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# MICROFILTRATION AND ULTRAFILTRATION OF *Bacillus thuringiensis* FERMENTATION BROTH: MEMBRANE PERFORMANCE AND SPORE-CRYSTAL RECOVERY APPROACHES

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**Abstract -** Recovery of spores and crystals from the fermentation broth of *Bacillus thuringiensis* (Bt) was studied using the membrane separation technology. Four types of polymeric membranes, with different characteristics, in the range of microfiltration (MF) and ultrafiltration (UF) were used for evaluating their permeate flux and spore–crystal recovery capacity. Results indicated that both MF and UF membranes are effective for spore–crystal recovery. The hydrophobic MF membrane made of polyvinylidene fluoride (PVDF) achieved a better performance compared to the one made with hydrophilic cellulose acetate (CA). Both had a 0.22 µm pore size, under the condition of an upper range of feed pressure. Also, with the increase of the feed flow rate, a higher flux was achieved for the PVDF membrane. A UF membrane made of polyethersulfone (PES) polymer was also used effectively for spore/crystal recovery from the broth, but under a higher operating pressure. In the entire experiment, a 99.9% rejection factor was measured with the applied membranes for the spore/crystal in the fermentation broth.

*Keywords*: *Bacillus thuringiensis* (Bt); Microfiltration (MF); Ultrafiltration (UF); Fermentation broth; Permeate flux.

## INTRODUCTION

About three million tons of chemical pesticides worth 20 billion dollars are sold all over the world annually. Only half a billion dollars worth of the pesticide trade is biopesticides, and more than 60% of this amount is related to the commercial products of *Bacillus thuringiensis* (Bt). More than 50 species of pests sensitive to Bt, belonging to the three orders of Lepidoptera, Coleoptera, and Diptera, have been reported. At present, various Bt-based biopesticides and transgenic plants form an important part of Integrated Pest Management (Marzban, 2012).

*Bacillus thuringiensis* are gram-positive, spore forming, and soil-inhibiting bacteria widely used in agriculture, forest, and public health sectors as effective biopesticides (Brar *et al*., 2006). *B. thuringiensis* is characterized by the production of insecticidal spores and crystal proteins. There have been extensive studies on the efforts made to improve the biological efficiency of Bt-based biopesticides, using alternative substrates and additives in the formulations, to try and enhance the entomotoxicity and optimize the fermentation process (Bryant, 1994; Brar *et al*., 2006b; Zhuang *et al*., 2011). However, it must be highlighted that the penultimate step in Bt bi-

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*To whom correspondence should be addressed opesticide production, which can directly affect the formulation and consequently the efficiency, is the effective recovery of the broth components, which has not yet been addressed comprehensively.

During the downstream step of the Bt production process, the main broth components, that is, the spores and crystals, must be recovered effectively. Various unit operations have been used for this issue. Table 1 compares the various recovery methods for Bt and their characteristics. Earlier, studies were conducted on the recovery of the spores and crystals using precipitation, adsorption, evaporation, and centrifugation (Brar *et al*., 2006a and 2006b; Adjalle *et al*., 2007). Other methods of recovery currently employed, such as immobilization (Prabakaranand Hoti, 2012), take time and are laborious, with a complicated scale-up process. In this context, membrane-based recovery processes, such as microfiltration (MF) and ultrafiltration (UF), have been considered to be effective techniques for various applications (Mirtalebi *et al*., 2014; Shirazi *et al*., 2013a and 2013b), particularly for biological solution treatment (Kristic *et al*., 2001; de la Casa *et al*., 2007; Li *et al*., 2008; Tang *et al*., 2010). MF and UF are pressure-driven membrane processes for the separation of microorganisms from suspended solutions (e.g., biological solutions) and emulsions. The applied membranes in these processes have microporous structures (Shirazi *et al*., 2014a). MF and UF are the oldest membrane technologies that need lower operating pressures compared to nanofiltration and reverse osmosis (Davis, 1992). Therefore, the required energy for MF/UF-based separations is significantly lower than that required for other conventional processes.

In this study, cross-flow MF and UF processes were applied to observe the recovery of spores and crystals from the Bt fermentation broth. Microporous polymeric MF and UF membranes were comprehensively characterized, using atomic force microscopy (AFM), and used for the experiments. To the best of our knowledge, this is the first attempt to investigate the effect of membrane characteristics on the recovery performance of Bt fermentation broth.

