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Scaling *E. coli* disruption for a continuous mill design for plasmid recovery

Escalamiento de la ruptura de *E. coli* para el diseño de un molino continuo para la recuperación de plásmido

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Abstract

The production of plasmid DNA (pDNA) is fundamental for creating DNA and RNA vaccines, which have proven to be a novel and effective alternative to traditional vaccines. The pDNA production process includes the stages of fermentation, primary recovery, intermediate recovery, and purification. Primary recovery involves cell harvest and rupture, which is a critical step in the bioprocess. In this research, a bead milling operation was employed as an alternative to the conventional alkaline lysis technique to enhance the efficiency of the primary recovery stage. Batch kinetic studies were performed with milling chambers with *L/D* of 0.51, 0.93 and 1.67. By fitting a kinetic model to the experimental data, the specific kinetic constants of 0.46, 0.44 and 0.21 min^{-1}, respectively, were determined. Electrophoretic analysis shows that more than 60% of the supercoiled plasmid was recovered in less than 5 min, with no RNA content. The results of the milling kinetics in batch mode were used to analyze the behavior of a continuous mill using the Perfectly Agitated Tanks in series model. The results indicate that the highest overall efficiency is achieved with a chamber having an *L/D* ratio of 0.51 in 4 stages.

Keywords: mechanical lysis, plasmid, *E. coli*, bead milling.

Resumen

La producción de ADN plasmídico (ADNp) es fundamental para la fabricación de vacunas de ADN y ARN, las cuales han demostrado ser una alternativa novedosa y efectiva frente a las vacunas tradicionales. El proceso de producción de ADNp incluye las etapas: fermentación, recuperación primaria, recuperación intermedia y purificación. La recuperación primaria consiste en la separación y ruptura de las células, el cual es un paso crítico del bioproceso. En esta investigación se empleó una molienda con perlas como alternativa a la técnica convencional de lisis alcalina. Se realizaron estudios cinéticos en lote con cámaras de molienda con relaciones *L/D* de 0.51, 0.93 y 1.67. Mediante el ajuste de un modelo cinético a los datos experimentales, se determinaron las constantes cinéticas específicas, que fueron 0.46, 0.44 y 0.21 min^{-1}, respectivamente. El análisis electroforético muestra que se logró recuperar más del 60% del plásmido superenrollado en menos de 5 min, sin presecia de ARN. Los resultados de la cinética de molienda se utilizaron para analizar el comportamiento de un molino continuo, empleando el modelo de Tanques Perfectamente Agitados en Serie. Los resultados muestran que la mayor eficiencia global se logra con la cámara con una relación *L/D* de 0.51 en 4 etapas.

Palabras clave: lisis mecánica, plásmido, *E. coli*, bead milling.

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1 Introduction

Vaccination is the most effective and least expensive technique for disease prevention and eradication (Juárez *et al*., 2021). However, recent outbreaks have led scientists to develop new types of vaccines, such as third-generation vaccines based on plasmid DNA (pDNA). These vaccines offer clear advantages over other technologies in terms of safety, easy manufacture, and stability (Abdulrahman & Ghanem, 2018).

Plasmids play a crucial role in the biotechnology industry, with their application increasing in recent years due to their potential in gene therapy and vaccine development (Jaén *et al*., 2021). Therefore, meeting the demand for GMP-grade plasmid production has proven challenging (Ohlson, 2020). Designing a sustainable bioprocess for the recovery and purification of pDNA with efficient unit operations is a significant challenge. Bioprocesses for pDNA recovery are divided into upstream and downstream operations. Upstream operations include inoculum preparation, sterilization, and culture medium preparation for fermentation. Downstream operations involve recovery, concentration, purification, and finishing (Tejeda Mansir *et al*., 2011). Specific challenges in pDNA recovery and isolation arise due to its unique shape and conformation. Generally, the bioprocess design for obtaining pDNA relies on heuristic rules. After cell harvest, cell disruption is a critical step, as it releases the intracellular content. The method used for cell rupture significantly impacts the quantity and quality of the desired product (Prazeres, 2011). There are various methods for cell rupture, which can be categorized into mechanical and non-mechanical techniques. Mechanical methods include bead milling, homogenization, and the French press, while non-mechanical include alkaline treatment, osmotic shock, lipid dissolution and enzymatic digestion, which allows selective release of intracellular contents (Tejeda Mansir *et al*., 2011). Not all *E. coli* rupture methods are suitable for industrial-scale applications. To be used at this scale, methods must meet the following criteria: high rate of disintegration, low cost of operation and maintenance, and preservation of the desired product's integrity (Aires-Barros & Azevedo, 2017). Some methods are not easily scalable, and regulatory agencies like the the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) do not recommend the use of solvents, toxic chemicals, or animal-derived enzymes for clinical use (Roush & Lu, 2008).

