

Optimization of Clarification Step in Pneumococcal Polysaccharide Conjugate Vaccine Manufacturing: Evaluating the Performance of Depth Filters

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ABSTRACT

Pneumococcal infections, stemming from *Streptococcus pneumoniae*, present a substantial health risk, especially among vulnerable populations. To counter this, vaccines employing bacterial capsular polysaccharide (CPS) have been developed. However, the purification of CPS is a pivotal vaccine manufacturing step, with the clarification process playing a crucial role in achieving optimal product yield and purity. This research focuses on refining the clarification step by assessing the performance of various depth filters. The study explores both conventional cellulose-based and innovative synthetic depth filters, utilizing pneumococcal fermentation harvests from two challenging serotypes. Evaluation criteria include hydraulic performance, throughput, and filtrate turbidity. Employing the constant flow rate sizing method for scale-up, the study determines that synthetic depth filters with suitable pore sizes demonstrate low resistance and high throughput, suggesting their efficiency in impurity removal. Overall, optimizing the clarification step is crucial for improving downstream processing of pneumococcal polysaccharide conjugate vaccines, and this study provides valuable insights into selecting and optimizing depth filters for efficient clarification, ensuring high product quality and process efficiency in vaccine manufacturing.

Pneumonia is caused by *Streptococcus pneumoniae* (*S. pneumoniae*), a gram-positive coccus that also causes, otitis media, meningitis, and bacteremia in pediatric, elderly, and immunocompromised populations (1). The leading cause of pneumonia in children, worldwide, is pneumococcal infections (2).

Pneumococcal infections also occur frequently in at-risk populations including individuals with diabetes, asthma, chronic obstructive pulmonary disease, cardiovascular disease, human immunodeficiency

virus (HIV), and sickle cell disease. Pneumococci express chains of one of 100 immunologically distinct polysaccharide subunits anchored to the cell wall surface. These polysaccharide chains protect pneumococci from complement-mediated opsonophagocytosis, the main mechanism of clearance of pneumococci from the lungs. Production of this polysaccharide chain is essential for pneumococcal colonization and virulence, making it an obvious early target antigen (3). To elicit a protective immune response in children

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under the age of two, pneumococcal conjugate vaccines are developed using the CPS and different carrier proteins such as cross-reacting materials (CRM197), tetanus toxoid, diphtheria toxoid, and other proteins. These conjugated antigens generated a T-cell-dependent antibody response that was effective in children under two years of age. They also have proven efficacy at generating higher antibody titers in high-risk individuals immunized with the unconjugated vaccine. The first 7-valent pneumococcal polysaccharide conjugate vaccine (PCV7- 4, 6B, 9V, 14, 18C, 19F, and 23F) was developed in 2002. The second conjugate vaccine that was developed, the 13-valent pneumococcal conjugate vaccine (PCV13-1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F), contained the seven serotypes of PCV7, five serotypes found in PPSV23 (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F). All these conjugate vaccines require purified polysaccharide produced by fermentation followed by purification. The high purity required for polysaccharides specific to medical and drug manufacturing has led to the development of new purification methods based on fractional precipitation, ion exchange chromatography, gel filtration, and affinity chromatography (4). A clarification step is essential post fermentation and before subsequent downstream purification steps. A suitable clarification step primarily removes cell debris, colloids, and large aggregates to reduce burden on the downstream processing for its final purification. In certain cases, clarification also reduces insoluble impurities, host cell proteins (HCPs), and host cell nucleic acids. Like any other purification step, the clarification step needs to be optimized to achieve maximal product yield and purity while accommodating for vaccine specificities and manufacturing constraints (5).

Diverse technologies, such as centrifugation, or alternative filtration technologies, such as depth filtration, are used for clarification due to the heterogeneity of vaccine types because pneumococcal vaccines require multiple sero-specific strains for producing sero-specific polysaccharides. Several series of operations are often required to achieve desired clarification before further processing. The first operation is aimed at removing larger particles (primary clarification) and a second one for removing colloids and other sub-micron particles (secondary clarification). Depth filters are easy to scale-up, inexpensive, and high capacity but may be prone to recovery issues. Concern over extractables and leachables and sizing needs to account for inherent variability in product (5).

