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OPTIMIZATION OF VERO CELL EXPANSION AND FLAVIVIRUS ANTIGEN PRODUCTION IN A BIOREACTOR USING SERUM-FREE MEDIUM

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ABSTRACT

Japanese Encephalitis (JE) remains a significant global public health challenge in Asia, necessitating the development of efficient and scalable vaccine production methods. Traditional vaccine manufacturing often relies on serum-containing media and static culture systems, which pose risks of contamination and limited scalability. Vero cells are widely accepted mammalian cell lines used in research and commercial applications, particularly for vaccine production in both human and veterinary medicine(s). This article presents a refined methodology using serum-free media and microcarrier-based Vero cell bioreactor systems to enhance the productivity and safety of the SA 14-14-2 JEV strain. By employing Vero cells cultivated on Cytodex-1 microcarriers within a 3L bioreactor and utilizing serum-free media (VP-SFM and Cellin-1), we established a robust platform for high-titer vaccine manufacturing. Comparative analysis between static (CS-10) and suspension bioreactor cultures revealed that the bioreactor system significantly enhanced cell yields by approximately 30% (2.69×10^5 cells/cm². Furthermore, viral titers in the bioreactor reached 2.20 x 10^7 PFU/mL, representing a 10–15% increase over static conditions. These findings provide a standardized framework for industrial-scale, serum-free production of JE vaccines with improved efficiency and quality control. This optimised upstream process offers a scalable, safer, and more cost-effective platform for manufacturing flavivirus vaccines, thereby improving global access and pandemic preparedness.

KEYWORDS: *Japanese encephalitis virus*, Vero cells, Microcarriers, *Flavivirus*, Serum-free media, Bioreactor.

1. INTRODUCTION

Japanese Encephalitis Virus (JEV) is a neurotropic arthropod-borne flavivirus of the *Flaviviridae* family and *Ortho flavivirus* genus. It remains a major predominant cause of viral encephalitis and has a wide distribution in many countries across Asia and the Western Pacific and in northern Australia (Chugh et al., 2025; Solomon et al., 2003; Burke et al., 1988). JEV is a mosquito-borne flavivirus (Simmonds et al., 2017) and was first identified in 1871 in Japan. It was first isolated in 1935 from the brain of a fatal case of JE (Srivastava et al., 2023). The virus is primarily transmitted by *Culex* mosquitoes and maintained in an enzootic cycle involving birds as reservoir hosts and pigs as amplifying hosts (Samy et al., 2018). Symptomatic cases are uncommon and occur in approximately 1 in 250 subclinical infections. JEV is a major threat, with a case fatality rate up to 30% among those with disease symptoms. The infection causes a spectrum of clinical illnesses that begins with flu-like symptoms, neck stiffness, disorientation, coma, seizures, spastic paralysis, and eventually death. Although humans are incidental, dead-end hosts, JEV infection can lead to several neurological diseases, particularly in children, with high rates of mortality and lifelong neurological sequelae among survivors (Schuh et al., 2013). The newborns and the children below the age of 15 years are more vulnerable to JEV with the increased threat of neurological complications over adults (Campbell et al., 2011). Almost 2 billion people living in endemic countries face a constant threat of JEV, and an upsurge in

the mosquito populations poses a risk of expansion into new geographic areas.

In India, the first evidence of JEV was obtained through serological studies in 1952, and the first recorded JEV case was in 1955. Later on, frequent disease outbreaks were reported in all parts of India at regular intervals (Tiwari et al., 2012; Kulkarni et al., 2018). A major outbreak occurred in the Bankura district of West Bengal in 1973, with a fatality rate of 42.6% (Tiwari et al., 2012). The most prolonged epidemic of JEV was witnessed in the Gorakhpur district of Uttar Pradesh in 2005 (Parida et al., 2006; Halstead and Thomas, 2011), with more than 5500 documented cases of viral encephalitis and ~23% fatalities (Tiwari et al., 2012; Prakash et al., 2021; Solomon, 2006). Several reports have indicated the expansion of the virus to non-endemic areas, including the northern and north-eastern parts of the Indian continent. Cases have been reported, signifying the spread of the virus, including in urban areas such as New Delhi (Walsh et al., 2022; Singh et al., 2019). It is one of the major public health problems not only because of a large number of deaths but also due to severe neuro-psychiatric sequelae that necessitate lifelong support, amounting to a considerable socioeconomic burden (Campbell et al., 2011; Solomon, 2006).

