Incubation Temperature and Period During Denarase Treatment and Microfiltration Affect the Yield of Recombinant Adenoviral Vectors During Downstream Processing

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Abstract
Adenoviral vectors (AV) are commonly used as vaccine and gene therapy vehicles because of their ease of construction, ability to grow to high titers in the large-scale production process, and safety for human applications. However, the efficiency rate of downstream processes for adenoviral vectors still varies greatly. In the current study, we aimed to investigate the effect of the downstream treatment protocol and microfiltration of the harvested upstream material on viral vector yield. We compared the performance of the repeated freeze–thaw (RFT) and the Tween-20 detergent lysis (DLT) methods. In addition, the effects of the cell lysis method, incubation temperature, and time on viral yield were investigated. The samples were incubated at either room temperature or 37 °C for 1-, 2-, and 4-h periods. Samples were filtered with PES and SFCA membrane. Virus yield and infectivity were assayed by qPCR and immuno-titration. In conclusion, our results suggest that 2-h incubation gives the best results when incubated at 37 °C for denarase activity when Tween-20 is used for virus recovery. If the room temperature is preferred, 4-h incubation could be preferred. A phase 1 clinical trial (NCT05526183, January 21, 2022) was started with the recombinant adenovirus used in the study.

Keywords Downstream processing · Bioprocess development · Viruses · Vaccines · SARS-CoV-2

Introduction
Viral vectors such as adenoviruses, adeno-associated viruses, or retroviruses are becoming increasingly crucial in vaccines and gene therapy to deliver genetic material to the target cell [1]. Adenoviral vectors have several properties that make them ideal candidates as gene delivery systems in gene therapy and recombinant vaccine design. Contrary to lentiviral vectors, adenoviruses do not integrate into the host genome, with the viral genome remaining episomal [2, 3]. This property makes adenoviral vectors safer for mass vaccination against viral diseases. In addition, many features of adenoviral vectors make them advantageous in gene therapy, including their ease of construction, their ability to grow to very high titers in cell culture, the availability of certified cell lines for large-scale production and purification, and their safety for human applications. They also induce high levels of transgene expression and high levels of antigen-specific humoral and cell-mediated immune (CMI) responses by activating innate immunity [4, 5]. Adenoviral vectors also have the advantage of systemic or mucosal administration.

Abbreviations
SARS-CoV-2 Severe Acute Respiratory Syndrome Coronavirus-2
rAd5 Recombinant adenovirus type 5
RFT Repeated freeze–thaw method
DLT Tween-20 detergent lysis method
VvDNA Viral DNA
qPCR Real-Time Quantitative Polymerase Chain Reaction
PES Polyethersulfone
SFCA Surfactant-free cellulose acetate

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Adenoviruses (Ad) are a diverse group of double-stranded DNA viruses with more than 50 distinct human adenoviral serotypes [6]. The most common human adenoviral vector serotypes used in clinical studies are Ad serotype 5 (Ad5), Ad26, Ad35, and Ad4 [7–9]. Human Ad serotype 5 (Ad5) has a non-enveloped icosahedral nucleocapsid containing a linear, double-stranded DNA genome consisting of “early” and “late” regions of approximately 36 kb. Typically, gene insertion into an Ad vector occurs in any early region (E), mainly in the E1 region. E1, E2, and E4 regions critical for virus replication can be deleted to increase transgene insertion capacity. E3 genes are not required for virus replication. Adenoviral vectors with only the E3 gene are replication-competent; vectors with deletion of E1, E2, E4, or any combination are replication-defective [10]. Therefore, a cell line expressing gene cassettes capable of providing viral replication functions is needed [11]. Ad5 has been widely investigated as a gene delivery vector because it can be easily produced at high titers. A wide variety of studies are available to improve its efficacy and safety for clinical use [12–15]. With the COVID-19 pandemic, four adenoviral vector-based vaccines were approved for emergency use [16]. In addition, many adenoviral vector-based COVID-19 vaccines are being tested in clinical trials [3, 17, 18]. Improvement studies are still ongoing to reduce production cost and increase the stability of the vaccine.