The first step post-fermentation is clarification, which removes cell debris and colloidal impurities to protect and enhances the performance of the subsequent downstream process. The clarification step, being at the interface between the upstream and downstream processes, is sometimes neglected during vaccine process

development as time and resources are preferentially allocated to other purification steps, such as chromatography, density gradient centrifugation, or ultrafiltration. The limited literature available reflects the lack of systematic efforts on this topic. Even vaccine manufacturing process patents often omit clarification steps. However, in the case of a pneumococcal vaccine being multi-serotype and highly viscous in nature, clarification efficiency directly influences the performances of the downstream process. A poorly optimized clarification step can negatively impact the capacity of sterilizing-grade filters or may shorten a chromatography resin lifetime (5). Depth filtration is widely used in downstream bioprocessing to remove particulate contaminants and is therefore applied to harvest clarification and other processing steps. Depth filters remove soluble materials, suspended particles, submicron particles, and colloidal material by taking advantage of the depth of a particular media to enable high levels of contaminant removal. During depth filtration, either the particles in harvest

Table 1. List of depth filters with materials of construction (MOC) and pore sizes.

Sr. No	Filter family	Filter type	Manufacturer	Filter media	Pore size (μm)
1	Millistak+ HC Pro	Filter-1	Merck Millipore	Silica and poly acryl based	0.6-8
2	Millistak+ HC Pro	Filter-2	Merck Millipore	Silica and poly acryl based	0.2-2
3	Millistak+ HC	Filter-3	Merck Millipore	Cellulose and diatomaceous earth based	0.2-2
4	NA	Filter-4	Competitor-A	Cellulose, inorganic filter aid, and binding resin	0.2-5
5	NA	Filter-5	Competitor-A	Cellulose, inorganic filter aid, and binding resin	0.5-5
6	Millistak+ HC	Filter-6	Merck Millipore	Cellulose and diatomaceous earth based	0.6-8

process fluid are in a thicker media or multiple layers of media form a tortuous path.

Depth filtration by multiple porous layers with depth are used to capture the solid contaminants from the liquid phase. Due to the tortuous and channel-like nature of the filtration medium, the particles are retained throughout the medium within its structure, as opposed to on the surface.

Depth filters are nominal in pore-size rating as opposed to absolute and are characterized as a range. A “depth” matrix is used to trap suspended particles, separating them from their carrying fluid. Interception of solids occurs in depth media due to loss of energy. Solids must pass through a tortuous path while crossing the depth of the filter media. The loss of energy experienced traps solids within the matrix.

Adsorption is the adhesion of molecules to the solid structure with which they are in contact. Adsorption usually occurs because of physical attraction between molecules (van der Waals force), chemical attraction at the surface/molecule interface, or, more commonly, in-depth media through electro-static attraction. One major advantage of depth media is the ability to impregnate the medium with filter aids to assist in filtration and resist blinding.

Typically, conventional depth filters used for biological purification processes contain cellulose media embedded with diatomaceous earth. But, recently, novel synthetic depth filters are available that have more consistent depth filtration media in terms of hydraulic performance over cellulosic depth filtration media. Synthetic depth filter media are primarily made of poly acrylic fiber and silica filter aid. Using synthetic depth filters for processing has an advantage of having no beta glucans to indicate a false positive limulus amoebocyte lysate (LAL) testing for bacterial endotoxins, which could be an important process quality

Table II. List of abbreviations and full form.