Alkaline lysis has been a widely used method for pDNA extraction, however, it involves chemical agents that generate difficult-to-dispose residues (e.g., precipitates of chemical agents with proteins, genomic DNA, cellular remains). Studies have reported the use of different cell rupture methods to obtain the most efficient technique for the recovery of pDNA, nevertheless, more research is required to determine a cost-effective and scalable method.

Bead milling has proven to be an efficient technique for *E. coli* rupture to achieve high recovery of high-quality plasmid DNA (Padilla-Zamudio *et al*., 2018). Cell rupture in bead milling is based on the high stress generated by the collision of the beads with the biomass, causing high shear stress that ruptures cell membranes and releases intracellular contents. Various bead mill designs and sizes exist, operating in either batch or continuous modes, with horizontal or vertical chamber (Delran *et al*., 2024). The size of the cell fragments produced significantly impacts subsequent processing (Carlson *et al*., 1995).

Batch-operated bead mills are used for low-volume solution or to generate experimental data. The milling chamber is loaded with beads and cell suspension, and cell rupture follows first-order kinetics. In continuously operated bead mills, cell suspension is feed into the chamber at one end, where an agitator with mounted discs, rods, or rings transfers kinetic energy to the beads. The lysate is continuously removed from the opposite end. These mills are used on an industrial scale and require a cooling jacket due to the high frequency of collision. Generally, they require little maintenance and achieve high milling efficiency (Tejeda Mansir *et al*., 2011).

2 Materials and methods

2.1 *Host strain and plasmid*

The *E. coli* DH5α strain hosting pVAX1-NH36 (4.0 kbp) used in this work was obtained form CINVESTAV-IPN (Mexico City, Mexico) (Sanchez-Casco *et al*., 2013).

2.2 *Culture condition*

E. coli DH5α cultures hosting pVAX1-NH36 were grown in TB medium (glycerol 13 g/L; vitamins: yeast extract 24 g/L, tryptone 12 g/L; salts: KH_2PO_4 2.31 g/L, K_2HPO_4 12.54 g/L) in a shake flask containing 250 mL of medium, supplemented with kanamycin at 50 μg/mL. culture was incubated for 12 hours at 37°C and 350 rpm. The growth of *E. coli* is monitored by measuring the optical density at 600 nm (OD_{600}) of appropriately diluted samples taken at each time interval. The cells were harvested at the end of the exponential phase of growth. Biomass was recovered by centrifugation at 3500 g at 4°C for 15 min. The harvested biomass was then resuspended in TE1 buffer

Table 1. Experimental L/D.

Chamber vol. V_M (mL)	Inner diameter D (cm)	Operation vol. V_O (mL)	Inner chamber area AI (cm^2)	Operation height L (cm)	L/D
10	1.9	9.00	2.84	3.17	1.67
35	3.2	24.00	8.04	2.98	0.93
50	4.3	32.00	14.52	2.20	0.51

(Tris-HCl 25 mM, EDTA 10 mM, pH=8) for cell rupture in a bead mill and TE2 buffer (Tris-HCl 25 mM, EDTA 10 mM, glucose 50 mM, pH=8) to carry out the alkaline treatment.

2.3 Cell lysis

2.3.1 Alkaline lysis

The cells are resuspended in TE2 buffer to a concentration of 8 mL per gram of wet cells and mixed using a vortex until a homogeneous mixture is obtained. Lysis solution composed of 0.2 mM NaOH and 1% SDS (8 mL per gram of wet cells) is added to the resuspended cells and gently mixed until the mixture becomes viscous, then incubated for 10 minutes. Subsequently, cellular debris precipitates when the neutralization solution (3M CH_3CO_2K and glacial acetic acid, 8 mL per gram of wet cells) is added. The tube is gently shaken and allowed to cool for 10 minutes. Once the cells are lysed and release their intracellular contents, the cellular debris is removed by centrifugation at 13,000 g, for 30 minutes and at 4 °C. The liquid phase is recovered, and the cellular debris is discarded. The centrifugation operation is repeated under the same conditions. To remove smaller particles, the liquid is filtered through a 0.45 μm microfilter.