The effectiveness of vector control strategies is limited due to the complex eco-epidemiology of the virus. Vaccination is the most effective means of prevention, where JEV is a major public health problem. The lifecycle of JEV is maintained through an enzootic cycle involving avian reservoir hosts, and due to its ecological persistence across both avian and mammalian populations, the virus remains firmly established in nature, suggesting that global eradication is an improbable goal for the foreseeable future (Kulkarni et al., 2018). Thus, effective antiviral therapy and an ideal vaccine against JEV are of pressing priority. With no specific antiviral treatments available, vaccination is the only effective method to prevent infection and control outbreaks. Vaccines are available in various forms, including live-attenuated, inactivated, and recombinant subunit vaccines. Vaccines play a critical role in global public health by preventing infectious diseases in both humans and animals. Many viral vaccines are produced using mammalian cell culture systems, which can be cultivated either as adherent or suspension cultures. Commonly used adherent cell lines include MRC-5, MDCK, PK-15, CEF, and Vero cells. The manufacturing of many human viral vaccines utilises the Vero continuous cell line, derived from the kidney of an African green monkey (Yasumura and Kawakita, 1963). The regulatory bodies approve Vero cells for their safety profile and permissiveness to a broad range of viruses, including poliovirus, rabies virus, and various flaviviruses. Large-scale production involves technologies like roller bottles or microcarriers in stirred-tank bioreactors to provide the necessary surface area for cell growth.

Traditionally, Vero cells have been cultivated as adherent monolayers on the surface of roller bottles or cell stacks in media supplemented with fetal bovine serum (FBS). However, this method is fraught with significant limitations that are increasingly untenable for modern vaccine production. The reliance on FBS introduces inherent risks of adventitious agent contamination, notable batch-to-batch variability, and high costs, which compromise process consistency and regulatory compliance. Furthermore, the limited surface area of adherent culture systems severely restricts scalability, making it impractical to meet the multi-thousand-liter volumes required for global vaccine campaigns. However, a major challenge in using Vero cells is their anchorage-dependent nature, which complicates upstream processing, is labour-intensive, and limits scalability. The use of animal-derived components introduces variability, potential for contamination with adventitious agents, and increases production costs, creating a recognised need to transition to serum-free or chemically defined media. Currently, large-scale vaccine production using Vero cells is carried out with microcarriers to increase the available surface area for adhesion. Despite achieving remarkable productivity, the process remains limited by surface area.

To address all these issues, the current research has focused on adapting Vero cells using chemically defined, serum-free media. This approach allows for cultivation in bioreactors, which provides a more controlled environment, simplifies process automation, and enables a significantly higher cell density per unit volume, directly translating to increased viral antigen yield and reduced production costs. However, adapting Vero cells to grow in suspension presents challenges, including the formulation of appropriate serum-free media, maintaining high viability, and managing prolonged doubling times. This study aimed to optimize Vero cell growth for flavivirus antigen by adapting the cells to a serum-free suspension culture medium (SFM) environment using microcarriers in a bioreactor system, and to evaluate the impact on virus productivity. The key optimization parameters include the cell adaptation protocol, selecting an SFM that supports high cell densities while minimizing aggregation, and process intensification via perfusion culture. Furthermore, these adapted cells are highly productive for viral antigens, with high-density perfusion culture intensification increasing volumetric productivity. This optimized upstream process provides a scalable, safer, and more cost-effective platform for manufacturing flavivirus vaccines, improving global access and pandemic preparedness.

2. MATERIALS AND METHODS

2.1 Cell line sourcing: Vero cells used in the study were originally sourced from ATCC (CCL-81) and maintained

in the serum-free VP-SFM (Gibco) culture media at 37°C, 5% CO_2 conditions. A two-tier banking system, both master cell bank (MCB) and working cell bank (WCB), was established and stored in vapor phase of liquid nitrogen using serum-free media.

2.2 Viral Seed: The study utilized the SA 14-14-2 strain, a live, attenuated strain of the Japanese encephalitis virus (JEV) for viral seeding, and the research working virus bank used for this study had a seed stock titer of 8.38×10^6 PFU/mL.

2.3 Culture Media Formulation: The commercially available, powdered VP-SFM (Gibco) and Cellin-1 (Himedia) were formulated in-house by reconstituting in an ambient Water for Injection (WFI) with 4 mM L-glutamine as an energy source for rapid cell growth. The final pH was adjusted to a range of 7.0 and 7.3 using sodium bicarbonate. Neomycin was added at a concentration of 10 mg/L as a growth media supplement, and the media were sterilized using a 0.2 µm filtration system, followed by sterility testing using Tryptic Soy Broth (TSB) and Sabouraud Dextrose Broth (SDB), resulting in no signs of contamination.

2.4 Microcarrier Preparation: Cytodex-1 microcarriers, composed of cross-linked dextran polymers with positively charged DEAE groups, were utilized throughout the matrix. These microcarriers provide a surface area of 4400 cm^2 per gram of hydrated material. All materials available in presterilized form, the microcarriers underwent further processing, including overnight hydration and autoclaving, before being introduced into the bioreactor. The sterility was confirmed through direct inoculation into Fluid Thioglycolate Medium. Additionally, the mycoplasma contamination screening was assessed using a species-specific PCR assay that targets the *M. fermentans*, *M. orale*, *M. neurolyticum*, *M. hyopneumoniae*, and *M. capricolum* species. All the materials used were confirmed free of mycoplasma contamination before use.