Abbreviations	Full form and unit
A	Area of depth filter (m ²)
A _{guess}	Guessed area (m ²)
A _{min}	Calculated area minimum (m ²)
J _{avg}	Average flux (LMH)
VB	Batch volume (L)
tB	Batch time (Hour.)
ΔP	Pressure drop (psi)
P _{limit}	Process Δpressure endpoint (psi)
R _{calc}	Calculated resistance (psi/LMH)
R _{actual}	Process derived resistance (psi/LMH)

control parameter to determine efficient purification and final quality of purified polysaccharides. Further helping in deterrence of any adverse impact due to false positive results and improving patient safety. There are some more advantages while using synthetic depth filter over conventional depth filter such as reduced pre-use flush volume due to reduced total organic carbon (TOC) extractable, higher filtration capacity, improved removal efficiency of HCP impurity, and better lot-to-lot consistency.

MATERIALS AND METHODS

Depth filters

Various depth filters were evaluated for the clarification of pneumococcal fermentation harvest post-fermentation. Details are provided in **Table I**. Filtration parameters such as flush volume and flow rate were selected as per manufacturer’s specification of respective depth filters.

Depth filter study procedure

The constant flow method was performed for this study where feed flow was maintained constant using peristaltic pump until the pressure drop increased to a limit (20 psi was considered as pressure breakthrough point) or breakthrough in filtrate

turbidity (≥50% of load turbidity) observed.

Filter flushing was performed for all studied filters using water for injection (WFI) and/or equilibration buffer as defined by the manufacturer.

Pressure drop (psi) was determined by difference between input pressure and output pressure.

Filter resistance (psi/LMH) was calculated using pressure drop (psi) divided by permeate flux (LMH; flux in L/m²/h).

Throughput (loading capacity) was calculated by normalizing the studied filter area and its filtrate volume in L/m².

Load material

Centrifuged harvest from two different serotypes is used as depth filter load for this study. Load turbidity and viscosity data are mentioned as follows:

- **Serotype 3:** harvest supernatant with turbidity of 75.2 NTU and high viscosity of 22 cP (highest viscosity amongst all the serotypes).
- **Serotype 8:** harvest supernatant with turbidity of 440 NTU (highest turbidity, amongst all the serotypes) and viscosity of 16 cP.

Study design

Serum institute of India is working on a Pneumococcal polysaccharide conjugate vaccine project that con-

sists of multiple serotypes. Two challenging serotypes in terms of turbidity (High OD at 590 nm) and viscosity amongst all serotypes were selected for a depth filtration study wherein other depth filters fail for the removal of cell debris/aggregates, etc. Ceratin serotypes were selected for worst-case simulation in the study based on their viscosity and turbidity values compared to other serotypes.

Serotype-3 was observed to be more viscous than all other serotypes, and Serotype-8 was observed to have higher turbidity than all other serotypes.

Serotype 3 was evaluated using five different depth filters based on different materials of construction (MOC) and pore size rating to compare the hydraulic performance and output quality in terms of turbidity.

Serotype 8 was evaluated using two different depth filters of different MOC, which showed comparable hydraulic performance when challenged with Serotype 3. Depth filters used for this study were different in terms of pore size, which was chosen based on the target output quality required in terms of turbidity, and depth filter media (Cellulosic and Synthetic) were based on the filter performance with Serotype 3.

Higher throughput/loading capacity (L/m^2) and lower filtrate turbidity were the selection criteria for better depth filter.

Calculations

The constant flow rate P_{max} approach can be performed on all types of filters including membrane filters, surface filters and depth filters. However, as it does not assume any underlying mechanistic model of filter plugging, it is commonly used to size depth filters. P_{max} Sizing Method for known batch volume (V_b) and batch time (t_b) (Table II):

$$\text{Flux (LMH)} = L/m^2 \cdot h$$

$$\text{Loading (L/m}^2\text{)} = \frac{\text{filtrate vol (L)}}{\text{filter Area (m}^2\text{)}}$$

Figure 1. Comparative resistance profile of depth filters at constant flow.