The adenoviral vector production process consists of two steps: upstream and downstream. First, the upstream process of the adenoviral vectors is amplified in suitable cell lines (HEK293 or PERC.6) [19]. The downstream process mainly includes the viral particle release, clarification, and ultra/diafiltration steps. All the steps in the downstream process affect the yield of the final product. Likewise, the downstream processes cause the most loss of vectors in the adenoviral vaccine production process [20]. Replicated viruses in the cells release into the supernatant by a freeze–thaw method and the chemical cell lysis method also reduces the host cell DNA. While the cell lysis method performed at the beginning of the downstream process reveals the AV particles, Denarase applied in this process also reduces the host cell DNA. During cell lysis and enzyme incubation, protease enzymes released in the medium can affect the number of viral particles released from cells and their infectivity, depending on temperature and time [40–42]. Many viral products are less stable at culture temperatures (typically 37 °C) as compared to lower temperatures (between 4–25 °C) [43]. There is no consensus regarding lysis temperature and enzyme treatment time [29, 43]. Therefore, in this study, we aimed to compare the performance of the repeated freeze–thaw method as a physical lysis method and the chemical cell lysis method.
with Tween-20 detergent and determine the optimum incubation temperature and period with denarase endonuclease. In addition, we also studied the performance of microfiltration with SFCA and PES membranes.

Materials and Methods

Adenoviral Vector Amplification and Study Groups

HEK293 (human embryonic kidney) cells were purchased from Germany (DSMZ, ACC305). These cells adapted to suspension culture were amplified in Acti-Pro (Cytiva, MA) cell culture medium supplemented with cell boost 7A (Cytiva, MA). After cell density was reached at $1 \times 10^6$ cells/ml, cells were infected with a recombinant human type 5 replication-deficient adenoviral (rAd5) COVID-19 vaccine vector carrying spike gene of SARS-CoV-2 [44] at the multiplicity of infection (MOI) of 5 and incubated at 37°C and 5% CO$_2$ with shaking at 130 rpm in a shaker flask for three days. The development of suspended HEK293 cells’ cytopathic effects (CPEs) was observed daily. The cells were harvested when the ratio of cells with CPE was 85% on the third day of incubation and kept at −80°C till the downstream process. The harvested rAd5 vaccine vector was thawed on ice before starting the treatment protocol. The harvested sample was divided into 12 separate study groups with 40 ml each for treatment (Fig. 1).

Cell Lysis and Nuclease Digestion

Two cell lysis protocols were used: the Repeated Freeze–Thaw method (RFT) and the Tween-20 Detergent Lysis method (DLT) [45]. Due to the very low viral particle concentration of the supernatant, the harvested material was first kept at −80°C immediately following the harvesting procedure. As a reference treatment, the repeated freeze-thawing method was used. For freeze-thawing, 6 tubes from the harvested sample were frozen in a −80°C freezer for 10 min, followed by thawing at 37°C. This procedure was repeated twice. Following the RFT cycles, MgCl$_2$ at a final concentration of 1 mM and 30 U/ml of Denarase (cLEcta GmbH, Germany) was added to the samples. For the detergent lysis method, Tween-20 (0.5% v/v) was added to the other 6 tubes in addition to MgCl$_2$ and Denarase. The digestion proceeded for 1-, 2-, and 4-h either at room temperature (25°C) or 37°C in the incubator on a shaker with 100 rpm. Experiments are conducted at both temperatures.

After the incubation, samples were taken to analyze pH, cell viability, and remaining intact cells. Then, the samples...
were centrifuged at 5000×g for 5 min, and supernatants were collected for microfiltration.

**Microfiltration**

Clarification of the harvested material was performed by normal flow filtration (NFF), using syringe filters with a membrane diameter of 28 mm and a pore size of 0.45 µm, with Surfactant-free Cellulose Acetate (SFCA) (Corn-ing, NY) or Polyethersulfone (PES) (Sartorius, Germany) membranes for removal of cell debris and initial impurity reduction. Before use, filters were equilibrated with growth medium. Half of the samples (15 ml) from the same incubation were filtered with a PES membrane and half (15 ml) with an SFCA membrane using a 20 ml syringe with constant pressure for 60 s.