2.5 Schematic workflow and Scale-up: The scale-up workflow involved the sequential passage of Vero cells from CS-1 containers through expansion in CS-5 and CS-10 cell stalks and containers, followed by seeding into a 3L Eppendorf Bioflo 320 bioreactor with a 2.2 L working volume containing Cytodex-1 microcarriers, providing a total surface area of 30,800 cm^2, equivalent to 5 X CS-10. Cell stalks were used instead of traditional flasks to reduce manual manipulation and facilitate easier scale-up. Cells were infected with JEV at an MOI of 1:500 (0.002), and four harvests were collected over five days post-infection for analysis. The use of advanced cell stalk static containers rather than traditional flasks favoured higher scale-up options and reduced manual manipulation. The schematic scale-up workflow was represented in a flow chart, as shown in Figure 1.

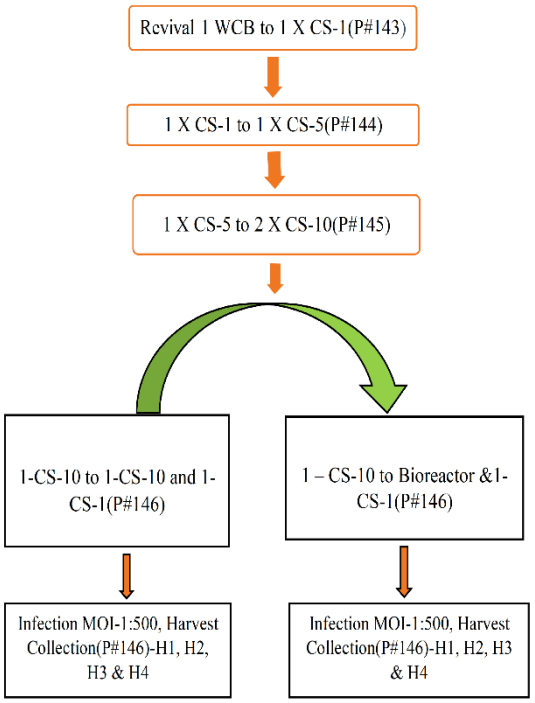

Figure 1: The schematic representation of the flow process and Scale-up.

2.6 Vero Cell Revival: The Vero cells were thawed from the research working cell bank (WCB) with $1.2 \times 10^{\wedge 7}$ cells/mL, centrifuged at 1000 rpm, 5 min, 25±2°C, and incubated at 36°C with 5% CO_2 for 6 days. The media was replenished between 24 and 48 hours. The monolayers with 70 - 80% confluency were detached using TrypLE Select Cell Detaching Solution and neutralized with VP-SFM. The viable cell counts were determined using trypan blue exclusion stain using a hemacytometer or Countess II cell and viability counter.

2.7 Subculturing and Bioreactor Cultivation Process: The subculturing and the bioreactor cultivation were performed in a 3L Eppendorf Bioflo 320 bioreactor, with a working volume of 2.2 L and a 30,800 cm^2 of total surface area. The Vero cells were seeded at ~15,000 cells/ cm^2. The cultural parameters were maintained at 37°C, pH 7.2, DO 20–40%, at 60–150 RPM. The process parameters were managed with Bio-command automation software, which ensured high-fidelity maintenance of the cellular environment. The software regulated pH levels at 7.2 through the precision-sparging of CO_2 gas and the automated addition of alkali.