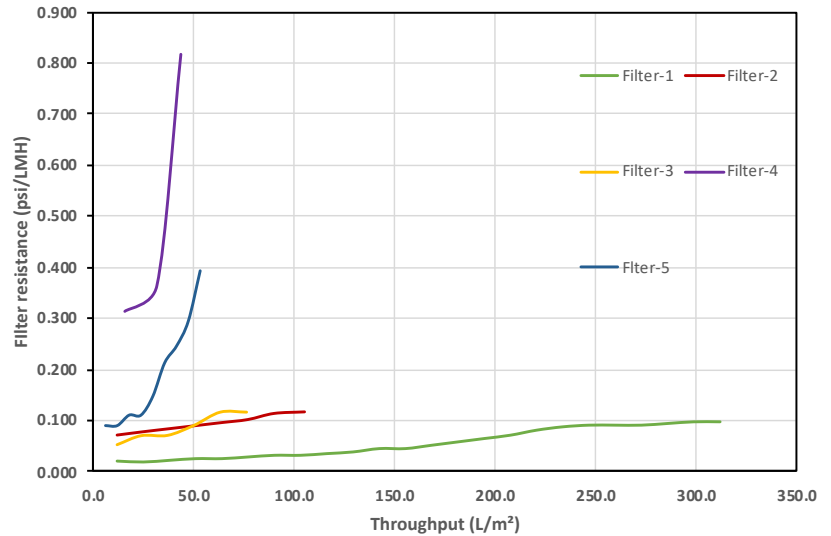


Figure 2. Comparative turbidity reduction profile of depth filters at constant flow.

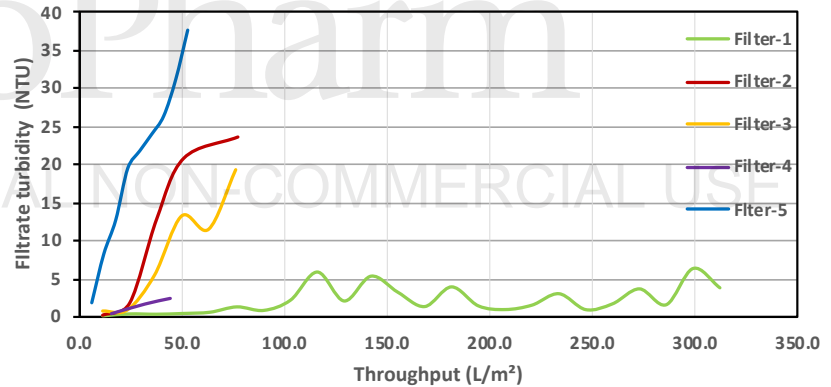
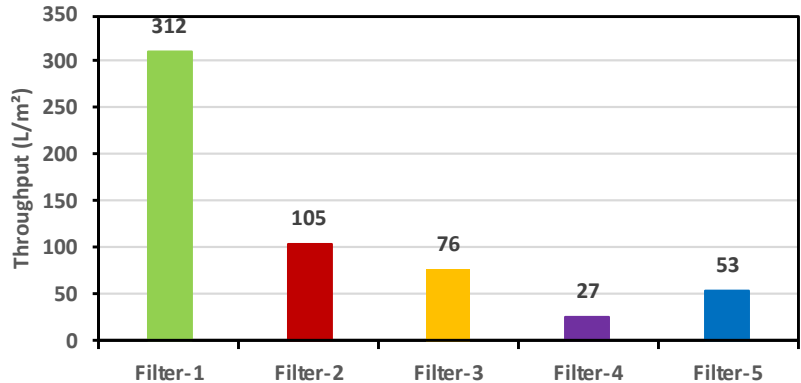


Figure 3. Throughput comparison of depth filters.



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Delta pressure, ΔP (psi) = input pressure - output pressure (psi)

Resistance [psi/LMH] = ΔP (psi)/flux (LMH)

Let us select an area, A_{guess} (start with batch volume divided by the final test loading) and select P_{limit} for process endpoint.