2.3.2 Mechanical lysis kinetics

The lysis was carried out in a RETSCH MM400 mill at a frequency of 30 Hz, using a cell suspension of 20 g wet cells/L and a proportion of 0.5 mL beads/ (1.0 mL suspension), at a room temperature. These condition where used in all experiments. The kinetics of pDNA release were studied in 2 mL eppendorf tubes placed in adapters in the mill, with each tube containing zirconium beads (200-400 μm) and cell suspension. Tubes were removed from the mill at various times within a range of 0 to 11 min. The lysate obtained from the milling was clarified by centrifugation at 13,000 g and 4°C for 20 min and analyze pDNA concentration by hydrophobic interaction chromatography (HIC) and pDNA quality by electrophoretic analysis.

2.3.3 Mechanical lysis in scale up milling

To determine mixing and rupture degree for Perfectly Stirred Tanks in Series, 10, 35 and 50 mL milling chambers (free volume of the mill V_M) were used with 200-400 μm diameter zirconium beads and a cell concentration of 20 g/L at a bead-to-cell suspension ratio of 0.5 mL beads/ (1 mL suspension), and a mill frequency of 30 Hz. The lysate obtained from the milling was clarified by centrifugation.

L/D was calculated for each milling chamber (V_M) to relate this parameter to the specific rate constant (k) (Table 1). The internal diameter (D) corresponds to that of the milling chamber. The operation volume (V_o) represents the volume of beads plus cell suspension. Operation height (L) represents the operation volume (V_o) divided by the inner chamber area (AI), and *L/D* the operation height/ (inner diameter).

2.4 Sample analysis

2.4.1 Plasmid concentration

According to Diogo *et al*. 2003, samples were analyzed using a 0.46×10 cm hydrophobic interaction chromatography column (HIC Source 15 PHE) (Diogo *et al*., 2003). The Äkta Purifier system was connected to the column and equilibrated with adsorption buffer (1.5 M ammonium sulphate $(NH_4)_2SO_4$, 10mM Tris-HCl, pH=8). An autosampler injected 30 μL of sample (diluted in adsorption buffer at 1:5 ratio). After sample injection, the column was washed for 1.4 min with the adsorption buffer with a volumetric flow rate of 1 mL/min. Afterward, isocratic elution was carried out with 10 mM Tris–HCl pH= 8, at 1 mL/min for 0.7 min to elute bound species. After elution, the column was re-equilibrated with the adsorption buffer for 5.5 minutes to continue analyzing samples. The first peak (at 0.68 minutes) corresponds to the pDNA species not adsorbed on the column (pDNA and its isoforms), while bound impurities were eluted as a series of peaks with retention times between 1.0 and 4.5 min. The pDNA was quantified by the eluted peak area at 0.68 min, using a calibration curve generated with purified plasmid standards, prepared in the concentration range of 10-60 μg/mL.

2.4.2 Agarose gel electrophoresis

To assess plasmid integrity in samples, electrophoretic studies were conducted using a 0.8% agarose gel in TAE buffer (Tris 40 mM, acetic acid 20 mM, EDTA 1 mM, pH 8.0). A DNA Ladder 1 kb by Sigma-Aldrich was used as a molecular size marker. The gel was run at 60 V for 110 min.

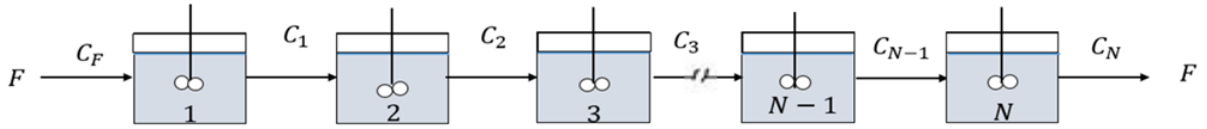

Figure 1. Conceptual rendering of a bead mill with the model of Perfectly Stirred Tanks in Series. Where F = feed flow to the mill [L/min]; N = number of stages in the series; C_N = tracer concentration at stage N [μg/mL]. Adapted from (Tejeda Mansir *et al*., 2011).