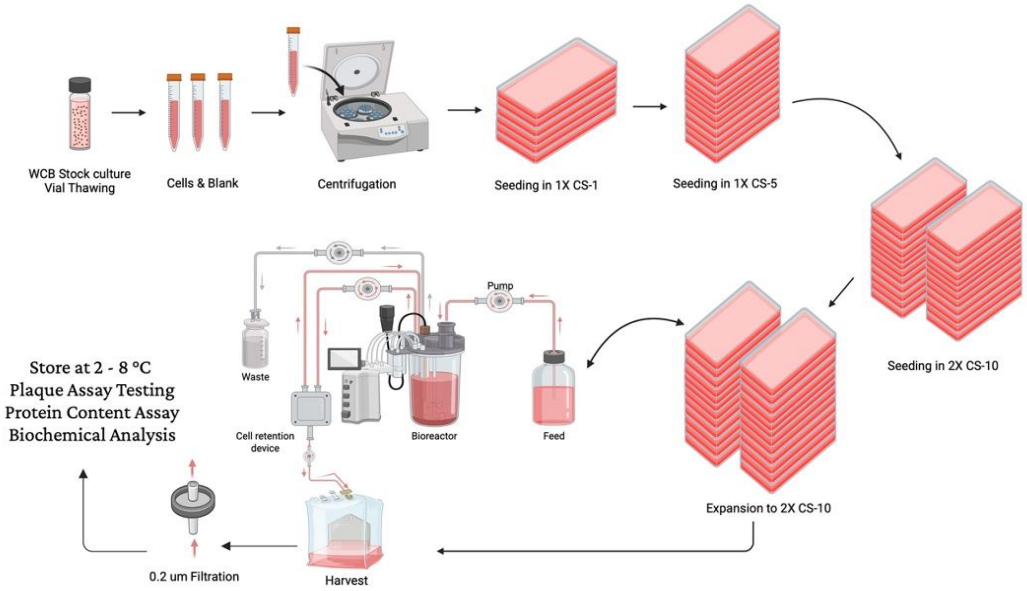

Figure 2: Pictorial representation of experimental flow with Cell stalks and bioreactor.

Dissolved oxygen was maintained at a setpoint of 20–40% saturation; oxygen demand was satisfied via an automated gas-mixing manifold using a combination of medicinal-grade oxygen, CO_2, and process air. Temperature was held constant at 37 °C, and agitation was modulated between 60 and 150 RPM to maintain microcarrier suspension while minimizing hydrodynamic shear stress. The overall experimental process from vial thawing to harvest using Cell stacking and a bioreactor system follows as shown in Figure 2.

2.8 Virus Infection and Harvesting: The viral Infection was initiated at a Multiplicity of Infection (MOI) of 0.002, followed by a 24-hour adsorption period at 36°C. The culture was washed twice with VP-SFM post-adsorption to remove the residual amount of FBS from the culture and unbound virus. Four sequential harvests (H1–H4) were collected every 24-hour intervals starting at Day 3 post-infection, followed by filtration through a 0.2µm filter as shown in Figure 2. The post-filtered samples were collected for the titer value and protein analysis, and the remaining volume was stored at 2- 8 °C for further analysis.

2.9 Quantitative and Analytical Methods

2.9.1 Plaque Assay Testing: The plaque assay was utilized as a quantitative method to measure the concentration of infectious virus particles. The procedure relied on identifying clear zones that form on a host cell layer following viral infection. The process involved the serial dilution of the virus followed by inoculation onto a cell monolayer. The diluted samples were added to the cells and subsequently covered with a semi-solid agar medium to restrict viral spread, as the virus infected and destroyed host cells, localized regions of cell death (plaques) developed. These plaques were counted to calculate infectious units per volume, with each visible plaque representing a single virus particle from the original sample.

2.9.2 Protein Content Assay: The protein concentrations of Japanese encephalitis virus (JEV) proteins were quantified using the Bio-Rad Protein Assay. This colorimetric method is based on the Bradford dye-binding technique, using IgG as the standard. The protein sample was mixed with a dye reagent that binds to the proteins, including a shift in the dye's absorbance maximum. The procedure was standardized using purified IgG, which shares chemical and structural similarities with many viral antigens, thereby ensuring high accuracy in JEV protein quantification. A linear range of different concentration solutions from 1.2 to 25µg/mL was established using the IgG standard. Samples were mixed with dye reagent, vortexed, and incubated at room temperature for 10 minutes. The absorbance was read at 595 nm using a spectrophotometer to extrapolate concentrations from the standard curve.

2.9.3 Biochemical Analysis: Glucose, lactate, and ammonia levels were measured in bioreactor samples using a YSI 2950D Biochemical Analyzer, a benchtop instrument for rapid quantification and monitoring of cell culture processes. Spent media samples were collected from the bioreactor and, if necessary, centrifuged to remove debris. Samples were loaded onto the analyzer via substrate-specific enzymatic reactions and electrochemical detection. All results were displayed and exported within one minute.

3. RESULTS

3.1 Cell growth and metabolite analysis

The bioreactor system significantly demonstrated superior cell growth and outperformed static cultures in cell expansion. The suspension bioreactor culture system achieved a higher yield of 2.69 × 10^5 cells/cm², compared to static CS-10 cultures with a yield of ~2.0 × 10^5 cells/cm², representing approximately 30% higher increase over the CS-10 static culture. Metabolic activity was higher in the bioreactor, leading to faster glucose consumption. Media changes were performed based on metabolite levels to remove toxic by-products. Metabolic monitoring ensured that ammonia and lactate levels remained below inhibitory thresholds within acceptable non-cytotoxic ranges <5 mM and <15 mM, respectively. The faster glucose consumption in the bioreactor necessitated strategic media changes to replenish nutrients like L-glutamine and remove used metabolic byproducts. While static containers lack real-time visualization of pH and metabolites, the bioreactor allowed for precise media changes based on glucose consumption, enhancing doubling rates. The stages of cell expansion across different containers, including a bioreactor system, were tabulated in Table 1.