Therefore, Flux, $J_{\text{avg}} = V_B / A_{\text{guess}} * t_B$

Capacity = V_B / A

Resistance, $R_{\text{calc}} = \Delta P / J_{\text{avg}}$

Actual resistance is determined from the graph (polynomial fit), R_{actual} .

Check to see if, $R_{\text{calc}} = R_{\text{actual}}$
then $A_{\text{guess}} = A_{\text{min}}$.

If $R_{\text{calc}} \neq R_{\text{actual}}$, then adjust A_{guess} until $R_{\text{calc}} = R_{\text{actual}}$.

RESULTS AND DISCUSSION

Serotype 3

Figure 1 shows comparative filter performance of evaluated depth filters after plotting throughput (capacity L/m²) with filter resistance (psi/LMH). Filter-1 exhibited the best hydraulic performance among all the evaluated filters (i.e., lowest filter resistance and hence highest throughput was observed). Higher resistance to flow was observed in Filter-4 and Filter-5. Filter resistance in Filter-2 and Filter-3 was moderate; however, the throughput observed was low due to pressure breakthrough in Filter-2 and turbidity breakthrough in Filter-3.

Figure 2 shows the turbidity reduction profile of evaluated filters. Filter-1 exhibited higher turbidity reduction amongst all evaluated filters. Filter-2 and Filter-3 showed lower turbidity reduction. Filter-4 showed low capacity; whereas, Filter-5 exhibited turbidity breakthrough/low retention.

Figure 3 shows that the throughput obtained with Filter-1 far sur-

Table III. Performance comparison of evaluated depth filters.

Filters evaluated	Filter manufacturer	Filter family	Filter media	Capacity (L/m ²)	Final turbidity (NTU)
Filter-1	Merck Millipore	Millistak+ HC Pro	Silica and poly acryl based	312	1.22
Filter-2	Merck Millipore	Millistak+ HC Pro	Silica and poly acryl based	105	2.87
Filter-3	Merck Millipore	Millistak+ HC	Cellulose and diatomaceous earth based	76	9.43
Filter-4	Competitor-A	NA	Cellulose, inorganic filter aid, and binding resin	27	1.40
Filter-5	Competitor-A	NA	Cellulose, inorganic filter aid, and binding resin	53	20.50

passes the filtration capacity of all the other evaluated filters. This could be due to the low flow resistance and higher retention capacity of silica and poly acrylic media. Even though the pore size rating of Filter-2 is one of the lowest among all the evaluated filters, it is observed to have a better throughput compared to Filter-3, Filter-4, and Filter-5 due to lower flow resistance and marginally better capacity.

Table III summarizes the filter performance of studied depth filters for clarification of Serotype 3. Among the evaluated depth filters, Filter-1 observed to have highest capacity followed by Filter-2 with moderate capacity; whereas, Filter-3, Filter-4, and Filter-5 exhibited low capacity.

Serotype-8

Figure 4 shows the flow resistance profile of Filter-2 and Filter-6. Both exhibited same throughput, but the flow resistance is marginally higher in Filter-2 compared to Filter-6.

In terms of filtrate turbidity, which is one of the selection criteria for the suitable depth filter, Filter-2 exhibited higher turbidity reduction compared to Filter-6.

Throughput data for evaluated filters, Filter-2 and Filter-6, were comparable and showed similar capacity in terms of throughput.

Table IV summarizes the filter performance of studied depth filters for serotype-8.

Figure 4 shows the comparative filter performance of Filter-2 and Filter-6, plotting throughput (L/m²) with filter resistance (psi/LMH).

Figure 5 shows the comparative filter performance of Filter-2 and Filter-6, plotting throughput (L/m²) with filtrate turbidity (NTU), and **Figure 6** displays the filter throughput (L/m²) summary for evaluated filters (Filter-2 and Filter-6). In Serotype-8, **Figures 4 and 5** indicate that Filter-2 is showing marginally higher resistance but lower filtrate turbidity, indicating better filter performance.