Subsequently, it was stained with ethidium bromide (0.5 μg/mL) for 30 min, followed by immersion in deionized water. The gel was then analyzed and photographed using the Multi-Doc software on the Digital Imaging System, BioRad's Trans UV photodocumenter.

2.5 *Theoretical framework*

2.5.1 *Biomass growth model*

The growth curve of *E. coli* was described by logistic equation. This equation characterize growth in terms in carrying capacity. The usual approach is based on a formulation in which the specific growth rate is related to the amount of unused carrying capacity (Eq. 1).

$$\mu_g = c\left(1 - \frac{X}{X_\infty}\right) \tag{1}$$

Thus:

$$\frac{dX}{dt} = cX\left(1 - \frac{X}{X_\infty}\right) \tag{2}$$

The integration of Eq. (2) with the initial condition $X(0) = X_0$ yields the logistic equation, Eq. (3) (Estrada-Martínez *et al*., 2023; Selvaraj & Vytla, 2017; Shuler & Kargi, 2017) where: X = cell mass concentration [OD_{600}]; X_0 = initial mass concentration; X_∞ = maximum concentration; μ_g = specific growth rate; c = load capacity coefficient. The logistic model was fitted to the experimental data of biomass growth curve. The parameters c and X_∞ were determined using the function *nlinfit* of MATLAB. The specific growth rate μ_g was determined using Eq. (1) (Shuler & Kargi, 2017).

$$X = \frac{X_0 e^{ct}}{1 - \frac{X_0}{X_\infty}(1 - e^{ct})} \tag{3}$$

2.5.2 *Bead mill model*

The Perfectly Stirred Tanks in Series model (Figure 1) is employed to simulate deviations of plug flow in commercial bead mills. Pulse tracer experiments help determine the equivalent number of Perfectly Stirred Tanks in Series for the degree of mixing in a continuous mill (Tejeda Mansir *et al*., 2011). Plasmid recovery is feasible using mechanical methods according to various studies. For this research, the scaling *E. coli* disruption will be investigated for a continuous mill design, recovering pDNA using zirconium beads due to their hardness and toughness properties.

Cell rupture in batch-operated bead mills follows first-order kinetics, and the mass balance of released plasmid can be expressed as shown by Eq. (4).

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

where: V_M = free volume of the mill [L]; R = concentration of plasmid released in time t [μg pDNA/ (g wet cell)]; R_m = maximum obtainable plasmid concentration [μg pDNA/ (g wet cell)]; k = first-order specific rate constant [min^{-1}]; t = operating time [min]. The integration of Eq. (4) with the initial condition $R(0) = 0$ gives:

$$R = R_m(1 - e^{-kt}) \tag{5}$$

The kinetics were studied by placing a set of eppendorf tubes in the batch-operated bead mill, with one tube being removed at each time point t to measure the concentration of released pDNA. Using the concentration data as a function of time, the parameters k and R_m of Eq. (5) were calculated using the *nlinfit* function in MATLAB.

The results of the milling kinetics were used to analyze the behavior of a continuous mill using the Perfectly Agitated Tanks in series model. In the continuously operated bead mill the free volume of each stage (V_R = reaction volume corresponds to the volume of cell suspension inside the milling chamber), the stage number ($N = V_{MC}/V_R$) (where V_{MC} the free volume of the continuous mill and t_R residence time of the continuous mill, data taken from literature (Günerken *et al*., 2017)), the residence time of the stage ($t_{Re} = t_R/N$) were calculated. Milling was conducted for each volume of reaction and residence time. Subsequently, the concentration of plasmid (R) released during the operation time was determined.

The maximum pDNA concentration (R_m) was determined from the kinetics of pDNA release (Eq. 5). The specific rate constant (k) was then calculated based on mechanical lysis performed in milling chambers of 10, 35, and 50 mL (corresponding to the free volume of the total chamber, V_M). The value of

k was obtained from the slope of the linear plot of $\ln[R_m/(R_m - R)]$ versus time (t).