**Characterization Assays**

To test the cell viability, the trypan blue exclusion method was performed just after harvesting, following the first thawing, repeated freeze–thaw cycles, and denarase treatment of RFT and DLT.

**Viral DNA Quantification**

For the determination of rAd5 vector genome copies, quantitative Real-Time Polymerase Chain Reaction (qPCR) was used. According to the manufacturer's instructions, the viral DNA was extracted using a commercial kit (PureLink™ Viral RNA/DNA Mini Kit, Thermo Scientific, USA). The viral genomes were quantified in triplicate by qPCR using the Adenovirus type 5 specific primers (forward: 5′-CCA CCGATAGCAGTACCCCTT-3′, reverse: 5′-GACCTTTGCTACGGTCAA-3′) and TaqMan probe (5′-FAM-TGC CCAAGCTACCATGGCAGT-TAMRA-3′). PCR reaction was performed in CFX96 Touch Real-Time PCR Detection System (Bio-Rad) under the following conditions: hold at 95 °C for 12 min, and 40 cycles at 95 °C/15 s (denaturation) and 60 °C/1 min (hybridization). Titration results are given in viral DNA/ml (vDNA/ml). A standard sample of known titers was used for quantification.

**Infectious Particles**

Infectious particles were quantified using the Immuno-titration assay kit (Cell Biolabs, CA). HEK293 cells in DMEM supplemented with 10% FBS were incubated in 24 well plates in an incubator with 5% CO₂ at 37 °C for 1-h. The cells were then infected with diluted viral samples and incubated for two days. Each sample was performed in duplicate. Positively stained cells (brown) were counted for at least five separate fields per well using a light microscope with a 10X objective. Viral titer was calculated using the formula Viral Titer (Infectious Unit (ifu)/ml) = (average positive cells/field) × (79 fields/well) × (dilution factor).

The methods used in the current study are standard in the literature [46, 47]. The methods were repeated three times with similar results.

**Statistical Analysis**

Statistical analyses were conducted with the Statistical Package for Social Sciences (SPSS) software, version 10.0. Mann–Whitney U test and Kruskal Wallis H test were used for non-parametric comparisons, and one-way ANOVA was used for appropriate parametric comparisons.

**Results**

The Cell Lysis Method and Incubation Conditions Affect the Viral Particle Yield

The effects of the cell lysis method, incubation temperature, and period on total viral particle yield were investigated. For cell lysis, freeze-thawing and treatment with the 0.5% Tween-20 methods were used. The ratio of viable cells was assessed by trypan blue assay at the end of each incubation period. The ratio of the viable cells by trypan blue test was 15% at the harvest time. The ratio of the viable cells on the trypan blue test was 2% following the gradual thawing of the harvested material and was less than 1% in the first hour of treatment protocols. There was no significant difference between the treatment methods. No viable cell was detected after 2-h of incubation of the samples in any treatment methods.

The viral genome copies in the harvested material were determined by the qPCR method. It was found that the total copy number of vDNA (3.75 × 10⁸ vDNA/ml) in the repeated freeze-thawed samples significantly increased compared to pre-treatment samples (2.5 × 10⁸ vDNA/ml, p ≤ 0.001). The more lysed the cells, the more the viral genomes increased.

To investigate the effect of incubation temperature and period on the total viral genome copies, the samples were incubated at either room temperature or 37 °C for 1-, 2-, and 4-h and virus yield was assayed by qPCR before filtration. As shown in Fig. 2, the percent viral genome copies (vDNA) obtained by qPCR in the RFT method decreased at the 1st-hour of incubation at room temperature (p ≤ 0.001). At the end of the 2nd-hour incubation, the percent of vDNA yield in the RFT sample showed a statistically non-significant increase compared to the 1st-hour incubation. However, at the 4th-hour RFT incubations, the vDNA yield increased significantly compared to the 1st-hour (p = 0.003) and the 2nd-hour (p = 0.029) incubation periods.
A significant decrease was observed in RFT samples following 1-h incubation at 37 °C compared to the pre-incubation \( (p \leq 0.0001) \). Similar to the incubation results at room temperature, a slight increase was observed for RFT in the 2nd-hour incubation compared to the 1st-hour incubation \( (p = 0.069) \). The 4th hour incubation results showed a statistically non-significant change compared to the 1st and 2nd-hour incubations at 37 °C.

