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Purification of Filamentous Bacteriophage M13 by Expanded Bed Anion Exchange Chromatography

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In this paper, we investigated the development of a simplified and rapid primary capture step for the recovery of M13 bacteriophage from particulate-containing feedstock. M13 bacteriophage, carrying an insert, was propagated and subsequently purified by the application of both conventional multiple steps and expanded bed anion exchange chromatography. In the conventional method, precipitation was conducted with PEG/NaCl, and centrifugation was also performed. In the single step expanded bed anion exchange adsorption, UpFront FastLine™20 (20 mm i.d.) from UpFront Chromatography was used as the contactor, while 54 ml (H_o=15cm) of STREAMLINE DEAE (ρ=1.2 g/cm^3) from Amersham Pharmacia Biotechnology was used as the anion exchanger. The performance of the two methods were evaluated, analysed, and compared. It was demonstrated that the purification of the M13 bacteriophage, using expanded bed anion exchange adsorption, yielded the higher recovery percentage, at 82.86%. The conventional multiple step method yielded the lower recovery percentage, 36.07%. The generic application of this integrated technique has also been assessed.

Key words: expanded bed chromatography, adsorption, protein purification, bacteriophage M13, anion exchanger

The filamentous bacteriophages, including M13, fd and f1, are closely related phages which infect *Eschericia coli* via the F pili. The phage particles normally manifest as filaments of approximately 900 nm in length and 7 nm in diameter, with a molecular weight of 12×10^6 Da (Berkowitz *et al.*, 1980). These phages have played significant roles in the development of molecular biology and biotechnology, and are crucial in biological particles including DNA sequencing, cloning and phage display. Despite rapid progress in the technology of phage display, particularly with regard to the use of filamentous phage, the purification of phages has been rather a neglected field, until recently. Hence, there an urgent need exists to design a large-scale process for the purification of the phages, simultaneously maintaining high yield and pure product while minimising purification cost.

The initial purification of any molecule has traditionally involved precipitation, centrifugation, microfiltration, or some combination thereof. The efficiency of initial solid-liquid separations (i.e. centrifugation and filtration) depends on factors such as particle size, density difference between particles and surrounding solution, and feedstock viscosity (Becker *et al.*, 1983). The standard clarification processes which have been currently adopted suffer from some limitations, especially in terms of applying the process on a production scale. Continuous centrifugations are necessary for the processing of large volumes of feedstock (e.g. 1000 liters), as part of the clarification procedure, but the removal of biomass during such production operations is much less effective than laboratory-scale centrifugation. Consequently, centrifugation usually must be performed twice, while an additional depth or microfiltration step is commonly necessary, in order to ensure a particle free (99-99.9% in terms of cell clearance) solution which can be fractionated via traditional packed-bed chromatography (Anspach *et al.*, 1999). The effectiveness of filtration is usually diminished as a result of membrane fouling during operation (Geankoplis, 1983). Furthermore, the combination of precipitation, centrifugation and microfiltration often results in long process time, high operational and maintenance costs, and significant product loss due to deg-

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radation.

A possible alternative to the conventional method is the adsorption of the target molecules to adsorbents in an expanded bed column, a process which obviates the necessity for particulate removal. Expanded bed adsorption (EBA) is an integrated operation which combines clarification, concentration, and initial purification (Nygren *et al.*, 1995). This is achieved by creating distance between adsorbent particles, which allows for the unhindered passage of particulate matter such as cells, cell debris and other solids during the application of crude feed to the contactor. By operating in a single pass operation, the decrease in the number of steps reduces both processing time and production cost.

In this study, M13, carrying an insert of C-WSFFSNI-C at the gpIII protein, which inhibits the association of hepatitis B surface (HBsAg) and core (HBcAg) antigens (Ho *et al.*, 2003), was propagated and subsequently purified by the application of both conventional multiple steps and expanded bed anion exchange adsorption. The aim of this study was to develop a simplified method for the cost-effective recovery of target protein from unclarified feedstock.

Materials and Methods

Phage propagation

Phage was prepared according to the method described by Sambrook *et al.* (1989).

Phage titration

Phage titration method was adapted from the methods of Sambrook *et al.* (1989).