Thus, the parameter $R_m/(R_m - R)$ of the batch process is calculated for each milling chamber rearranging Eq. (5) into Eq. (6). This equation allows to assess how effectively the plasmid is released relative to its maximum obtainable concentration.

$$\frac{R_m}{R_m - R} = e^{kt_{Re}} \tag{6}$$

By defining the bead mill factor $Q = kt_{Re}/N$, the plasmid released for N stage is given by (Eq. 7):

$$\frac{R_m}{R_m - R} = (1 + Q)^N \tag{7}$$

Finally, batch and continuous $R_m/(R_m - R)$ values were obtained using the number of perfectly stirred tanks in series equivalent to the mixing degree of a bead mill. The milling efficiency was computed as R/R_m.

3 Results and discussions

3.1 Cells growth kinetics

Figure 2 displays the average values of OD_{600} experimental data from the growth curve, demonstrating a strong fit (r^2 = 0.99) to the logistic equation. Using the *nlinfit* function of MATLAB, the carrying-capacity coefficient c = 0.72 h^{-1} and the maximum OD_{600}, X_∞ = 14 OD were calculated using Eq. (3). Furthermore, Eq. (1) allowed to calculate the specific growth rate (μ_g). During the adaptation phase, the specific rate remains constant before gradually varying until it reaches a minimum.

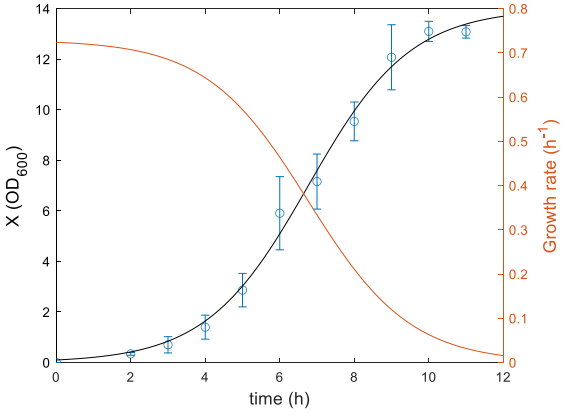

Figure 2. Growth curve of *E. coli* (in left), (o) average value with standard deviation bar, (-) logistic model. Growth rate (in right).

3.2 Plasmid release kinetics

Plasmid release kinetics in Eppendorf tubes through the milling process are shown in Figure 3.

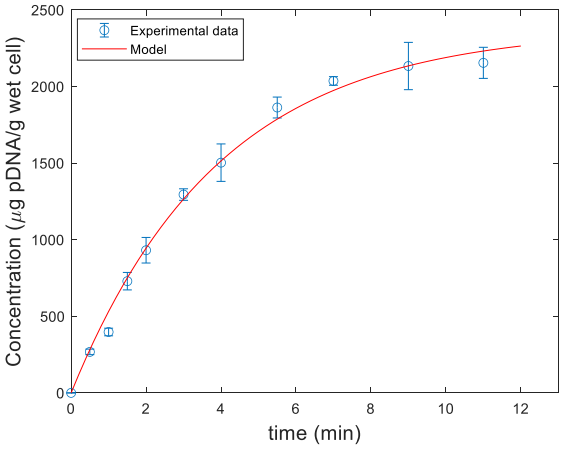

Figure 3. Kinetics of pDNA released in eppendorf tubes using zirconium beads, a cell concentration of 20 g/L at a grinding frequency of 30 Hz. The solid line represents the nonlinear fit (using the MATLAB *nlinfit* function) of Eq. (5) to the average experimental data.

Eq. (5) was adjusted to the experimental data from runs that were made quadruplicate (average concentration of plasmids released at each time) for a cell suspension of 20 g/L and a mill frequency 30 Hz by using *nlinfit* the MATLAB, and the following parameters were calculated: a maximum concentration R_m = 2,378 μg pDNA/ (g wet cell), and the first-order specific rate constant k = 0.253 min^{-1}. The model exhibits a good fit to experimental data (r^2 = 0.99). Another study (Padilla-Zamudio *et al.*, 2018) reported a specific rate constant of 0.19 min^{-1}, where the specific rate constant is influenced by bead size. Slow kinetics can be achieved by using a high cell concentration (20 g/L) and larger diameter beads, a behavior like that mentioned by Haque *et al.* (Haque *et al.*, 2016). The plasmid concentration obtained through alkaline lysis was 499.50 μg pDNA/ (g wet cell).