## MATERIALS AND METHODS

### Materials

Commercial Bt fermentation broth was supplied by a local manufacturer (Nature Biotechnology Co., Iran).

The membranes used for the experiments included two MF membranes made of cellulose acetate (CA) and polyvinylidene fluoride (PVDF), with the same pore size of 0.22 μm, supplied by Membrane-Solutions and Sepro companies, and two UF membranes made of polyethersulfone (PES) with different molecular weight cut-offs (MWCO) of 6 kDa and 10 kDa, supplied by Membrane-Solutions Co. Sodium hydroxide (Merck, Germany) was used to sterilize the system.

### Apparatus and Procedure

A cross-flow experimental apparatus was used for the MF and UF experiments. Figure 1 shows a general scheme of the employed system. The apparatus consisted of a plate and frame module, a diaphragm pump, stainless steel tubes and connections, a precise flow meter, a pressure gauge, and a regulator to adjust and control the flow rate and pressure, respectively.

**Table 1: Comparative study of conventional methods for recovery of Bt from fermentation broth.**

| Method | Equipment | Advantage | Disadvantage | Cost analysis |
|---|---|---|---|---|
| Precipitation | No device is needed, only acid (mostly HCl) is needed for the precipitation procedure | Very cheap | Low efficiency<br>Heavy wastage<br>Handling of corrosive chemicals | Initial investment is less, however, low efficiency makes it non-preferable for downstream processing |
| Evaporation | The largest tool is evaporator, piping, pump(s), utility or re-boiler for hot steam generation | Available on an industrial scale | Thermal sensitivity of feed<br>High energy consumption<br>Large equipment needed | Initial investment is high<br>Using vacuum to decrease the operating temperature increases the operating costs<br>Costly operation and maintenance |
| Centrifugation | Centrifuge | Work in continuous mode | Moderate wastage<br>Costly maintenance<br>High mechanical shear stress for biological solutions | Initial investment is high |

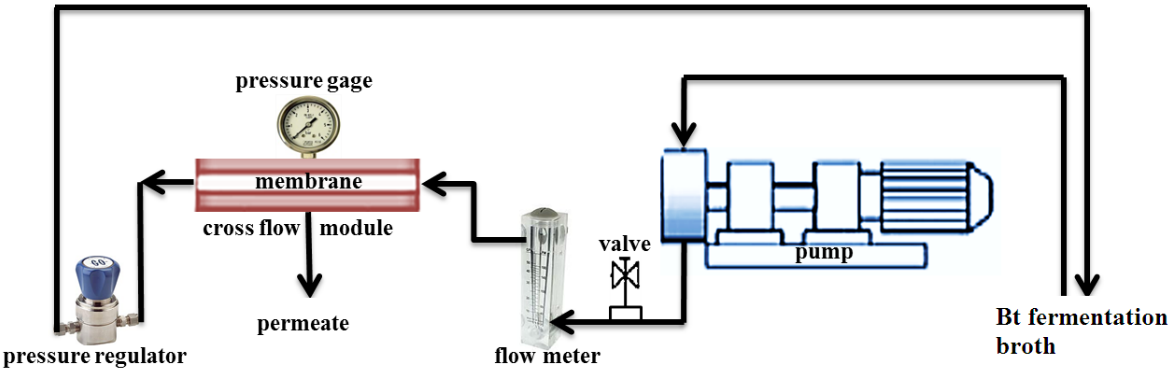


**Figure 1:** A general scheme of the cross-flow filtration apparatus applied in this work.

The overall performance of the MF and UF processes for spore and crystal recovery from the Bt fermentation broth was evaluated based on two major factors – the permeation flux (kg.m$^{-2}$.h$^{-1}$) and solute (i.e., spore/crystal) rejection (%) – as described in our previous study (Marzban *et al*., 2014).

0.1 mol/L NaOH was used for at least 15 minutes to clean the system after each experiment. Following this, the system was flushed with deionized sterile water twice (each run for 15 minutes).

## Analysis

To evaluate the filtration performance of the MF and UF membranes, samples of the feed, permeate, and retentate streams were analyzed for colony forming units (CFU), based on the standard method described previously (Marzban *et al*., 2014).

The applied membranes were characterized for their topographical features using atomic force microscopy (AFM) analysis, based on the method described recently (Shirazi *et al*., 2013a; Mirtalebi *et al*., 2014). The AFM was performed with the non-contact mode using a DUALSCOPE 95-200E apparatus equipped with a DS95-200 E scanner and DUALSCOPE C-21 collector (DEM, Denmark). Table 2 presents the specifications of the cantilever and its tip.