Table 1: Different stages of Cell expansion with seeding density and Total cells achieved.

Stage	Container	Surface area(cm²)	Seeding Density (cells/cm²)	Total Cells achieved/cm²
Revival	CS-1	636	17,800	217000
Passage-1	CS-5	3180	20000	232666
Passage-2	CS-10	6360	20000	225666
Passage-3	CS-10	6360	15000	205000
Passage-3	Bioreactor	30800	15000	269333

In the bioreactor, the consumption of Glucose started post 24 hrs, and increased in the later stages based on set points bioreactor system maintained critical parameters like pH and DO. In static containers, tissue culture flasks, roller bottles, and Cell stalks/factories, they also help in culturing monolayered cell lines, with facilitating features like Cell Bind surfaces to scale up to some extent. The basic disadvantage of static containers is fewer chances to scale up, it requires more technical labour to perform aseptic manipulations, and the chances to visualize and to control monitoring of pH and metabolite parameters and their accumulation are very limited compared with the Bioreactor system. In the data table, the average yield of three consecutive experiments at each stage was considered to plot the graph, as shown in Figure 3, which is the graphical representation showing average values of initial seeding of cells /cm² versus the final output total cells achieved per cm² yield, i.e., the input and output details and different stages of cells scale-up. Glucose consumption was faster in bioreactor cultures due to higher metabolic activity, necessitating more frequent media changes. The data performed is from a single media change in a bioreactor due to targeting cell growth mimic with the CS-10 process, for comparative analysis.

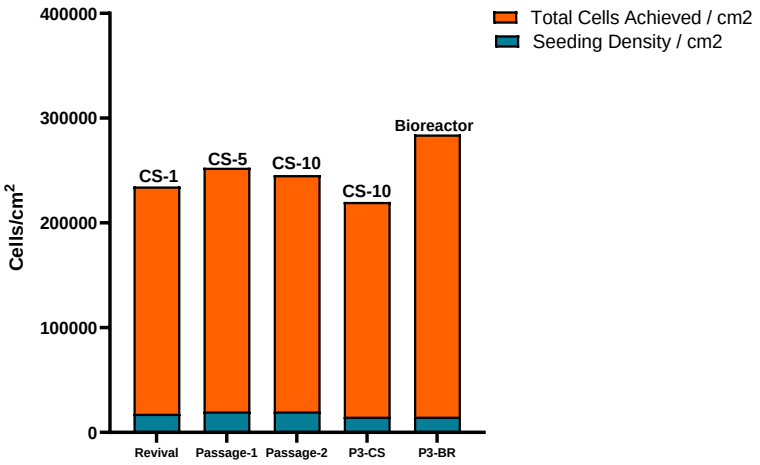

Figure 3: A graphical view of cell yield seeding/cm² with total cells achieved per cm².

3.2 Vero cells growth pattern in a Bioreactor: The Vero cells in the bioreactor system will attach onto the micro carrier upper surfaces and divide and crawl the entire surface in a day-wise manner. The microcarriers were synthetic materials designed for cell scale-up and batch processing of adherent cell lines in suspension. The microcarriers facilitate large surface areas in a limited volume. Optimal cell growth was achieved by maintaining appropriate growth nutrients in the media, and media changes were made based on metabolite levels measured with the YSI 2950 biochemical analyser. The metabolite changes based on glucose consumption

enhance cell growth in terms of cell doubling. The overall Vero cell growth pattern in a bioreactor system from days 1 to 6 is shown in Figure 4.