Precipitation and centrifugation

500 ml of culture containing the propagated bacteriophage was distributed equally into two 250 ml centrifuge tubes (duplicate) and centrifuged at 8,000 rpm for 15 min at 4°C (type rotor JA 14, J2-M1 Beckman, USA). The supernatant was transferred to an 1 liter conical flask and precipitated overnight (6 h) with 75 ml of PEG/NaCl (20% w/v PEG 8000/2.5 M NaCl) at 4°C. The mixture was then centrifuged at 8,000 rpm for 30 min at 4°C (type rotor JA 14, J2-M1 Beckman, USA). The precipitate obtained was collected by removing the supernatant. The precipitate was then dissolved in 30 ml of TBS (100 mM Tris-Cl, 0.9% w/v NaCl, pH 7.5) and centrifuged at 15,000 rpm for 10 min at 4°C (type rotor JA 14, J2-M1 Beckman, USA) to precipitate any insoluble matter. The supernatant was then transferred to a new centrifuge tube and re-precipitated with 45 ml of PEG/NaCl (20% w/v PEG 8000, 2.5 M NaCl) for 1 h at 4°C. The precipitated phage was recovered by centrifugation at 15,000 rpm for 30 min at 4°C (type rotor JA 14, J2-M1 Beckman, USA). The supernatant was removed by aspiration, and the pellet was re-suspended in 10 ml of TBS at a pH of 7.5.

Expanded bed adsorption

Expanded bed adsorption was conducted using UpFront FastLine^{TM}20 (20 mm i.d.) with a magnetic stirrer as the EBA column, and 54 ml (H_o=15 cm) of STREAMLINE DEAE as the anion exchanger. Equilibration buffer (50 mM Tris-HCl buffer at pH 7.5) was pumped into the column with a pump speed of 8 rpm corresponding to a flow rate of 13.07 ml/min. This generates an ascending flow rate of 250 cm/h in the column. The bed is considered stable if the bed height remains constant for 15 min with no channels, in the expanded bed. The feedstock was then applied while maintaining a flow rate of 13.07 ml/min. The adsorbent was then washed with equilibration buffer, in order to remove any non-bound materials. The flow rate of the buffer was maintained at 13.07 ml/min. Elution buffer (2 M NaCl in 50 mM Tris-HCl buffer at pH 2.2) was then applied with a flow rate of 6.54 ml/min corresponding to the ascending velocity of 125 cm/h in the column. When the bed expansion was stabilized, the outlet pipe was adjusted to minimize the headspace. Elution was carried out, until all the desired bound products had been eluted out.

Results and Discussion

The phage concentration and total plaque forming units (pfu) in both the propagated phage and purified phage, by both precipitation and centrifugation methods, are shown in Table 1. The extraction efficiency associated with the precipitation and centrifugation methods was determined as follows:

$$\text{Efficiency of extraction (\%)} = \frac{\text{pfu of purified phage}}{\text{pfu of propagated phage}} \times 100 \quad \cdots\cdots (1)$$

From Table 1 and equation 1, the extraction efficiency is:

$$\frac{1.01\times10^{13}}{2.80\times10^{13}}\times100 = 36.07\%$$

The initial phage concentration in the feedstock, final equilibrium concentration of the phage, and the eluted phage from the expanded bed anion exchange adsorption are shown in Table 2. The efficiency of adsorption, A', and elution, E', for expanded bed adsorption was deter-

Table 1. Phage concentration and total pfu in propagated and purified phage by conventional method (precipitation and centrifugation)

	Propagated phage	Purified phage
Concentration (pfu/ml)	2.80×10^{10}	1.01×10^{12}
Volume (ml)	1000	10
Total pfu	2.80×10^{13}	1.01×10^{13}

Table 2. Phage concentration and total pfu in various stages of EBA

	Initial phage concentration in feedstock (c_F)	Final equilibrium concentration of phage in solution (c)	Eluted phage
Concentration (pfu/ml)	2.05×10^{10}	3.20×10^{8}	6.80×10^{10}
Volume (ml)	1000	1300	250
Total pfu	2.05×10^{13}	4.16×10^{11}	1.70×10^{13}

mined as follows:

$$\text{Efficiency of adsorption, } A'(\%) = \left(\frac{c_F - c}{c_F}\right)\times 100 \quad \cdot(2)$$

$$\text{Efficiency of elution, } E'(\%) = \left(\frac{q'_F - q'}{q'_F}\right)\times 100 \quad \cdots (3)$$