**Table 2: The specifications of cantilever and tip in the applied AFM.**

| Cantilever | |
|---|---|
| Length (µm) | 160 |
| Width (µm) | 45 |
| Thickness (µm) | 4.6 |
| Spring/force constant (N/m) | 42 |
| Resonance frequency (kHz) | 285 |
| Slope (o) | 10 |
| Tip | |
| Material | Silicone nitrate |
| Height (µm) | 10~15 |
| Tip curvature radius (nm) | 10> |

Scanning electron microscopy (SEM) (VEGA3, TESCAN) was used to observe the morphologies of the membranes and Bt, based on the standard techniques (Shirazi *et al*., 2015).

## RESULTS AND DISCUSSION

Four types of commercial polymeric membranes, two MF membranes with 0.22 µm pore size (labeled as CA and PVDF) and two UF membranes made of PES with 6 kDa and 10 kDa pore size (labeled as PES-6 and PES-10) were selected. They were chosen for their excellent performance, high permeate flux, and low protein adsorption, as recommended in the literature (Van Reis and Zydney, 2001; Charcosset, 2006).

The membranes were used for separation of the spore–crystal complex from the fermentation broth, under various operating conditions. In our previous study (Marzban *et al*., 2014), it was shown that feed temperature had a negligible effect on the separation performance and permeate flux; therefore, in the present study, the effects of two major parameters, pressure (10–30 psi) and flow rate (40–120 liter per hour (LPH)) were studied. Figure 2 presents the flux performance of the MF membranes when used under various operating pressures (P: psi) and flow rates (Q: liter per hour, LPH).

It is observed in Figure 2 that, under a constant feed pressure of 30 psi, the overall performance of the PVDF membrane as well as its initial permeate flux are better and higher than those of the CA membrane, respectively. As the feed flow rate increases from 40 to 120 LPH under a constant pressure of 30 psi, the initial flux also increases from 61.44 to 119.64 kg.h$^{-1}$m$^{-2}$ for the PVDF membrane and from 49.44 to 66.84 kg.h$^{-1}$m$^{-2}$ for the CA membrane, respectively. Whenever the feed pressure and the flow rate of 30 psi and 40 LPH are used, after almost 10 minutes, both the PVDF and CA membranes show the same performance and flux decline. However, increasing the feed flow rate, a further flux decline is observed for the CA membrane. A higher permeate flux with an increase in the feed flow rate can be explained by the fact that, under constant feed pressure, the higher feed can pose a higher shear velocity to the deposited cake layer on the membrane surface (Zulaikha *et al*., 2014; Shirazi *et al*., 2014b). As a result, more open pores are made available for the transport phase, which can achieve a higher flux, as is observed in Figure 2-c.

When the feed flow rate was kept constant at 120 LPH, using a lower feed pressure of 10 psi, it led to a different result. Under such conditions, a higher initial flux was observed for the PVDF membrane; however, after approximately 5 minutes of filtration, a higher permeate flux was observed for the CA membrane (Figure 2-d). Similar results were observed in Figure 2-f, when operating parameters with lower values, that is, P = 10 psi and Q = 40 LPH, were used, although the initial flux of the CA membrane was higher, ~44.52 kg.h$^{-1}$.m$^{-2}$. Table 3 shows the maximum and minimum values of the permeate flux for the MF membrane under various operating conditions. This behavior is in good agreement with previous studies, in which the authors used various operating conditions and polymeric membranes with various pore sizes (Chang *et al*., 2012; Marzban *et al*., 2014).

On the basis of the present results, it can be concluded that, under constant pressure (in the upper range), the permeate flux will increase with an increase in the feed flow rate. In this case, the PVDF membrane, which is hydrophobic, shows a better performance. On the other hand, with a lower range of operating pressure and higher and lower ranges of feed flow rates, the CA membrane shows a better performance, as compared to the PVDF membrane.