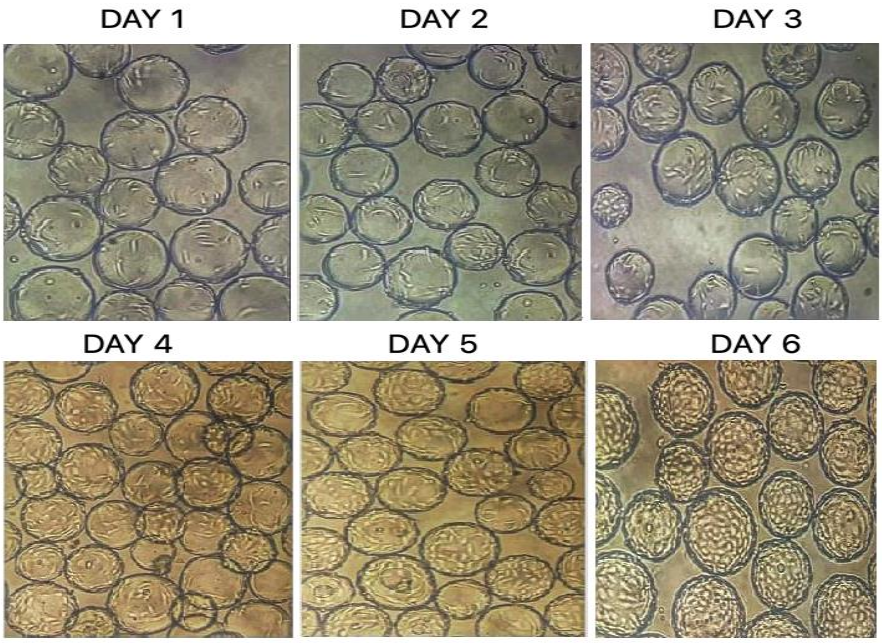

Figure 4: Vero cells' growth pattern in a bioreactor on different days of culture.

3.3 Virus Productivity Assessment: Viral harvesting of Vero cells occurred in a bioreactor, and Cell stalks in four sequential batches (H1–H4). The bioreactor consistently yielded higher titers and protein concentrations compared to the Cell stalks CS-10. The average viral titer enhancement in the bioreactor was estimated at 10–15%, as shown in Table 2.

Table 2: Viral yield productivity assessment of Bioreactor and CS-10.

Container	H1	H2	H3	H4
Bioreactor (Pfu/mL)	1.83E+07	1.96E+07	2.20E+07	2.05E+07
CS-10(Pfu/mL)	1.60E+07	1.81E+07	2.00E+07	2.04E+07

3.3.1 Plaque Assay analysis: In continuation of the post-cell scale-up in the bioreactor, the cell culture was infected with the Japanese Encephalitis virus (JEV) SA 14-14-2 virus strain, and to evaluate the efficiency of the bioreactor system, viral titers from collected harvests were quantified using the plaque assay across the three independent experimental runs, as shown in Figure 5. The bioreactor system consistently outperformed the static CS-10 cultures, resulting in peak titters between 1.83 x 10^7 pfu/mL and 2.20 x 10^7 pfu/mL. In comparison, the static cell cultures produced lower yields, ranging from 1.60 x 10^7 pfu/mL to 2.0 x 10^7 pfu/mL. On average, the transition to a bioreactor-based system resulted in a ~10-15% enhancement in viral titer compared to traditional static methods, resulted in demonstrating the superior scalability and productivity of the optimized upstream process.

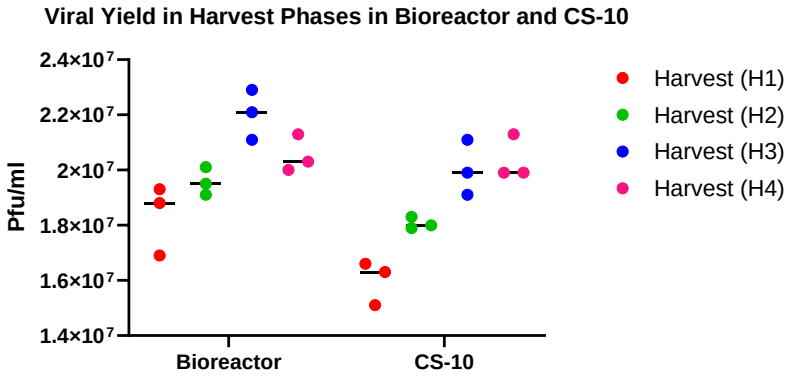

Figure 5: Viral Yield in Harvest Phases in Bioreactor and CS-10.

3.3.2 Protein Content Analysis: As protein content measurement was a critical metric for monitoring the vaccine production, the total protein concentration of the Japanese encephalitis virus harvests was determined, and all the continuous harvests' protein concentrations were quantified using a Bio-Rad protein assay with IgG as the reference standard. Corroborating the plaque assay data, the protein content analysis reflected higher productivity in the bioreactor-derived cultures. These findings indicate that suspension-adapted Vero cells maintained in a bioreactor under serum-free conditions support superior virus yields and volumetric productivity compared to traditional static substrates. Overall protein content values of harvests (H1 to H4) were tabulated as shown in the table 3.

Table 3: Viral protein content value in Harvest phases.

Container	H1	H2	H3	H4
Bioreactor(µg/mL)	86.7	95.3	106.3	91.7
CS-10(µg/mL)	71.7	81.1	89.0	70.0

4. DISCUSSION

Japanese Encephalitis Virus (JEV) is a neurotropic flavivirus of the family *Flaviviridae,* and the *Ortho flavivirus* genus remains a major predominant cause of viral encephalitis in many countries across Asia and the Western Pacific and in northern Australia (Chugh et al., 2025; Solomon et al., 2003; Burke et al., 1988). It is a vector-borne flavivirus that was reported for the first time in 1871 in Japan, and JEV was first isolated in 1935 from the brain of a fatal case of JE (Srivastava et al., 2023). It is one of the major public health problems not only because of infected cases and a large number of deaths but also due to severe neuro-psychiatric sequelae that necessitate lifelong support, amounting to a considerable socioeconomic burden (Campbell et al., 2011; Solomon, 2006).