Where c_F = initial phage concentration in feedstock
c = final equilibrium concentration of phage in solution
q'_F = initial concentration of phage adsorbed onto the adsorbent
q' = phage retained on the adsorbent after elution

From Equations 2 and 3, the efficiency of adsorption and elution for EBA are as follows:

$$\text{Efficiency of adsorption} = \left(\frac{2.05\times10^{13}\text{pfu} - 4.16\times10^{11}\text{pfu}}{2.05\times10^{13}\text{pfu}}\right)\times 100 = 97.97\%$$

$$\text{Efficiency of elution} = \left(\frac{2.01\times10^{13}\text{pfu} - 3.10\times10^{12}\text{pfu}}{2.01\times10^{13}\text{pfu}}\right)\times 100 = 84.58\%$$

The value of 2.05×10^{13} (see Table 2) is the initial phage concentration in the feedstock (c_F). The value of 2.01×10^{13} represents the initial concentration of phage adsorbed onto the adsorbent (q'_F), which is the value of ($c_F - c$). The overall efficiency of extraction was expressed as follows:

$$\text{Efficiency of extraction} = A'\times E' \quad \cdots\cdots (4)$$

From Equation 4, the overall extraction efficiency of bacteriophage for EBA is as follows:

$$97.97\%\times84.58\% = 82.86\%$$

This study shows that the recovery of phage by conventional methods, including precipitation and centrifugation, resulted in a yield of 36.07% while the recovery of phage by expanded bed adsorption resulted in a yield of 82.86% (Table 3). The difference in yields can be considered significant, with a margin of 46.79%. In the conventional method, PEG salts out the phage by reducing the solubility of the particles beyond a critical value (Ingham, 1984). High speed centrifugation ($10,000\times g$) spins out particulate matter such as *E. coli* cells, cell debris, and other solid matter while a higher speed centrifugation ($28,000\times g$) spins out and collects the "salted out" phage (Becker *et al.*, 1983). However, the recovery of the phage from crude feedstock using these conventional methods was demonstrated to be unsatisfactory (see Table 1). The recovery of phage by expanded bed anion exchange adsorption using STREAMLINE DEAE as the anion adsorbent yielded a recovery of 82.86%, which can be considered an excellent result, compared to that obtained by the conventional method.

Table 3. Recovery efficiency of phage by conventional method compared to EBA

	Conventional	EBA
Adsorption efficiency (%)	not determined	97.97
Elution efficiency (%)	not determined	84.58
Total yield (%)*	36.07	82.86

*Total yields of conventional and expanded bed chromatograph method were determined by equation 1, 2, 3, and 4 respectively.

Fig. 1 summarizes the various stages involved in the purification of phage by precipitation and centrifugation, and by expanded bed anion exchange adsorption. In the purification of phage by the conventional method, the time spent for the entire process exceeded 18.5 h, with 16 h being spent on the overnight precipitation, an additional h for the second precipitation, and a total of 1 h and 25 min dedicated to centrifugation. The adoption of expanded bed chromatography permitted the rapid capture of target protein from the unclarified feedstock. Here, the time spent for the entire process was 228 min, or the equivalent of 3.8 h. In the conventional method, PEG and other host contaminants are usually removed by cesium chloride (CsCl) density equilibrium ultracentrifugation. The centrifugation is performed at approximately $175,000\times g$ for 18 h at 4°C (Smith and Scott, 1993). Although this further step gives rise to a higher purity in terms of phage preparation, it is also time-consuming and labor-intensive, therefore directly increasing the effective cost of the purified product. Furthermore, in the classical process, the amount of phage particles must be maintained at lower than 10^{14} particles per 12 ml of production, whereas in expanded bed chromatography purification, the process feedstock can be increased simply by scaling up the diameter of the column.