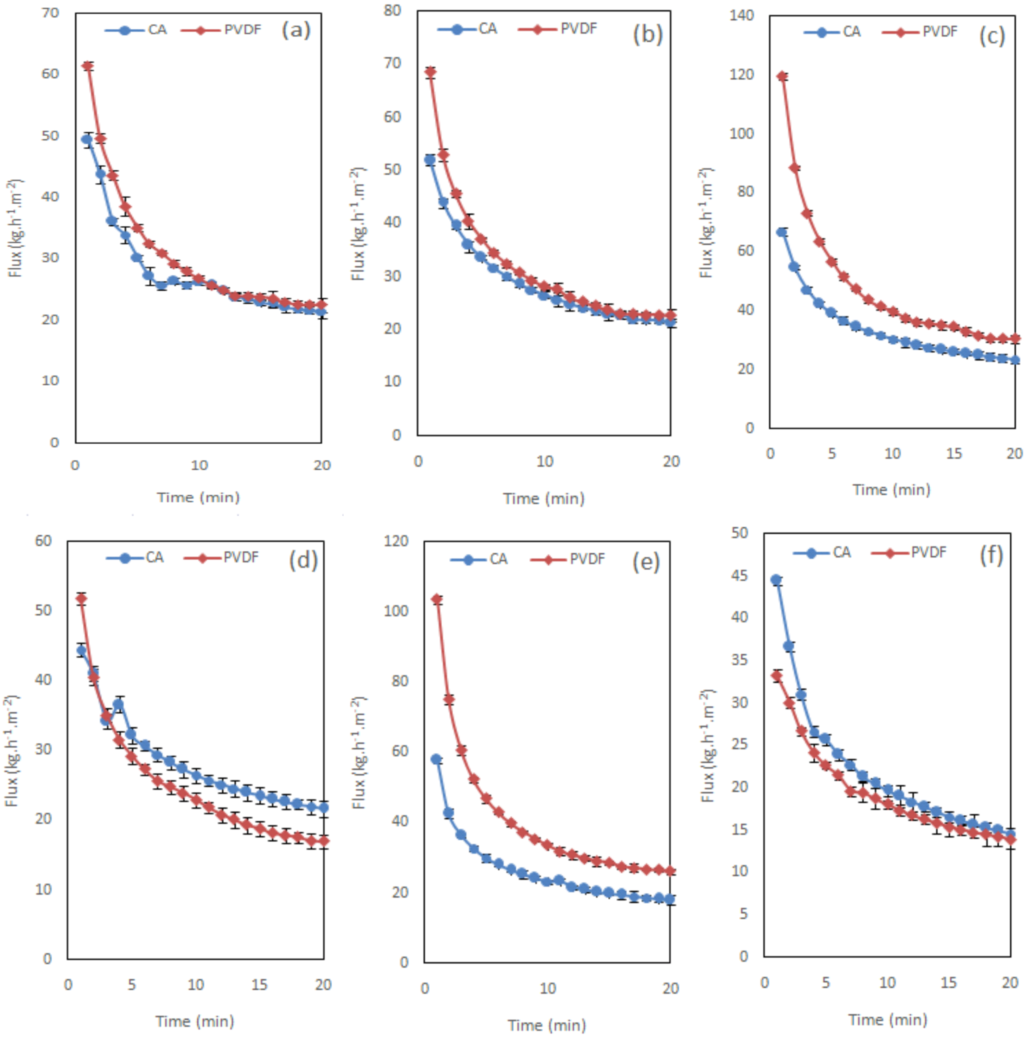


**Figure 2:** Permeate flux versus filtration time for MF membranes under various pressure (P) and flowrate (Q) conditions; (a) P = 30 psi and Q = 4 LPH, (b) P = 30 psi and Q = 80 LPH, (c) P = 30 psi and Q = 120 LPH, (d) P = 10 psi and Q = 120 LPH, (e) P = 20 psi and Q = 120 LPH, and (f) P = 10 psi and Q = 40 LPH.

