, by comparing the results of Experiments 1 and 8 presented in Figure 1.

3.2. High Performance Hydrophobic Interaction Chromatography

The HPLC-HIC analysis was conducted using the nonstop lysate sample after 4 min in the bead mill of Experiments 1 and 8 as well of an alkaline lysate sample of the same culture. The corresponding chromatograms are shown in Figure 2. For comparative purposes, the analytical chromatogram from the bead mill lysate was multiplied by 4 when a 10 g/L of cellular suspension was used and by 2 when a 20 g/L cellular suspension was used, since in the alkaline treatment (AT) a cell suspension of 40 g/L is used, in accordance with [26]. The area of the pDNA(t) peak at 0.7 min in Figure 2A corresponds to concentrations of 9.91 mg pDNA(t)/(g dcw) and 7.91 mg pDNA(t)/(g dcw) for the bead milling and alkaline treatment, respectively. The bead milling lysate obtained had a 4.75% pDNA(t) HPLC purity, while the alkaline lysate sample had a 2.25% HPLC pDNA(t) purity, like those found in several studies [33–35]. This effect is mainly due to the smaller area of the peak associated with the RNA at 3.23 min. In Figure 2B, the area of the pDNA(t) peak at 0.7 min corresponds to concentrations of 6.12 mg pDNA(t)/(g dcw) and 4.22 mg pDNA(t)/(g dcw) for the bead milling and alkaline treatment, respectively. The bead milling lysate obtained had an 11.83% pDNA(t) HPLC purity, while the alkaline lysate sample had a 2.25% pDNA(t) HPLC purity. As discussed above, this effect is mainly due to the smaller area of the peak associated with the RNA at 3.23 min.

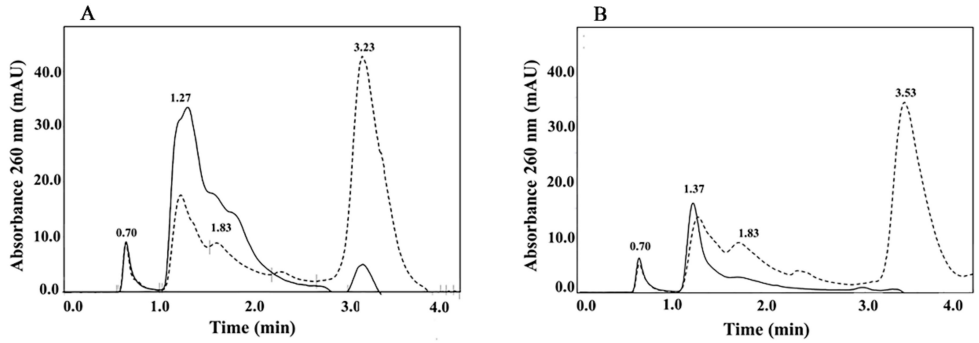

Figure 2. Analytical chromatograms (HPLC-HIC). (**A**) Sample of *E. coli* disruption by a bead mill (x4) in Experiment 1 (—) and cellular disruption by alkaline treatment (- - -). (**B**) Sample of cellular disruption by a bead mill (x2) in Experiment 8 (—) and cellular disruption by alkaline treatment (- - -).

3.3. RSM Analysis

Table 2 shows the average percentage of two replicates of the pDNA(t) and pDNA(sc) released in the bead mill used as response variables in the RSM analysis.

Table 2. Average percentage of the pDNA(t) and pDNA(sc) released in the bead mill.

Experiment	pDNA(t) %	pDNA(sc) %
1	66.06	100.00
2	71.87	67.30
3	80.56	79.52
4	100.00	74.93
5	44.26	38.94
6	61.37	45.90
7	40.20	48.94
8	42.80	35.20
Avg	63.39	61.34

Estimates from the pDNA(t) and pDNA(sc) percentages were obtained with Equation (2) using the data in Table 1. The ANOVA analysis was conducted with a significance value of $\alpha = 0.1$, considering the sample sizes [18]. Using the Matlab function *fd23*, it was concluded that the significant treatments for the pDNA(t) release were mill frequency, bead size, and the combination of cell concentration and bead size. In the release of pDNA(sc), the significant treatments were mill frequency, bead size, and the combination of the three variables used. With these results, the not-significant coefficients were removed from Equation (3) to obtain

$$y_{pDNA(t)} = 63.39 + 5.62x_1 - 16.24x_3 - 8.16x_2x_3 \quad (6)$$

$$y_{pDNA(sc)} = 61.34 - 5.51x_1 - 19.10x_3 - 6.10x_1x_2x_3. \quad (7)$$

Figure 3 shows the correlation between the model obtained by Equations (6) and (7) and the experimental data. The correlation between the predicted response and the experimental data for pDNA(t) and pDNA(sc) in the bead mill showed a good fit with an R^2 of 0.95 for both responses, indicating the accuracy of the model for predicting system behavior. When a significance value of $\alpha = 0.05$ was used, a good model fit was obtained only for pDNA(t).