This study clearly demonstrates that the long sequence involved with the traditional recovery method can be compressed into a single process (see Fig. 1). In conclusion, the integrated operation (i.e. expanded bed anion exchange chromatography) demonstrated herein is a scalable and cost-effective approach for the recovery of bacteriophage

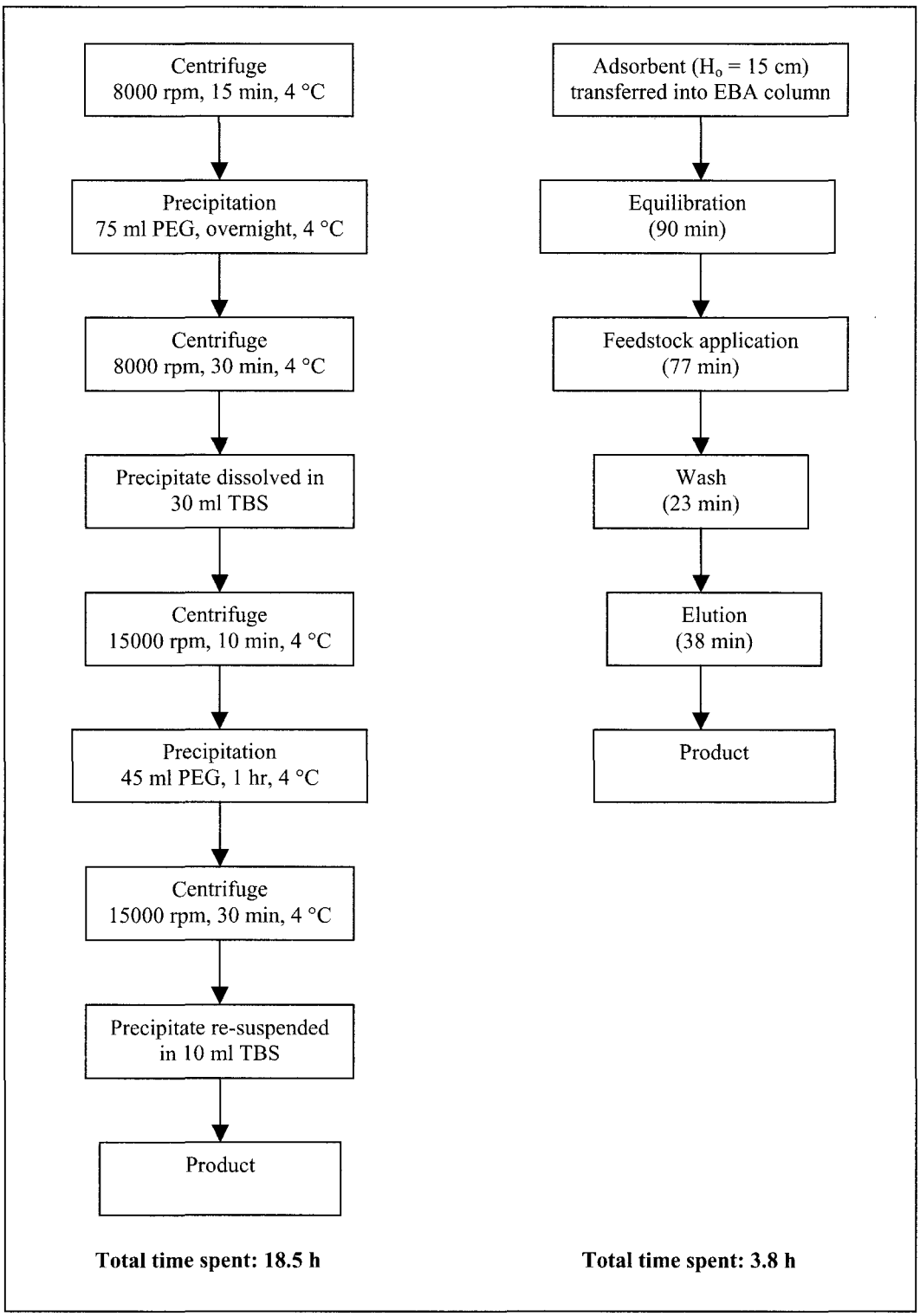

Fig. 1. Flowchart of purification of M13 bacteriophage. Left hand panel shows the steps involved in precipitation, centrifugation and microfiltration, while right hand panel shows the stages in expanded bed adsorption. Parameters listed are for feedstock volume of 1 litre.

M13 from unclarified *E. coli* feedstock. Expanded bed anion exchange chromatography, then, constitutes an appealing alternative method for the purification of filamentous bacteriophage.

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