In the DLT method, the percent vDNA yield in the cell suspension decreased significantly at the first hour of incubation compared to pre-treatment samples at room temperature \( (2.5 \times 10^8 \text{ vDNA/ml}, p \leq 0.001) \). However, a statistically significant increase in the 2nd-hour incubation compared to the 1st-hour incubation at room temperature \( (p \leq 0.0001) \). In addition, a significant increase in the vDNA yield at the 4th hour incubation was observed in the DLT sample compared to the 1st-hour \( (p = 0.0001) \) and the 2nd-hour \( (p = 0.001) \) incubation period at room temperature (Fig. 2).

A slight decrease was observed in DLT samples following 1-h incubation at 37 °C compared to the pre-incubation values \( (p = 0.141) \). In contrast, the vDNA yield increased significantly in the 2nd-hour Tween-20 incubation compared to the 1st-hour incubation \( (p = 0.0001) \). At the 4th-hour incubation at 37 °C for DLT, while the vDNA yield increased significantly \( (p = 0.005) \) compared to the 1st hour, a significant decrease was observed compared to the 2nd-hour incubation \( (p \leq 0.0001) \) (Fig. 2).

### Microfiltration with SFCA Membrane Yields More vDNA

After low-speed centrifugation, clarification was performed by filtrating the sample through PES and SFCA membranes to remove the cell debris from the virus suspension. The viral genome amplification after filtration was determined by qPCR (Fig. 3). The filtering efficiency was calculated by dividing the vDNA concentration before and after filtration. Also, the filtering efficiency was calculated for different incubation temperatures and periods using different methods for cell lysis.

While the efficiency of the PES membrane was between 10 and 50%, the efficiency of the SFCA membrane was between 20 and 55% (Fig. 2 and 3). The filtration efficiencies of the PES membrane with the RFT method after 1-, 2-, and 4-h of incubation were 18.13%, 19.72%, and 17.60% at room temperature, 28.07%, 10.02%, and 23.27% at 37 °C, respectively. The SFCA membrane filtration efficiencies of the same group of samples were 40.28%, 26.12%, and 32.94% at room temperature and 41.87%, 27.14%, and 20.76% at 37 °C, respectively. The PES membrane filtration efficiencies of the samples incubated with Tween-20 detergent were 80.36%, 49.75%, 21.37% at room temperature, and 76.02%, 22.81%, and 25.02% after 1-, 2-, and 4-h of incubation. However, the SFCA membrane filtration efficiencies of the same group of samples were 81.05%, 55.09%, and 34.73% at room temperature, while it was 65.49%, 35.24%, and 47.17%
at 37 °C. Although the filtration efficiency over the incubation period did not change significantly, a slight decrease was observed with longer than 1-h incubation with PES and SCFA membrane filters.

**Cell Lysis Method and Microfiltration Affect the Infectivity of the Vector**

The effects of the cell lysis method, incubation temperature, and incubation period on virus infectivity were investigated by the immuno-titration method. The results of the immuno-titration as infectious units or IFU performed in the study groups are shown in Fig. 4.

In the RFT method incubations at room temperature and 37 °C, the incubation period in the samples filtered with PES membrane caused a slight but not significant increase in the infectivity of the virus. In contrast, though not significant, a decrease in the 2nd hour and an increase in the 4th hour were observed in the samples filtered with SFCA (Fig. 4). The insufficient degradation of host cell DNA and free vDNA from lysed viral particles at 2 h of incubation with Denarase might decrease the infectivity of the vector in HEK293 cells [46].

When cell lysis was performed with the DLT method, the highest IFU value was obtained with 1-h incubation of PES membrane filtration at room temperature. At the same time, the infectivity of the virus decreased as the incubation period extended (Fig. 4). Similarly, for the SFCA membrane filtration, the highest infectivity ratio was achieved during the 1st-hour incubation at room temperature. A progressive decrease of virus infectivity was found at the 2nd and 4th-hour incubations. The incubation at 37 °C with an increasing incubation period decreased the infectivity of the virus during the 1-, 2-, and 4-h incubations after PES membrane filtration. The highest immuno-titration result in SFCA membrane filtration was obtained in the 2nd-hour incubation (Fig. 4). In the DLT method, more infectious vectors were obtained than in the RFT method (Fig. 4). However, both methods obtained better results in samples filtered with PES membrane.