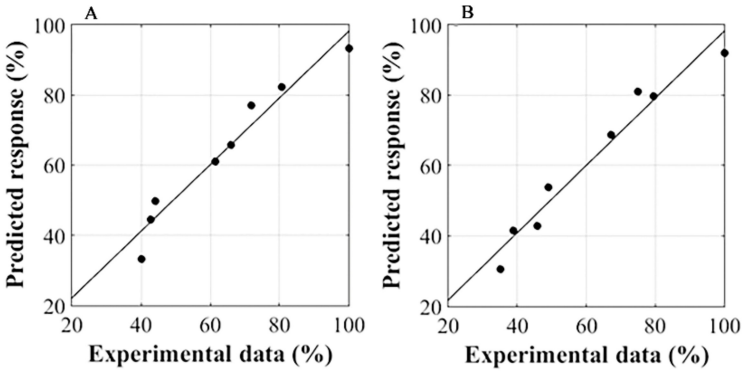

Figure 3. Correlation between experimental data and predicted response. Data represent the means of two experiments. (**A**) pDNA(t) and (**B**) pDNA(sc).

The RSM predicted responses for pDNA(t) and pDNA(sc), Equations (6) and (7), were plotted on three-dimensional graphs called "response surface plots" and are shown in Figure 4.

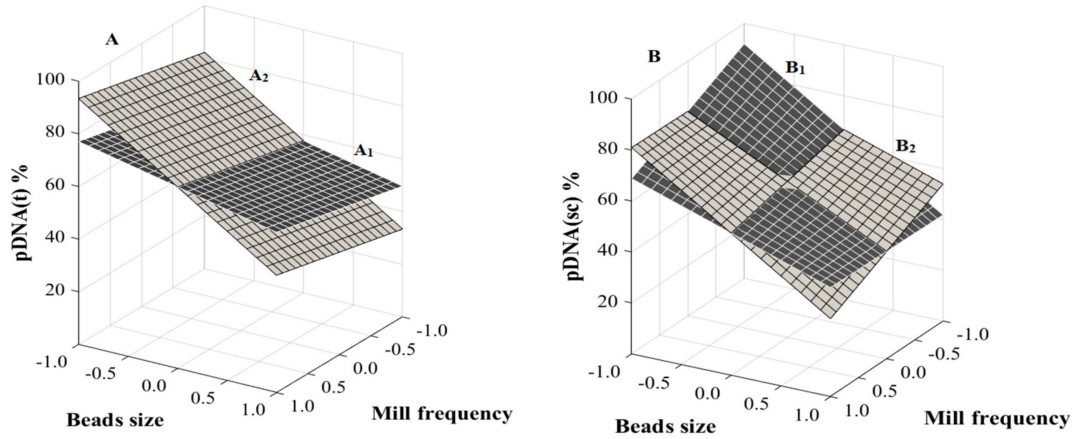

Figure 4. Response surface plots. (**A**) Effect of mill frequency and bead size in the release of pDNA(t). (A_1) Low cell concentration (10 g wcw/L) and (A_2) high cell concentration (20 g wcw/L). (**B**) Effect of mill frequency and bead size in the release of pDNA(sc). (B_1) Low cell concentration (10 g wcw/L) and (B_2) high cell concentration (20 g wcw/L).

Figure 4A shows that, in the experimental range studied, the pDNA(t) release is more sensible to bead mill parameters at the higher cell concentration of 20 g/L (Surface A_2). It can also be observed that, by using a high mill frequency (30 Hz) and the smallest bead size (0.10–0.25 mm), a maximum release of pDNA(t) of 77.10% for a cell concentration of 10 g wcw/L (Surface A_1) and 93.41% of maximum release for a cell concentration of 20 g wcw/L (Surface A_2) are obtained. Carlson et al. [36] also found that bead milling is the best processing method for intact plasmid recovery, in which over 90% of the plasmid is solubilized without substantial degradation. However, the target molecule is the pDNA(sc) and the corresponding behavior is subsequent discussed.