3.3 Electrophoretic analysis

Figure 4 displays the electrophoretic analysis of clarified lysates obtained during kinetics and alkaline lysate procedures. Lanes 1 and 13 show the DNA Ladder, while lanes 2 to 11 contain samples from mechanical lysate taken at different times. The intensity of bands increases with milling operation time, indicating the presence of plasmid DNA (supercoiled and open circular forms), with the latter in a lower proportion. Additionally, the release of genomic DNA (gDNA) can be observed. Lane 12 shows an alkaline lysate sample, where genomic DNA is absent due to molecule denaturation and precipitation, while pDNA and RNA are present. RNA was likely not released during milling with the experimental agitation rate used, a result consistent with the findings of (Padilla-Zamudio *et al.*, 2018). Experimental duplicates are shown in lanes 14 to 20 for comparison.

Table 2. Calculation of perfectly agitated tanks in series.

V_M (mL)	V_R (mL)	V_{MC} (mL)	N	t_{Re} (min)	L/D	k (min^{-1})	Overall Eff[a].
10	6	500	8	0.6	1.67	0.21	63
35	16	500	3	1.6	0.93	0.44	81
50	21	500	2	2.1	0.51	0.46	80

[a] Efficiency of the continuous system calculated using Eq. (7).

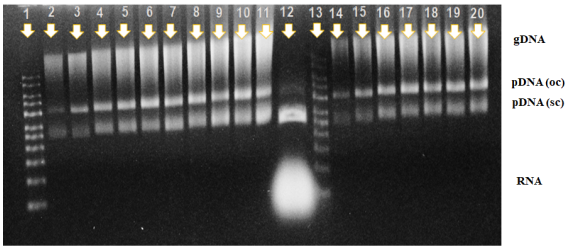

Figure 4. Electrophoretic analysis of milling. Lane 1) DNA Ladder. Lane 2) 0.5min. Lane 3) 1 min. Lane 4) 1.5 min. Lane 5) 2 min. Lane 6) 3 min. Lane 7) 4 min. Lane 8) 5.5 min. Lane 9) 7 min. Lane 10) 9 min. Lane 11) 11 min. Lane 12) Alkaline lysis. Lane 13) DNA Ladder. Lane 14) Duplicate 0.5 min. Lane 15) Dup. 1.5 min. Lane 16) Dup. 4 min. Lane 17) Dup. 5.5 min. Lane 18) Dup. 7 min. Lane 19) Dup. 9 min. Lane 20) Dup. 11 min.

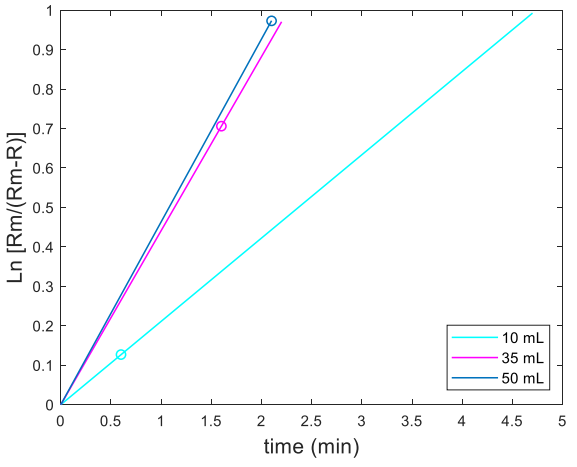

Figure 5. Release of pDNA from *E. coli* in batch experiments.

3.4 Bead mill scale up

Based on the equipment design data, scaling for a continuous mill was developed. A residence time (t_R = 5 min) and the free volume of the mill (V_{MC} = 0.05 L) were used (Günerken *et al.*, 2017). The specific rate constant (k) of each reaction volume (V_R) is shown in Table 2, as fitted in Figure 5. Table 2 also illustrates the relationship of L/D to the specific rate constant (k), indicating that the specific rate constant of rupture is higher at the lower L/D values. This may suggest better cell disruption due to the available space within the milling chamber, resulting in more shocks being generated.