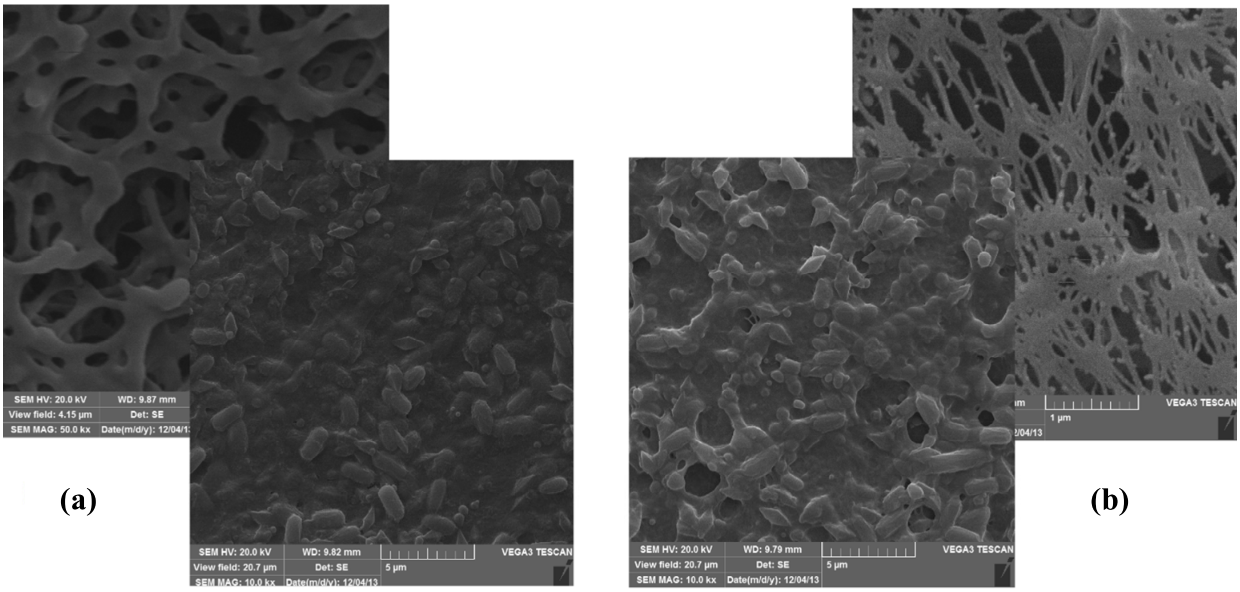
**Table 3: Permeate flux (max. and min.) for PVDF and CA membranes under various operating conditions.**

| Operation | Membrane | | | |
|---|---|---|---|---|
| | Flux for PVDF (kg.h$^{-1}$.m$^{-2}$) | | Flux for CA (kg.h$^{-1}$.m$^{-2}$) | |
| | *Max. ±se* | *Min. ±se* | *Max. ±se* | *Min. ±se* |
| **P** = 30 psi, **Q** = 40 LPH | 61.44±1.56 | 22.50±0.86 | 49.44±1.96 | 21.49±0.76 |
| **P** = 30 psi, **Q** = 80 LPH | 68.52±2.02 | 22.70±1.16 | 51.96±1.62 | 21.55±0.93 |
| **P** = 30 psi, **Q** = 120 LPH | 119.64±2.84 | 30.45±1.32 | 66.84±1.73 | 23.53±0.88 |
| **P** = 10 psi, **Q** = 120 LPH | 51.84±2.06 | 16.98±1.06 | 44.52±1.74 | 21.69±0.97 |
| **P** = 20 psi, **Q** = 120 LPH | 103.68±2.46 | 26.14±0.98 | 57.84±1.89 | 17.95±0.63 |
| **P** = 10 psi, **Q** = 40 LPH | 33.24±1.19 | 13.92±0.66 | 44.52±1.88 | 14.58±0.59 |

It is to be noted that the performance of the polymeric membranes can be directly related to their morphological and topographical features. Therefore, the MF membranes were characterized by using SEM and AFM analyses. Figure 3 shows the SEM images of the applied MF membranes, before and after using them for the experiments. As observed in Figure 3, after filtration there exist some open pores on the surface, which cause a higher flux; this observation was made for the PVDF membrane when compared with the CA membrane. Moreover, having some open pores can mean that the adsorption affinity of the spore and crystal to the PVDF polymer may be lower than that of the CA polymer.

Furthermore, surface roughness can affect pore fouling and cake formation on the membrane surface (Bowen and Doneva, 2000; Vrijenhoek *et al*., 2001; Boussu *et al*., 2005; Mirtalebi *et al*., 2014). Therefore, the applied membranes were characterized for their topographical features using AFM analysis (Figure 4). It is indicated in the literature that higher surface roughness can lead to a severe fouling effect when the hydrostatic pressure difference is the driving force, as it is in the MF and UF processes (Hashino *et al*., 2011; Van Wagner *et al*., 2011; Shirazi *et al*., 2013a). Table 4 presents the surface roughness of the applied MF membranes. A detailed description of these roughness parameters can be found in previous studies (Shirazi *et al*., 2013a and 2013c).

As observed in Table 4, the PVDF membrane has a surface that is smoother than the surface of the CA membrane; although they have the same pore size (0.22 µm). This difference can be explained by the fact that various manufacturers use different methods for the preparation of polymeric membranes. Moreover, the PVDF membrane has a more hydrophobic surface than the CA membrane. All these characteristics, in addition to having a rougher surface, lead the CA membrane to have a weaker performance in the separation of spores and crystals from the Bt fermentation broth in the current study.


