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Application Note

July, 2022

Keywords or phrases:
Sartoclear Dynamics® Lab, Bacterial cultures, bacterial isolates, Clarification, Extracellular Vesicles, Outer Membrane Vesicles, *E. coli*

Centrifuge-Free Clarification of Large-Volume Bacterial Cultures Using Sartoclear Dynamics® Lab

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Source publication:https://doi.org/10.1111/jam.15608

Abstract

Clarification of bacterial cultures is often performed by centrifugation and subsequent dead-end microfiltration, a cumbersome and time-consuming process, particularly when large volumes of cultures need to be treated. One example is the production of vaccine prototypes based on outer membrane vesicles (OMVs), a type of bacterial vesicle which has been shown to possess great immunogenic potential and has been a central focus of the research conducted in our group[1-9].

In this study we describe the optimization of a novel Sartoclear Dynamics® Lab protocol for the production of large volumes of cell-free bacterial isolates without employing any centrifugation step. The amount and quality of outer membrane vesicles (OMVs) present in the cell-free isolates produced was used as a benchmark parameter to assess for the efficiency of the protocol proposed, by comparing the results obtained to OMVs produced with a conventional centrifuge-based protocol.

Our results indicate that Sartoclear Dynamics® Lab can be effectively employed for the clarification of large volumes of high-density bacterial cultures without hindering yield and quality of OMVs. Additionally, the employment of Sartoclear Dynamics® Lab offered improved sample handling and a greater potential for scalability than traditional centrifuge-based approaches.

Introduction

Bacterial culture systems are routinely employed to produce metabolites, enzymes, and pharmaceutical precursors. An example of a bacterial extractable is represented by outer membrane vesicles (OMVs), a type of vesicle secreted by most Gram-negative bacteria which finds increasing applications for the development of pharmaceutical products such as vaccine prototypes and drug delivery systems [9–13].

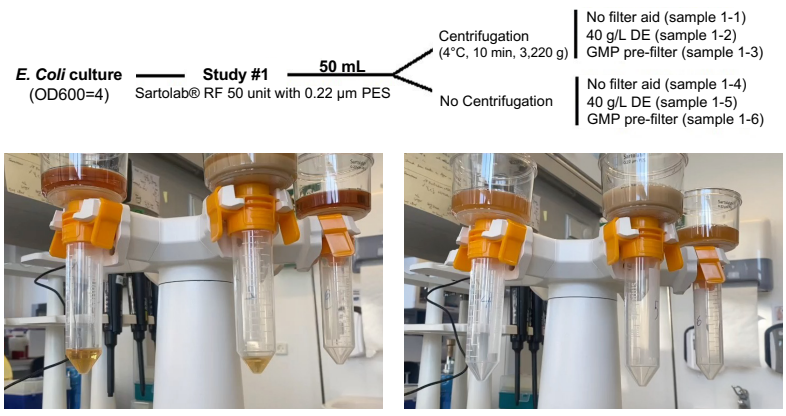
To obtain the derivative of interest, removal of bacterial cells often need to be removed from culture through a process defined as *clarification*. The majority of clarification protocols employ multiple centrifugation steps to separate the cellular biomass (pellet) from the culture medium (supernatant). Clarified supernatants are then subjected to filtration to produce crude, sterile products for downstream processing.

Although an overall effective and widely adopted approach, clarification of bacterial cultures by centrifugation presents some drawbacks:

- Equipment limitations – Although most laboratories are equipped with centrifuges, rotor capacities are typically limited to only a few liters per run (at most). This understandably poses an issue when larger volumes of cultures need to be treated, forcing several centrifugation runs that increase processing time and may challenge reproducibility.
- Filter clogging – After centrifugation, bacterial supernatants need to be separated from the sedimented pellet and residual cells that remain in suspension. The complete separation of supernatant from pellet is hard to achieve, often resulting in pellet carry-over. Residual cells in the supernatants can lead to filter clogging during downstream processing, due to the formation of a layer of cells on the filter surface (filter cake). This in turn affects reproducibility, increases processing costs and lengthens sample processing times.
- Scalability – Clarification by centrifugation and subsequent filtration is a difficult process to scale, compounding the issues previously mentioned when larger volumes of culture need to be treated. This poses a problem when scaling up production to pilot or industrial scale.
- Time – Centrifugation requires the preparation and balancing of samples to avoid damaging the centrifuge. This time-consuming process is aggravated by the need to re-balance samples in between the different centrifugation steps often required to avoid filter-clogging in downstream sample treatment.