The pH of the samples did not change significantly during the incubation and filtration processes. While the pH was 6.84 before the cell lysis process, it increased to 7.08 and 6.98 at room temperature and up to 7.04 and 6.90 during incubations at 37 °C in the RFT and DLT groups.

When the vDNA/IFU (viral DNA copy number / infectious units) ratios were compared after cell lysis by the RFT method, the most consistent and lowest rates were in samples filtered with PES membrane after 1-, 2-, and 4-h incubations at room temperature (Table 1). However, filtering the samples with SFCA increased the rate by almost double (Table 1). In addition, the PES membrane had better vDNA/IFU ratios than the SFCA membrane in the clarification of harvested vector material, in which cell lysis was performed either by RFT or DLT methods (Table 1).
The efficiency of downstream processing of adenoviral vectors varies significantly between 2 and 60% among different protocols [48, 49]. The cell lysis method, DNase-I and MgCl₂ concentrations, pH, incubation period, and incubation temperature are important parameters affecting the virus purification efficiency. Therefore, the current study investigated the effects of the cell lysis methods and microfiltration of the harvested upstream material on viral vector yield. Furthermore, the effect of different incubation temperatures (room temperature and 37 °C) and times (1-, 2-, and 4-h) in these processes were investigated.

The treatment protocols, including repeated freeze–thaw cycles and detergent lysis methods, have been used widely. Previously, it has been reported that the virus yields from host cells using the detergent lysis method were higher than that of the repeated freeze–thaw method [50, 51]. The repeated freeze–thaw method is the oldest method for cell lysis; however, it is only suitable for lysing small amounts of harvest and cannot be linearly scaled up to an industrial scale, unlike the detergent-based lysis method [22, 52]. Benzonase and Denarase have been routinely used in virus manufacturing studies to eliminate the host cell DNA and RNA from the recombinant vector product. The temperature and time during the treatment may affect the viral vector yield. Total vDNAs and infectious particles are critical parameters for comparing production processes [43, 53, 54].

In the current study, we compared the efficiency of the Tween-20 and the traditional repeated freeze–thaw method. The viral genome yields obtained by qPCR in both the RFT and DLT methods decreased significantly at the 1st-hour of incubation at room temperature (Fig. 2, p < 0.001). The decrease of vDNA yield in the first hour of incubation may result from the degradation of the vDNA outside the capsid of the vector by the denarase caused by osmotic shock.

![Infectious viral particles (IFU/ml) were obtained after cell lysis using repeated freeze–thaw and detergent-based methods. Infectious viral particles were determined by immuno-titration assay. In the DLT method, more infectious particles were obtained than in the RFT method. However, better results were obtained in samples filtered with PES membrane in both methods. Data are expressed as mean ± standard deviation.](image)

| Table 1 | The ratio of Viral Genomes to Infectious Units (vDNA/IFU) |
|----------------|---------------------------------|
| vDNA/IFU | Repeated Freeze–Thaw | Tween-20 |
| Room temperature | 37 °C | Room temperature | 37 °C |
| 1 h | 2 h | 4 h | 1 h | 2 h | 4 h | 1 h | 2 h | 4 h |
| PES | 3.95 | 4.08 | 4.00 | 9.64 | 3.69 | 6.19 | 9.62 | 33.91 | 7.77 | 10.90 | 8.35 | 12.95 |
| SFCA | 10.36 | 8.10 | 8.60 | 11.19 | 11.43 | 6.11 | 21.72 | 21.90 | 20.20 | 13.72 | 10.45 | 17.09 |

**Discussion**

The efficiency of downstream processing of adenoviral vectors varies significantly between 2 and 60% among different protocols [48, 49]. The cell lysis method, DNase-I and MgCl₂ concentrations, pH, incubation period, and incubation temperature are important parameters affecting the virus purification efficiency. Therefore, the current study investigated the effects of the cell lysis methods and microfiltration of the harvested upstream material on viral vector yield. Furthermore, the effect of different incubation temperatures (room temperature and 37 °C) and times (1-, 2-, and 4-h) in these processes were investigated.