In general, Figure 4B indicates that, at low cell concentration, the milling process to obtain pDNA(sc) improves as bead size and mill frequency decrease. For high cell concentration, the milling process to obtain pDNA(sc) improves as bead size decreases and mill frequency increases. It can also be observed that, by using a low mill frequency (15 Hz) and the smallest bead size (0.10–0.25 mm), a maximum release of pDNA(sc) of 92.05% is obtained for a cell concentration of 10 g wcw/L (Surface B_1); for a cell concentration of 20 g wcw/L (Surface B_2), using high mill frequency (30 Hz) and small bead size (0.10–0.25 mm), a maximum release of pDNA(sc) of 81.03% is obtained. Further discussion of this results is presented in combination with the kinetic studies.

3.4. Plasmid Release Kinetics

Figure 5 shows the experimental data of the release of pDNA(t) at various times of bead milling, using mill frequencies of 15 Hz and 30 Hz. The continuous line in the figures represents the best fit of the model given in Equation (5). The kinetic parameters R_m and k were determined using the experimental kinetic data and Equation (5) using a MATLAB code with a *nlinfit* function. The corresponding values were an R_{mt}-value of 14.16 mg pDNA(t)/g dcw and a k-value varied from 0.14 to 1.07 min^{-1} (Figure 5).

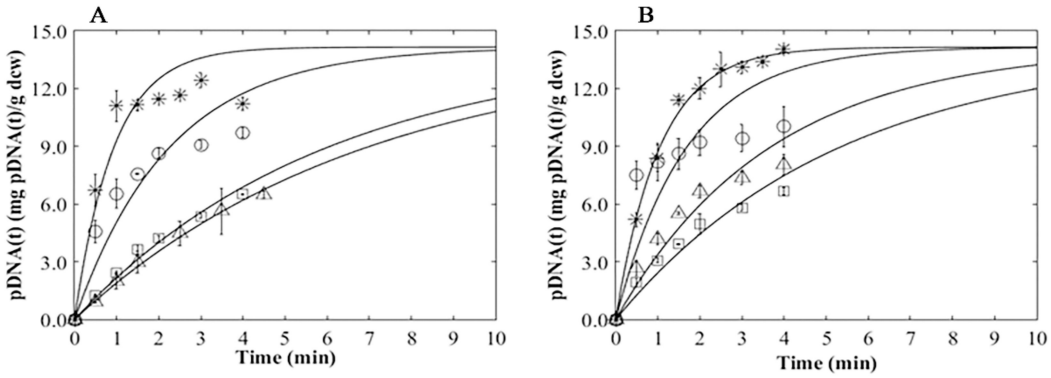

Figure 5. Kinetics of pDNA(t) release. (**A**) Mill frequency of 15 Hz. (o) Experiment 1: k = 0.44 min^{-1}; (*) Experiment 3: k = 1.07 min^{-1}; (Δ) Experiment 5: k = 0.14 min^{-1}; (□) Experiment 7: k = 0.17 min^{-1}; (—) model fit. (**B**) Mill frequency of 30 Hz. (o) Experiment 2: k = 0.59 min^{-1}; (*) Experiment 4: k = 0.96 min^{-1}; (Δ) Experiment 6: k = 0.27 min^{-1}; (□) Experiment 8: k = 0.19 min^{-1}; (—) model fit. Data represent the means of two replicates and error bars represent the standard deviation.

It can be observed in Figure 5A that the model provided a good fit to the release of pDNA(t) data obtained in the bead mill in Experiments 1, 3, 5, and 7 with R^2-values of 0.82, 0.87, 0.99, and 0.98, respectively. A faster kinetics of the release of pDNA(t) was obtained with high cell concentration and smaller beads. This could be due to the high probability of the collision between the beads against the cells. The opposite effect occurs when using a low cell concentration and large beads. This is probably because a greater bead creates greater void spaces, resulting in a reduced number of collisions against the cells.