CONCLUSION

Depth filters act by the mechanism of adsorption, entrapment, and size exclusion. Filter performance is negatively impacted by resistance; more resistance by a filter can be a symptom of filter clogging due to retention.

There are two methodologies that can be used for filter sizing through a scale-down study: the constant pressure sizing (V_{max}) methodology that measures decrease in flow as a function of throughput, and in which the endpoint is determined by flow rate or volume; and the constant flow rate (P_{max}) methodology that measures increase in pressure and decrease in filtrate quality as a function of throughput, in which the endpoint is determined by pressure limit or desired filtrate quality.

In this study, the constant flow rate sizing is used during small-scale filtration trials to evaluate the capacity of filters and to calculate the filter area needed for scale up. It is an empirical data (with a second- or third-order polynomial fit to resistance vs. normalized volume data) analysis method to analyze constant flow data. It is not based on any mathematical model. It will not predict the filter capacity from a short duration test such as the V_{max} method (constant pressure method), which in general means longer testing times. The advantage, however, is that it can be used for all filter types (membrane filters, depth filters) and independent of plugging model.

Clarification by depth filtration generally invokes two (three) separation mechanisms: mechanical sieving, (impaction) and adsorption. Some particles are removed from the solution by size exclusion (mechanical sieving) (and impaction) and on the other by binding to the internal surface of the depth filter media. Absorptive capture often is applied by making use of the native, net-negative, electrostatic charge that most biological solids carry at standard pH conditions. Therefore, depth filter media include a positively charged (cationic) surface (matrix) that will bind suspended particles by simple electrostatic attraction (ionic, hydrophobic, or other interactions).

Figure 4. S8-comparative resistance profile of depth filters at constant flow.

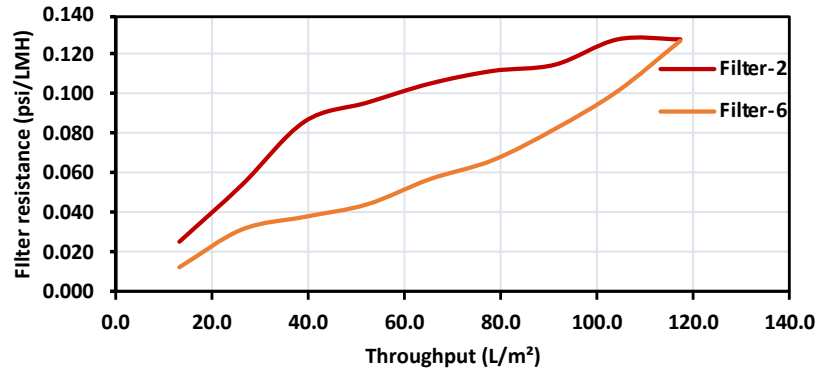


Figure 5. S8-comparative turbidity reduction profile of depth filters at constant flow.

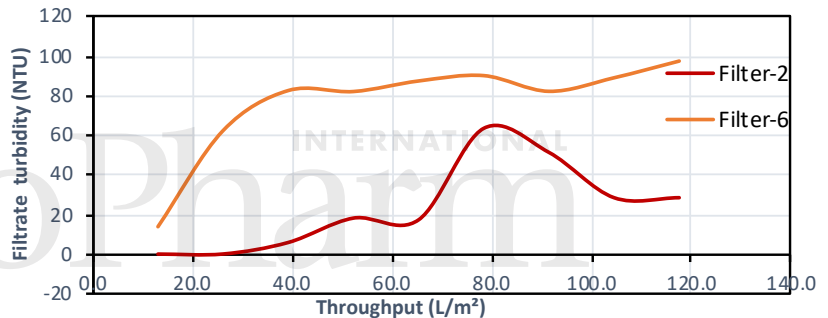
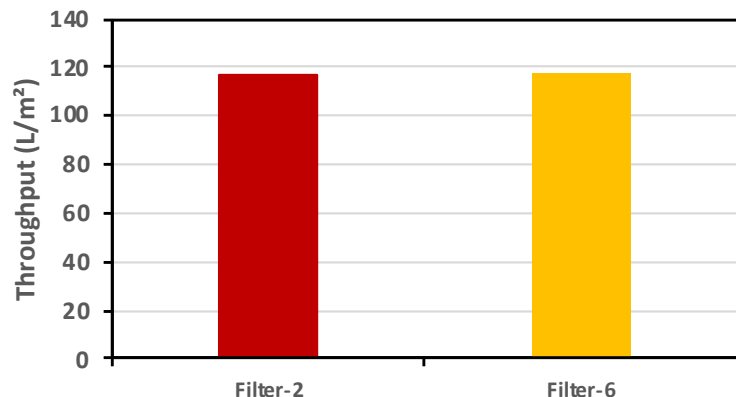