It is clear from these considerations that it would be highly desirable to develop an alternative process for the clarification of bacterial cultures. Although originally developed for mammalian cell cultures, our group hypothesized that the Sartoclear Dynamics® Lab system could be adapted to such a purpose. With this aim, we designed a series of experiments to verify the applicability of the system, comparatively assess its efficiency and produce a streamlined user protocol. The bacterium selected for testing was an *Escherichia coli* (*E. coli*) isolate, chosen for its ease of handling and wide employment in research and industrial applications. The amount and quality of isolated OMVs was used as benchmark to evaluate the effectiveness of the clarification procedure. The experimental design used in this study is detailed in Figure 1.

Figure 1: *Experimental design of this study. Adapted from [14].*



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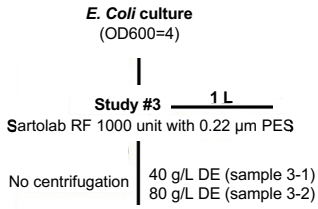

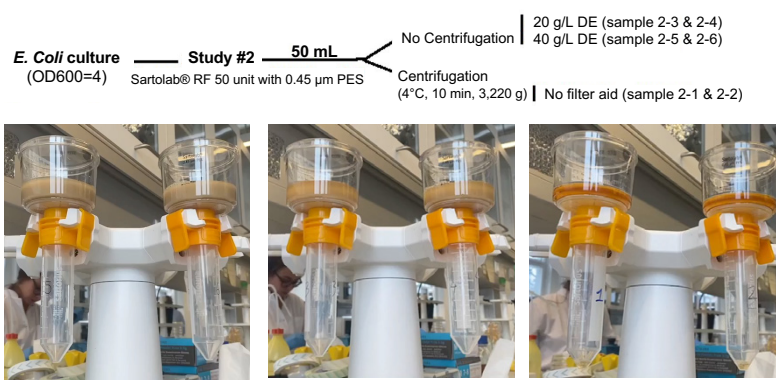

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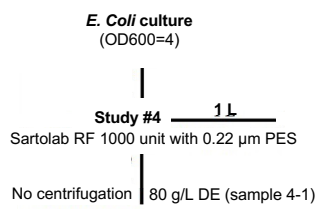


Study No.	Culture Volume	Filter Unit[a] pore size (µm)	Centrifugation[b]	Prefiltration Treatment	Sample ID
1	50 mL	0.22	+	None	1-1
				DE[d] (40 g/L)	1-2
				Glass microfiber pre-filter*	1-3
			-	None	1-4
				DE (40 g/L)	1-5
				Glass microfiber pre-filter*	1-6
2	50 mL	0.45	+	None	2-1; 2-2
			-	DE (20 g/L)	2-3; 2-4
				DE (40 g/L)	2-5; 2-6
3	1 L	0.22	+	DE (40 g/L)	3-1
			-	DE (80 g/L)	3-2
4	1 L	0.22		DE (80 g/L)	4-1

* MGA Grade (FT-3-1101-055, Sartorius, Göttingen, Germany). Adapted from 14.

Materials and Methods

Experiments were performed in small-scale (50 mL) and moderate-scale (800 mL) volumes using standard culturing conditions in glass Erlenmeyer flasks (1 L) and a Lambda Minifor bioreactor (7 L flask; Lambda, Brno, Czech Republic), respectively. Briefly, a single colony of *E. coli* was subcultured in brain-heart infusion medium (BHI; Gibco) and incubated overnight (ON) under aerobic conditions at 37 °C/160 rpm. On the following day, ON cultures were used to inoculate either 200 mL (0.2% v/v) or 5 L BHI broth (1% v/v). Cultures were incubated at 37 °C (Erlenmeyer flask: 160 rpm shaking; bioreactor: 5 Hz shaking) until reaching OD_{600} = 4. 5 L cultures were incubated under constant aereation (10 psi).

After reaching OD_{600} = 4, aliquots from harvested cultures were subjected to centrifugation or direct filtration using Sartoclear Dynamics® Lab filtration kits (Sartorius, Göttingen, Germany). Selected samples were filtered using MGA grade glass fiber filters (FT-3-1101-055, Sartorius, Göttingen, Germany; "GMP pre-filter") The centrifugation of selected samples was carried out at 3,220 g, 10 min, and 4 °C.