Nonetheless, JEV has an enzootic cycle involving mammals and birds, due to which the virus can persist in nature to such an extent that it might be next to impossible to eradicate the virus in the near future (Kulkarni et al., 2018). Thus, effective antiviral therapy and an ideal vaccine against JEV are of pressing priority. Vaccination is the most cost-effective therapeutic intervention. Despite challenges, such as a lack of an appropriate drug delivery system or a treatment plan independent of the stage of infection, JEV research has increased rapidly with growing technological advancements, with the primary aim of developing safe and cost-effective therapeutic vaccines for all age groups.

The existing studies on JE-VAX are formalin-inactivated virions prepared from JEV-infected mouse brain, which is expensive to manufacture and may carry potential risks such as allergic reactions to brain basic proteins. In addition, there are biosafety issues of propagating infectious JEV in mice. Therefore, the development of second-generation JEV vaccines has been promoted by the World Health Organization (Chambers et al., 1997). Earlier attempts have been initiated to produce high titers of the JEV vaccine strains in Vero cell cultures and to develop improved, inactivated whole-virion JEV vaccines (Srivastava et al., 2001; Sugawara et al., 2002). The Vero cell-derived vaccine is likely to have significant advantages in the absence of mouse brain proteins and in higher antigenicity than brain-derived JE-VAX (Chambers et al., 1997; Kinney and Huang, 2001).

Traditionally, Vero cells have been cultivated as adherent monolayers on the surface of roller bottles or cell stacks in media supplemented with fetal bovine serum (FBS). However, this method is fraught with significant limitations that are increasingly untenable for modern vaccine production. The reliance on FBS introduces inherent risks of adventitious agent contamination, notable batch-to-batch variability, and high costs, which compromise process consistency and regulatory compliance. Furthermore, the limited surface area of adherent culture systems severely restricts scalability, making it impractical to meet the multi-thousand-liter volumes required for global vaccine campaigns. To address these issues, research has focused on adapting Vero cells to suspension culture and using chemically defined, serum-free media. This approach allows for cultivation in bioreactors, which provides a more controlled environment, simplifies process automation, and enables a significantly higher cell density per unit volume, directly translating to increased viral antigen yield and reduced production costs. Traditional methods of cultivation in serum-containing media, and tremendous efforts have been made to adapt them to animal-component-free media. Indeed, removing serum reduces the risk of contamination with viruses, mycoplasmas, or prions (Paillet et al., 2009), as well as batch-to-batch variation and purification costs (Caron et al., 2018). However, removing serum can reduce cellular growth and density, necessitating medium optimization and cell adaptation.

The major concerns of the Vero cell-based vaccine are, however, the safety and cost during vaccine production. A large amount of JEV needs to be propagated in cultures, and then collected and purified before formalin-inactivation, procedures requiring high levels of biosafety containment according to the Good Manufacturing Practice. This may cause pressure on the cost and may prevent developing countries in the endemic areas from vaccinating large at-risk populations. Therefore, second-generation JEV vaccines available from more efficient, low-cost manufacturing are desirable for mass vaccination. Among the Continuous cell lines currently used in the vaccine industry, such as MDCK, CHO, PER.C6, and Vero cells, have been both the first to be approved by the WHO and the most widely used cell line for viral vaccine production in the last 25 years (Bourigault et al., 2025; Hussain et al., 2016; WHO, 2013). The Vero cells are a continuous adherent cell line isolated from African green monkeys' kidney

epithelial cells in 1962 (Yasumura and Kawakita, 1963). In 2014, genome sequencing revealed that the cells originated from a female *Chlorocebus sabaeus* (Osada et al., 2014). Vero cells were characterized by their spontaneous establishment and aneuploidy, with an average of 58 chromosomes in more than 65% of the cells. They have been used as a substrate for vaccine production since the 1980s. Their widespread use in viral vaccine production stems from their significant advantages in this application. The Vero cell line is susceptible to infection by viruses from various families, including the poliovirus (*Picornaviridae*), the rabies virus (*Rhabdoviridae*), and the influenza virus (*Orthomyxoviridae*) (Velcken et al., 2013). In addition, it facilitates viral replication due to a lack of type 1 interferon production (Hegde, 2015). Since its first US Food and Drug Administration approval in the 1980s (Duigou, 2010), the cell line has received greater attention and is currently deemed nontumorigenic and safe to use as long as it stays within the range of 150 passages (Grachev et al., 1998).