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During the gradual thawing of the frozen-harvested material [55]. We found a significant increase in the vDNA yield at the 4th-hour incubation of the RFT protocol compared to the 1st-hour and the 2nd-hour incubation periods (Fig. 2). Complete lysis of the cells remaining intact during the process and virus release seem to cause the increase of virus yield. Likewise, a significant increase in vDNA yield during the 4-h incubation was observed in the DLT samples compared to the 1st-hour and the 2nd-hour incubation periods. Incubation with the Tween-20 sample group at room temperature resulted in a higher vDNA yield than the samples in the RFT sample group (2nd and 4th-hour). The highest vDNA yield was achieved after 4-h of incubation at room temperature with the DLT method (Fig. 2).

Accordingly, we found a slight decrease in the viral DNA yield of samples in the first hour of incubation at 37 °C. Then a significant increase with 2-h and 4-h incubations was observed (Fig. 2). In both methods used for the cell lysis step to obtain viruses, there was lysis of the cells and the lysis of the viruses. The qPCR results of viral DNA released into the cell suspension and genomes isolated from viruses are shown after 1-h of incubation. The purpose of using the Denarase enzyme in these incubations is for the degradation of vDNA from lysed viruses. Therefore, after 1-h of incubation, the vDNA released into the medium by Denarase enzyme activity was degraded, and a decrease in the total vDNA yield was observed. The vDNA yield was significantly decreased at 37 °C compared to room temperature in both treatment methods. The vDNA yield of the DLT method was significantly higher than the RFT method at 2- and 4-h incubation periods (Fig. 2). 4-h incubation at room temperature yielded significantly more viral genomes than the 2-h incubation at room temperature and 37 °C (Fig. 2). However, the yields of 2-h incubation at both room temperature and 37 °C were similar. Our results show that the DLT treatment for 4 h at room temperature yields the best vDNA. By previous results (vDNA, IFU, and vDNA/IFU), a 2-h incubation period with Tween-20 at 37 °C seems the optimum period to lyse the host cells and release the virus completely [29].

Previously, it has been shown that virus viability decreased when the incubation temperature was above room temperature [56–58]. It has been reported that 2-h incubation is sufficient at 37 °C [54], which is the optimum temperature for denarase enzyme activity to eliminate host cell DNA better. In the study by Jardon and Garnier, they showed that low temperature increases cell viability, and the virus yield obtained at 35 °C is three times higher than at 37 °C [58]. Accordingly, we observed that the viability of the adenovirus type 5 vector decreased with increasing temperature (room temperature vs. 37 °C).

Clarification has received little consideration and is performed as an obligatory step before other DSP steps to reduce bioburden and increase the capacity of further downstream steps [59, 60]. The cell debris in the virus fermentation broth can easily be removed by filtration to attain the purpose of clarification using low-speed centrifugation to clarify the virus feed [52]. However, releasing intracellular content by cell lysis complicates the purification of viral vectors [38]. In membrane filtration, factors such as the structural properties of the membrane and the diameter of the membrane might affect the efficiency. One of the critical points in optimizing the DSP is the loss of viability and infectivity of the virus during filtration. Generally, PES, SFCA, and PVDF filters with 0.2 µm to 0.45 µm pore diameters are used for clarification [61]. There is no uniform consensus on the type of membrane used for adenoviral vector clarification. It has been shown that Tween-20 detergent is absorbed into the PES membrane surface and can reduce the throughput of adenoviral particles [22, 62]. Nestola et al. showed that PES-based membranes have a low yield recovery rate of total viral genomes (23–58%) [39]. In the current study, we have tested two widely used filters with SFCA and PES membranes. The filter efficiencies obtained in our study were also in the range of previous reports [39]. The vDNA yields of microfiltration with the SFCA were higher than the PES membrane in both RFT and DLT groups (Fig. 3). More vDNA yield was obtained in DLT samples filtered with the SFCA membrane compared to the PES membrane. SFCA caused the lysis of viruses in the filtered virus suspension due to the properties of the membrane, and the vDNA yield in the medium was determined by the qPCR method used. The results of the immuno-titration, in which the number of live viruses was determined, reflect this fact. On the other hand, the Denarase enzyme breaks down the viral DNAs released from the degraded viruses in the suspension. Figure 3 shows a higher vDNA yield at room temperature since room temperature (25 °C) is insufficient for the Denarase enzyme's activity.