All samples were filtered using Sartolab®RF vacuum filtration units (RF 50 and RF 1000 units; 0.22 and 0.45 µm filter pore size) according to the manufacturer's recommendations. Filters for the clarification of 50 mL samples (studies 1 and 2) were used in conjunction with a Sartolab® Multistation and Microsart® e.jet pump (Sartorius, Göttingen, Germany), and filtrates were collected into sterile 50 mL conical tubes. For 800 mL samples (studies 3 and 4) the Microsart® e.jet was replaced by a Microsart® maxi.vac pump (Sartorius, Göttingen, Germany), and sterile 1,000 mL Duran® pressure plus bottles (DWK Life Sciences, Melville, NY, USA) were used for sample collection. Different concentrations of diatomaceous earth (DE) filter aid were used during filtration (20, 40, 80 g/L). In studies 1-3, the DE filter aid was added to the empty receiving flasks prior to the culture samples. In study 4, the DE filter aid was added on top of the samples immediately before filtration, ensuring thorough mixing through the samples. Filtration flow rates of all samples were recorded (mL/min). Sterility of filtered isolates was tested by spotting (10 µL) on sterile BHI Agar plates.

The filtered isolates were loaded into cellulose ester dialysis membranes with a pore-size range of 1,000 kDa (Repligen, Waltham, Massachusetts, USA), and concentrated by Hydrostatic Filtration[4]. The concentrated crude filtrates were then dialysed twice 1:100 (4 h, 4 °C) in sterile phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA). 15 mL aliquots from each sample were then further concentrated (66-fold) using different types of centrifugal ultrafilters, as described in [14]. The concentrated isolates were then aliquoted and stored at –20 °C.

Concentrated isolates from studies 2-4 were analysed by: i) Qubit protein assay quantification; ii) SDS-PAGE; iii) Transmission Electron Microscopy (TEM); and iv) Nanoparticle Tracking Analysis (NTA). Analyses (i) and (ii) were aimed at assessing protein content of the samples, while analyses (iii) and (iv) were intended to verify quality and quantity of isolated OMVs. Only filtration flow data were recorded for samples in pilot study and study 1. SDS-PAGE data from study 4 are not shown.

Results and Discussion

Flow rate analysis (Figure 2): i) a concentration of ≥ 20 g/L of DE filter aid is sufficient for direct filtration of small volumes (50 mL) of bacterial cultures; ii) a concentration of ≥ 80 g/L of DE filter aid is necessary for the filtration of larger volumes (800 mL) of bacterial cultures; iii) The addition of DE filter aid on top of the samples immediately before filtration (instead of the other way around as in studies 1-3) coupled with vigourous sample agitation greatly improved flow rates (study 3 vs. study 4); iv) the addition of DE filter aid to centrifuged samples did not affect flow rates; v) the use of glass microfiber pre-filters in addition to DE also did not improve flow rates in any of the conditions tested; and vi) centrifugation followed by dead-end microfiltration can in some instances lead to filter-clogging, due to pellet carry-over ("Centr._no DE" samples, study 1 and 2, Figure 2).

Quantification of total protein yield (Figure 3): i) centrifugation appeared to reduce total protein yield as compared to non-centrifuged samples; ii) there were negligible differences in total protein yield between samples filtered using 20, 40 or 80 g/L DE filter aid; iii) the addition of DE filter aid immediately before filtration coupled with vigorous sample agitation significantly improved total protein yield (see study 4).

SDS-PAGE analysis (Figure 4): i) all samples presented a similar protein banding pattern; ii) protein bands from centrifuged samples (2-1, 2-2) were significantly fainter than non-centrifuged samples (2-3, 2-4, 2-5, 2-6, 3-1, 3-2), suggesting a lower protein concentration than the other samples, possibly due to some loss of OMV into the pellet during centrifugation. Protein banding from samples in study 4 was comparable to that observed in samples 3-1 and 3-2, albeit with greater band intensity (data not shown), in accordance with the total protein concentration data obtained. This suggested that the filtration protocol adopted did not negatively affect the protein composition of the isolated samples.

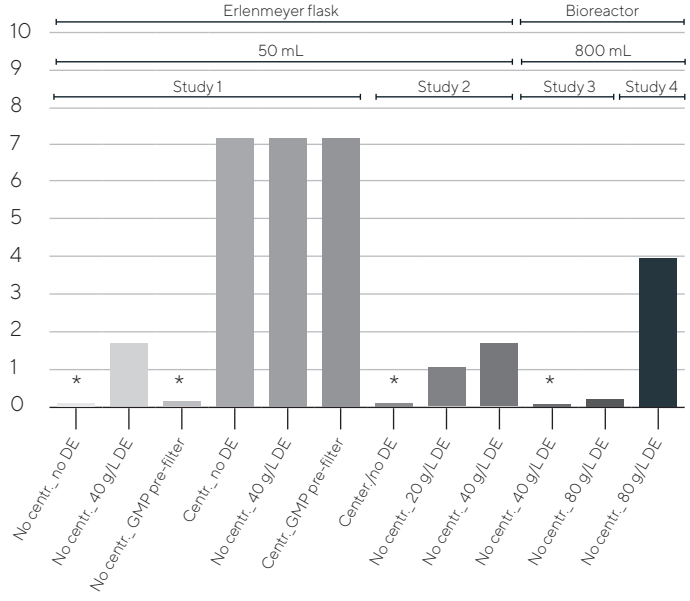
TEM analysis (Figure 5): i) microscopy imaging showed that OMVs were present in all samples and intact, suggesting that none of the processing procedures adopted had a negative impact on OMV integrity.