Figure 6. S8-throughput comparison of depth filters.



In sizing filtration trains, it is critical to know when the capacity of the filter is exhausted. With charged depth filter media there are two distinct capacity endpoints: pressure

limited (P_{max} method) and breakthrough limited (T_{max} method). The capacity endpoint is pressure limited when the pressure drop across the filter increases to an unacceptable

Table IV. Performance comparison of evaluated depth filters for Serotype-8.

Filters evaluated	Filter manufacturer	Filter family	Filter media	Throughput (L/m ²)	Final turbidity (NTU)
Filter-2	Merck Millipore	Millistak+ HC Pro	Silica and poly acryl based	117	24.09
Filter-6	Merck Millipore	Millistak+ HC	Cellulose and diatomaceous earth based	117	65.2

level (or defined endpoint). This is caused by progressive clogging of the pores of the filter media (primarily) by sieving. The adsorptive capture will not exhibit measurable change in pressure. The capacity depends on the number of binding sites available (contaminant load and 'residence time', though not to a great extent, i.e., once the binding sites are exhausted breakthrough is obvious). Once these are fully saturated, the solids content of the filtrate will increase, and breakthrough is a fact. Breakthrough will be monitored by measuring the turbidity of the filtrate. In most of the cases, size exclusion, impaction, and adsorption acts together rather than in isolation, so a decision on the breakthrough point can either be acceptable pressure or turbidity cut off' depending on 'occurrence order'.

As per the data observed for Serotype 3, one can infer that synthetic media and respective pore size are ideal for the hydraulic performance when used in clarification of PPCV as the resistance to flow is low and the throughput is high, along with low filtrate turbidity indicating high-capacity retention of impurities.

Filter-1 and Filter-2 having the same filter media performed well compared to the rest of the evaluated filters. However, because the pore size for Filter-2 is lower, the throughput capacity of Filter-2 was limited by pressure breakthrough. Similarly, Serotype 8 synthetic depth filter (Filter-2) outperformed cellu-

lose-based depth filters in terms of filtrate quality (turbidity) and throughput. Higher turbidity reduction was observed in filters with synthetic media, indicating retention of impurity (i.e., high filter capacity). This indicates that an appropriate combination of filter media and pore size can significantly improve the clarification filter performance. As per this study, synthetic depth filter media performs better than conventional cellulosic based depth filter media in terms of throughput (L/m²) and turbidity reduction/retention for both studied Serotypes 3 and 8. Removal of other impurities such as HCP and HC-DNA were not tested. However, clarified harvest from synthetic depth filters met the process requirements in terms of throughput, filtrate quality, and area requirement (data not shown) as per the process specifications.

Depth filter selection does not always have to be a tradeoff between throughput, filtrate quality, and filtration area; a feasible compromise can be achieved by selecting the ideal filter media and pore size. Higher throughput, good filtrate quality, and low filtration area by synthetic depth filter media may benefit the manufacturer in terms of easy scale up and better process economics. Nevertheless, this study can be extended by evaluating various feed materials from other serotypes to understand the robustness of the studied filters. Constant flow rate sizing will typically be used for depth filters.

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