Figure 5b shows that the model given in Equation (5) provided a good fit to the release of pDNA(t) data obtained in the bead mill in Experiments 4, 6, and 8 (R^2-values of 0.99, 0.90, and 0.94, respectively) except for Experiment 2 (R^2 = 0.54). Considering the results of the analysis of Experiments 1 and 2, a poorer model fit is obtained with low cell concentration and smaller beads, suggesting that, under these experimental conditions, the probability of disruption decreases more rapidly than a first-order kinetics as the process takes place. A faster kinetic of the release of pDNA(t) at 30 Hz (Figure 5B) can be achieved using a high cell concentration and small beads analogous to the results at 15 Hz (Figure 5A). On the contrary, slower kinetics is obtained using a high cell concentration and large beads. This behavior can be explained as discussed by Haque et al. [18]: large beads create larger void spaces in the milling chamber, resulting in a reduced number of collisions against the cells. Moreover, given a high cell concentration, the release of intracellular components increases the viscosity of the cell solution, so the impaction between beads is reduced, which results in an incomplete lysis.

Figure 6 shows the experimental data of the release of pDNA(sc) at various times of bead milling, using a mill frequency of 15 Hz. The continuous line in the figure represents the best fit of the model given in Equation (5). The corresponding fitted values were an R_{msc1}-value of 8.98 mg pDNA(sc)/g dcw and a k-value varied from 0.14 to 1.24 min^{-1} (Figure 6). It can be observed that the model provided a good fit of the release of pDNA(sc) data obtained in the bead mill in Experiments 1, 3, 5, and 7, with R^2 values of 0.99, 0.78, 0.88, and 0.99, respectively. One atypical behavior was obtained in Experiment 3 and requires deeper research. Faster kinetics of the release of pDNA(sc) at 15 Hz can be achieved using a low cell concentration and small beads. Haque et al. [37] reported that smaller beads are effective at lower viscosities, but these become fluidized when run at higher cell loads, at higher viscosities, or at a high agitation frequency, which in turn results in a lower momentum of impaction and incomplete cell lysis, and negatively affects the process productivity.

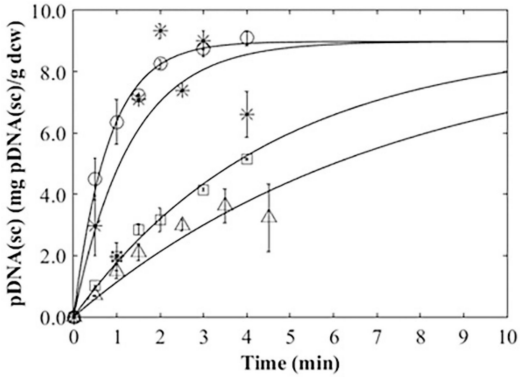

Figure 6. Kinetics of pDNA(sc) release with a mill frequency of 15 Hz. (o) Experiment 1: k = 1.24 min^{-1}; (*) Experiment 3: k = 0.76 min^{-1}; (Δ) Experiment 5: k = 0.14 min^{-1}; (□) Experiment 7: k = 0.22 min^{-1}; (—) model fit. Data represent the means of two replicates and error bars represent the standard deviation.

Figure 7 shows the experimental data of the release of pDNA(sc) at various times of bead milling, using a mill frequency of 30 Hz. The continuous line represents the best fit of the model given in Equation (5) in each figure, with R^2-values of 0.95, 0.84, 0.99, and 0.90 for Experiments 2, 4, 6, and 8, respectively. Faster kinetics of the release of pDNA(sc) can be achieved using low cell concentrations because the effectiveness of collisions between beads and the cells is not limited by the viscosity of the cell solution.