NTA analysis (Table 1): particle quantification showed similar OMV concentration in all samples, suggesting that none of the processing procedures adopted had a negative impact on the amount of OMV isolated.

Conclusions

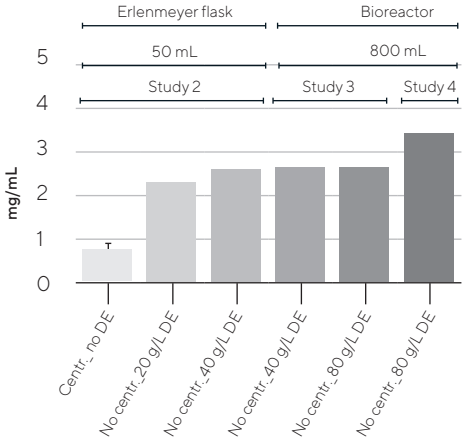
Our results showed that the Sartoclear Dynamics® Lab system can be successfully employed for the clarification of moderate volumes of bacterial cultures without prior centrifugation. The protocol proposed did not negatively affect the quality of the isolates in any detectable manner, leading on the other hand to increased protein yield as compared to the centrifuge-based alternative protocol tested in this study.

Figure 2: *Flow rate*



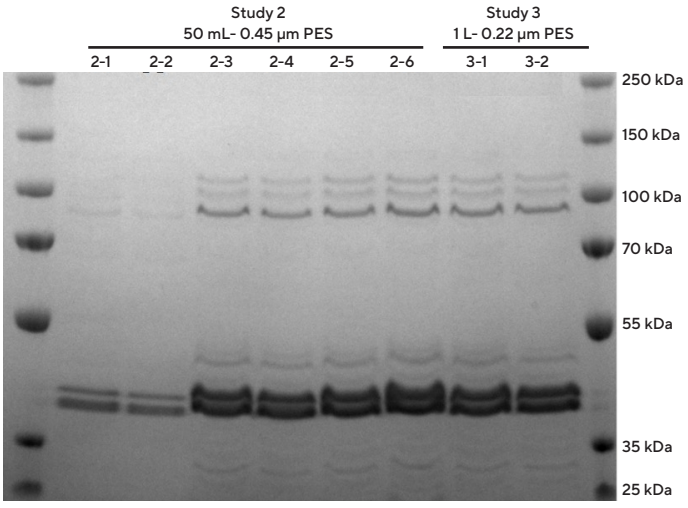
Flow rate (mL/min/cm^2) in all the studies undertaken. DE: Diatomaceous Earth; GMP: Good Manufacturing Practices. Asterisks (*) indicate filter clogging and incomplete filtration. Note that the definition (800 mL) refers to the volume of culture used, while the total volume of the sample filtered was 1,000 mL, due to the addition of DE filter aid (200 mL wet volume). The volumetric contribution of DE filter aid was negligible on smaller samples and concentrations. Adapted from [14].