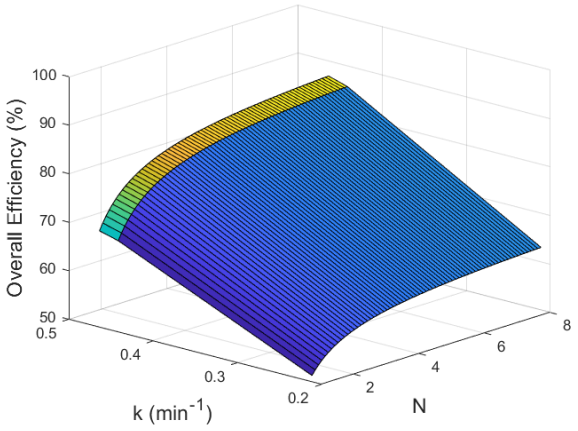

Figure 6. Continuous milling overall efficiency as a function of specific rate constant (k) and number of stages (N), t_R = 5 min.

Cell disruption in agitated bead mills is determined by the number of stress events (SN) that the cell undergoes during milling time and by the stress intensity (SI) of each of those events. SN increases proportionally with milling time and frequency. The bead/cell ratio (mill fill fraction), porosity and decrease in bead diameter, lead to an increased number of beads within the milling chamber, causing an increase in collision frequency. However, increasing the cell concentration within the chamber decreases the probability that each cell will be exposed to a stress event. The increase in SI is proportional to the milling rate, density, and diameter of beads (Taylor *et al.*, 2020).

The estimated pDNA release efficiency was calculated for a continuously operated mill. The rupture efficiency in a continuous mill is improved when the length-to-diameter ratio (L/D) is 0.51, as this condition corresponds to a higher specific rate constant k. Consequently, this leads to greater pDNA release, requiring only two stages (N) to achieve best results (as shown in Table 2).

The scaling study for *E. coli* rupture, based on the overall efficiency of plasmid release calculated using Eq. 7, is presented in Figure 6. The graph displays the specific rate constant (k) in relation to the corresponding number of stages (N). The results indicate that increasing (k) leads to a higher plasmid release, with peak efficiency reaching 84% at four stages. Further increasing the number of stages does not significantly improve efficiency. Given a residence time of t_R = 5 minutes, this setup allows

for effective product recovery without damaging the pDNA. Therefore, a design for a continuous mill with four stages is proposed.

Conclusion

In this study, cell rupture was achieved using bead milling, which resulted in a significantly higher plasmid yield per gram of cells compared to alkaline lysis, the most used cell rupture technique in laboratories. The cultivation process at the shake flask level provided the necessary biomass for pDNA production, enabling primary recovery studies using bead milling. During plasmid release kinetics with zirconium beads, a maximum pDNA yield, Rm of 2378 μg pDNA per gram of wet cells was observed after approximately 11 minutes of processing. This yield is notably higher than the concentration obtained through alkaline lysis, which was around 500 μg pDNA per gram of wet cells. The electrophoresis gel analysis demonstrated the kinetics of pDNA release, indicating satisfactory plasmid quality, particularly in its supercoiled (sc) form. It was observed that increasing the milling time resulted in higher pDNA release, but also led to an increase in genomic DNA (gDNA) contamination. Based on these findings, a residence time of 5 minutes was identified as the most efficient. Notably, RNA was not detected in the electrophoresis gel, suggesting that it may not have been released during the mechanical lysis process. This rupture method could be advantageous in subsequent stages of plasmid recovery and purification. The study conducted using perfectly agitated serial tanks for designing a continuous mill proved highly beneficial, as it enabled an in-depth analysis of the mixing degree, which is directly related to the degree of cell rupture. The milling process efficiency was found to increase with a decreasing length-to-diameter ratio (L/D), due to the increased space within the milling chamber, leading to higher collision rates and enhanced pDNA release. The scaling study, based on experimental data, allowed for the estimation of operational behavior across multiple stages, with four stages being sufficient to achieve an 83% recovery. Currently, there is a need to explore methods for scaling certain operations, as most processes have only been adapted to an industrial level thanks to previous laboratory studies. The bioprocess for plasmid production is not yet fully optimized to achieve the desired product purity. Bead milling has gained momentum as a viable alternative for recovering supercoiled pDNA compared to alkaline lysis, with residence time emerging as a crucial factor in the operation.

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