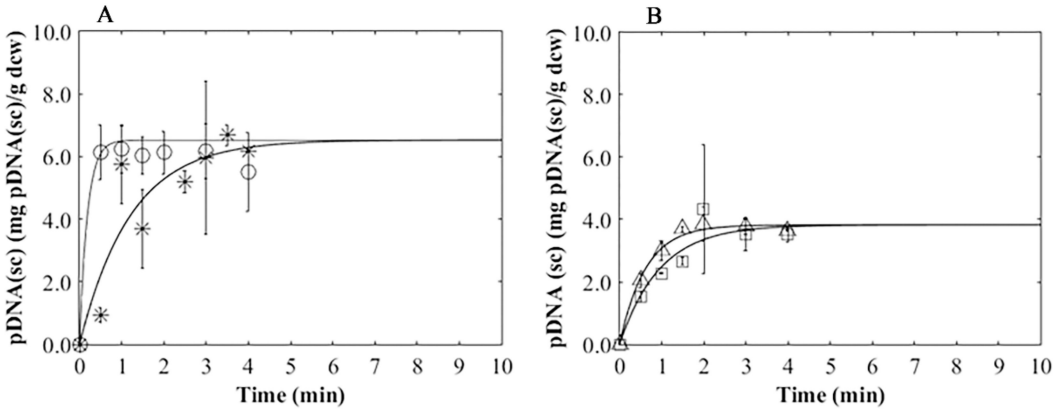

Figure 7. Kinetics of pDNA(sc) release with a mill frequency of 30 Hz. (**A**) (o) Experiment 2: k = 5.40 min^{-1}; (*) Experiment 4: k = 0.82 min^{-1}. (**B**) (Δ) Experiment 6: k = 1.62 min^{-1}; (□) Experiment 8: k = 1.04 min^{-1}; (—) model fit. Data represent the means of two replicates and error bars represent the standard deviation.

The maximum pDNA(sc) obtained for Experiments 2 and 4 was an R_{msc2}-value of 6.53 mg pDNA(sc)/g dcw (Figure 7A), whereas for Experiments 6 and 8 the maximum was an R_{msc3}-value of 3.83 mg pDNA(sc)/g dcw (Figure 7B). The corresponding k-values are shown in the legend of Figure 7. At a high mill frequency, the release kinetics becomes faster causing a faster increase of the viscosity of the solution [38] and affecting maximum concentration of pDNA(sc) obtained, due to greater pDNA denaturalization. This effect is more pronounced when larger beads are used (Figure 7B). The R_{mt}-value of 14.16 mg/g dcw suggests that all the plasmid content may be released if proper conditions are used [39]. However, the R_{msc1}-value of 8.98 mg/g dcw obtained at a low mill frequency represents the maximum concentration attainable and a lower pDNA(sc) purity. Since a higher purity and lower recuperation is obtained in shorter time periods, these results imply a compromise between pDNA(sc)

purity and recuperation in process development. At a high mill frequency with an R_{msc2}-value of 6.53 mg/g dcw and an R_{msc3}-value of 3.83 mg/g dcw, this behavior is more evident.

4. Conclusions

The RSM and release kinetics of plasmid pVAX1-NH36 by disruption of *E. coli* cells in a bead mill was studied for the very first time. The electrophoresis analysis of the *E. coli* lysates from all the experiments was used to obtain the pDNA(sc) percentage in each sample. These percentages along with the HPLC-HIC chromatograms determinations of pDNA(t) allowed us to obtain the pDNA(sc) concentration using ImageJ. The obtained data was used to perform RSM and kinetic pDNA release analysis. RSM proved to be successful for the prediction and modeling of the release of pDNA(t) and pDNA(sc). The significant treatments for pDNA(t) release were mill frequency, bead size, and the combination of cell concentration and bead size. For pDNA(sc), the significant treatments were mill frequency, bead size, and the combination of the three parameters studied in this research. Although ANOVA for RSM demonstrated that cellular concentration per se is not a significant parameter in this operation, as it was also concluded by other authors in the disruption of *E. coli* cells [40] and yeast cells [20], it was found in this work that it is a crucial parameter for effective cell lysis. Since the best results of the release of pDNA(sc) were obtained with a small bead size and low cell concentrations, in order to optimize the disruption of *E. coli* for the release of pDNA(sc), we also suggest the use of a denser material with higher specific gravity than glass, e.g., zirconium beads, as reported by Haque et al. [18], to increase cellular concentration and productivity. The description of pDNA(t) release by a first-order kinetic gave good fit to the data obtained in most of the experiments performed with the bead mill. The description of the pDNA(sc) release process by a first-order kinetic gave good fit to the data only at low mill frequencies. The maximum pDNA(sc) concentration values obtained in the experiments suggest a compromise between pDNA(sc) purity and plasmid recuperation in process development.

Acknowledgments: This research was supported by a grant provided by the National Council of Science and Technology (CONACYT) under the project CB2016-257411. We also appreciate the support given by the Strengthening Program Quality in Educative Institutions (PROFOCIE) 2017 and the University of Sonora. The authors declare no conflict of interest exists.

Author Contributions: Adriana Padilla-Zamudio performed all experiments and contributes in the manuscript writing. J. Armando Lucero-Acuña and Jaime Ortega-López contributed reagents/materials/analysis tools and discussion for all experiments. Patricia Guerrero-Germán and Armando Tejeda-Mansir conceived and designed the experiments and contributed to manuscript writing.

Conflicts of Interest: The authors declare no conflict of interest.

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