Currently, large-scale vaccine production using Vero cells is carried out with microcarriers to increase the available surface area for adhesion. Despite achieving remarkable productivity, the process remains limited by surface area. The current study established a robust, serum-free manufacturing process for the Japanese encephalitis virus (JEV) vaccine using the SA 14-14-2 strain. By utilizing Vero cells anchored to Cytodex-1 microcarriers and maintained in specialized serum-free media (VP-SFM and Cellin-1), we established a scalable upstream process. This process provides a standardized framework for the industrial-scale production and demonstrates an optimized, scalable upstream process to produce the Japanese encephalitis virus (JEV) vaccine using the live-attenuated SA 14-14-2 strain. By employing Vero cells cultivated on Cytodex-1 microcarriers within a 3L bioreactor and utilizing serum-free media (VP-SFM and Cellin-1), we established a robust platform for high-titer vaccine manufacturing. Comparative analysis between static (CS-10) and suspension bioreactor cultures revealed that the bioreactor system significantly enhanced cell yields by approximately 30% (2.69×10^5 cells/cm²). Furthermore, viral titers in the bioreactor reached 2.20×10^7 PFU/mL, representing a 10–15% increase over static conditions.

These findings provide a standardized framework for industrial-scale, serum-free production of JE vaccines with improved efficiency and quality control. Furthermore, these adapted cells are highly productive for viral antigens, with high-density perfusion culture intensification increasing volumetric productivity by over tenfold. This optimized upstream process provides a scalable, safer, and more cost-effective platform for manufacturing flavivirus vaccines, improving global access and pandemic preparedness. The results indicate that suspension-adapted Vero cells grown in a microcarrier-based bioreactor under serum-free conditions support significantly higher virus and protein yields compared to traditional static cultures. The bioreactor system offers better visualization and control of critical parameters such as pH and dissolved oxygen, which are limited in static containers like Roller bottles or Cell stalks. Although static containers are useful for initial scale-up, they require more intensive manual labour and carry a higher risk of aseptic manipulation failure. The integrated use of YSI biochemical analysis and automated bioreactor controls allows for optimized media conditioning, resulting in a more efficient and predictable production cycle. The transition from static "Cell Stalk" containers to a microcarrier-based bioreactor provides several advantages. Static systems suffer from limited scale-up potential and high labour requirements for aseptic manipulation. Conversely, the bioreactor system utilizes automated control of pH, DO, and temperature, ensuring a more stable environment for the Vero cell proliferation. The increased surface-area-to-volume ratio provided by the Cytodex-1 microcarriers allows for high-density growth in a compact footprint. Our data confirms that suspension-adapted Vero cells in serum-free media support superior viral replication, likely due to optimized nutrient availability and efficient metabolite removal through programmed media changes. The highest virus yield was observed using microcarriers with Vero cells resulted high yield in the Bioreactor system compared to cell stalks. Furthermore, to streamline Japanese encephalitis virus cultivation, we employed a serum-free medium devoid of animal-derived components. Accordingly, this study aimed to enhance viral yield by employing an efficient substrate, optimizing conditions for viral replication, and simplifying the bioprocess using a serum-free medium and modern technological approaches.

5. CONCLUSION

This study successfully established a robust and scalable serum-free production process for the JEV SA 14-14-2 vaccine. The transition from static cultures to an optimized 3L bioreactor system utilizing Cytodex-1 microcarriers increased cell density by 30% and viral yields by up to 15%. This methodology provides a robust foundation for high-capacity vaccine manufacturing while maintaining stringent quality and purity standards. With higher cell yields, enhanced viral titers, and better metabolic control, this process provides a robust framework for large-scale vaccine manufacturing. Beyond yield improvements, the integrated bioreactor platform offers superior metabolic process control and improved scalability, which are essential for maintaining stringent quality and purity standards in vaccine production. By utilizing serum-free media, the process further minimizes biological variability and reduces contamination risks, making it a safer and more cost-effective solution for the global market. Ultimately, the methodologies presented in this study provide a promising and versatile platform for the large-scale production of flavivirus antigens. This optimized upstream process not only strengthens the infrastructure

for current JEV vaccination efforts but also serves as a critical model for rapid-response vaccine manufacturing in the face of emerging global health threats and future pandemics.

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Author Contributions
The authors, PKC and PCM, designed the experiments. PKC, PCM, and PB carried out the experimental work. PKC and PCM conceptualised the complete work and contributed to writing and developing the manuscript. SP and PCM helped in the design and critically reviewed the manuscript. All the authors read and approved the final copy of the manuscript.

Consent for publication
Not applicable.

Ethical Clearance
The institutional ethical committee approved the study.

Conflict of Interests
The authors declare that they have no conflict of interest.

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