Although the vDNA yield quantification is mainly used for the formulations of final drug products of recombinant vectors, the virus infectivity is the most critical parameter of the biological activity of those preparations. Therefore, we also studied the vector infectivity rates of the processed samples with an immuno-titration assay (Fig. 4). Incubation temperature and period, pH of the harvested material, shear forces from stirring, centrifugation of fermentation broth, and filtration can damage the structure of adenoviruses, thereby reducing the infectivity of adenoviruses [63]. In the current study, we found that the infectivity of the rAd5 vector decreased with the increase in temperature (Fig. 4 and Table 1). The pH values at different incubation temperatures and periods in our study were very close to the optimum pH (7.2) value [58, 64].

On the other hand, biological factors, including various proteases, are primarily released from host cells. These
proteases can hydrolyze adenovirus surface proteins, leading to structural damage and reduced infectivity of adenovirus particles [65]. This explains the difference between total viral genome yield and immuno-titration results (Fig. 3 and Fig. 4). The denarase treatment used in the study did not destroy the ability of the infectivity of viruses [66, 67].

The most exciting and surprising effect of incubation with Tween-20 detergent is the production of rAd5 vectors with higher yields and better infection efficiency than vectors obtained by the classical cell lysis method (Fig. 4). The denarase treatment used in the study did not affect the total viral genome yield and immuno-titration results (Fig. 3 and Table 1). Using several cycles of freezing/thawing, the physical cell disruption method can alter the integrity of viral capsids without destroying the genome-full particles that can still be quantified by quantitative PCR. In other words, some of these vectors could be released in supernatant long before the end of the production phase. The progressive acidification of the culture medium during the upstream process, resulting from the cell metabolism, and the presence of proteases originating from producer cell lysis both provide an unfavorable environment for released rAd5 particles and lead to structural alteration of the supernatant-derived vectors [38]. These findings can have a significant impact on large-scale production. The results of the current study suggest that 2-h of incubation gives the best results when incubated at 37 °C for denarase activity when Tween-20 is used for virus recovery in the cell suspension. If the room temperature is preferred for this suspension, 4-h incubation could be used.

Although the methods of cell lysis and clarification affect the virus yield, the purity of the product depends on the chromatography and ultrafiltration steps, which are relatively more standard methods in the later stages. This study mainly targeted the detergent lysis method, incubation temperature and period, and then the microfiltration method. The methods investigated in our study are those used on an industrial scale. However, the effects of incubation temperature, period, and membranes used in microfiltration on adenovirus yield before moving to further purification steps have yet to be well known. HEK293 cells or their derivatives are generally used to produce rAd vectors. We used the RFT and DLT methods in our study of vector production that can be effective in all mammalian cells [46, 54]. Especially the DLT method used in this study as a bench scale is also used in large-scale production. The data we obtained on the bench scale regarding incubation temperature and period can also be applied on a large scale. The data we obtained on the bench scale for microfiltration, especially the parameters such as sample volume, flow rate, and membrane diameter, can also affect the filtration efficiency at a large scale. The lack of purification steps after microfiltration in this study is a limitation. In addition, empty to full ratio and agglomerates affect virus infectivity. Another limitation is that microfiltration is not carried out at different pressures. However, the pressure applied during microfiltration during large-scale production might affect the virus yield. Therefore, the microfiltration step should be optimized for large scale.

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Data Availability All data generated or analyzed during this study are included in this article.

Declarations

Conflict of interest The authors declare no commercial or financial conflict of interest.

Ethical Approval Our study did not require an ethical board approval because it did not contain human or animal subjects.

Consent for Publication Not applicable.

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