Figure 3: *Total protein yield by Qubit protein quantification*



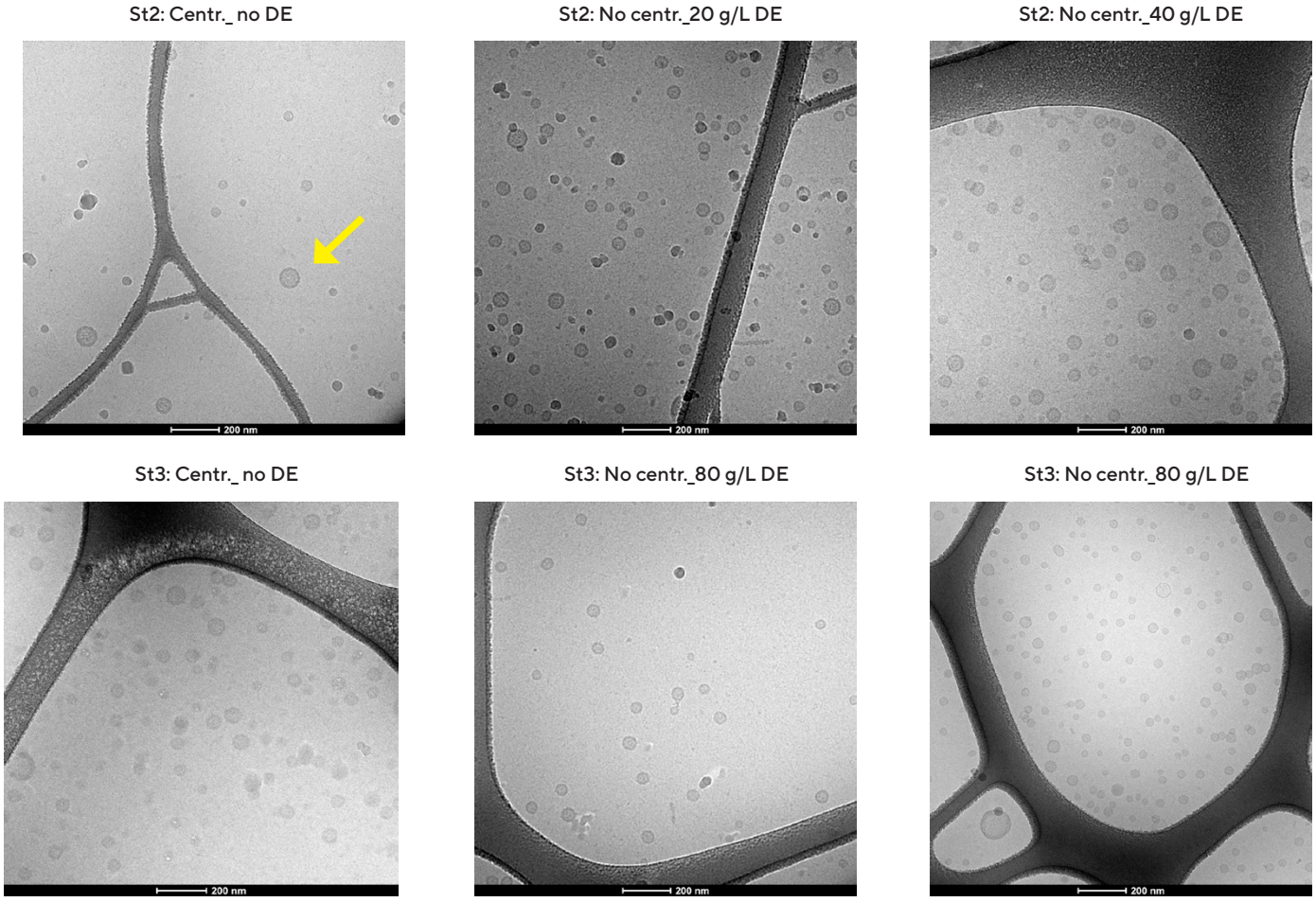
The definition (800 mL) refers to the volume of culture used, while the total volume of the sample filtered was 1,000 mL, due to the addition of DE filter aid (200 mL wet volume). The volumetric contribution of DE filter aid was negligible on smaller samples and concentrations. Adapted from [14].

Figure 4: *SDS-PAGE analysis*



Data from study 1 and 4 not shown. Adapted from [14].

Figure 5: *TEM analysis*



OMVs are visible as clear spherical particles (yellow arrow). Adapted from [14].

Study No.	OMV batch	Particles diameter (nm) Mean	Median	OMVs concentration (particles/mL)
2	2-1	95.9 (±1.0)	66.5 (±3.7)	2.64×10^{12}
	2-2	90.4 (±0.9)	68.4 (±2.0)	1.44×10^{12}
	2-3	96.0 (±1.6)	78.7 (±2.4)	2.32×10^{12}
	2-4	96.2 (±1.2)	64.4 (±1.0)	3.32×10^{12}
	2-5	91.3 (±0.8)	71.8 (±2.1)	2.67×10^{12}
	2-6	115.1 (±4.1)	84.1 (±8.4)	2.49×10^{12}
3	3-1	98.0 (±1.7)	+72.5 (±4.6)	4.47×10^{12}
	3-2	107.6 (±1.2)	75.0 (±2.2)	4.04×10^{12}
4	4-1	105.5 (±1.6)	82.6 (±3.3)	3.59×10^{12}

Table 1: Standard error is indicated in brackets. Adapted from [14].

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Status: 07 | 2022