(a)

(b)

**Figure 3:** SEM images of the virgin and used MF membranes, (a) CA membrane with 0.22 µm pore size and (b) PVDF membrane with 0.22 µm pore size.

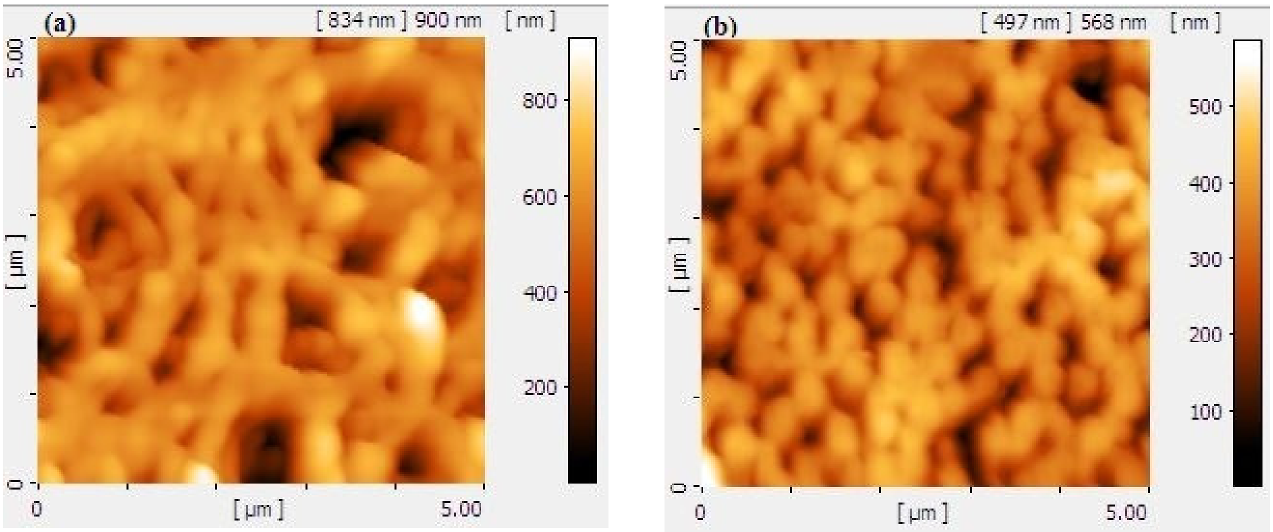


**Figure 4:** AFM images of the virgin membranes, (a) CA and (b) PVDF with 0.22 µm pore size.

**Table 4: The roughness parameters and their expressions that could be directly obtained using AFM analyses.**

| Roughness parameter | Expression | CA | PVDF |
|---|---|---|---|
| Average roughness ($R_a$) | $R_a = \frac{1}{n}\sum_{i=1}^{n} Z_i$ | 98 nm | 61.6 nm |
| Root-mean-square roughness ($R_q$) | $R_q = \sqrt{\frac{1}{n}\sum_{i=1}^{n} Z_i^2}$ | 128 nm | 77.3 nm |
| Peak-to-valley height ($R_z$) | $R_z = Z_{\max} - Z_{\min}$ | 771 nm | 426 nm |
| $Z_i$ | The height at point $i$ | | |
| $N$ | Number of points in the image | | |
| $Z_{max}$ and $Z_{min}$ | The highest and the lowest $Z$ values | | |

It is worth noting that 99.9% spore–crystal recovery was obtained for the applied membranes in these experiments, while Chang *et al.* (2012), in their study, reported 99% and 95% recoveries for spores and crystals, respectively, when a cross-flow MF process was used. The higher rejection rate obtained in this study could be explained by the fact that the separation in the MF process was based on the size exclusion of solutes (Belfort *et al.*, 1994). The average size of the spores and crystals in the applied Bt fermentation broth was observed using SEM analysis. The fermentation broth was filtered through a membrane sample at an ambient temperature and at a fixed transmembrane pressure of 10 psi. Figure 5 shows the scaled size of the spore and crystal deposited on the membrane surface. As observed in the present study, the sizes of the spore and crystal (i.e., ~1.7 µm in length and ~0.85 µm in width) are much larger than the membrane pore sizes of 0.22 µm. This fact supports the hypothesis of complete rejection of the target items, that is, spores and crystals.

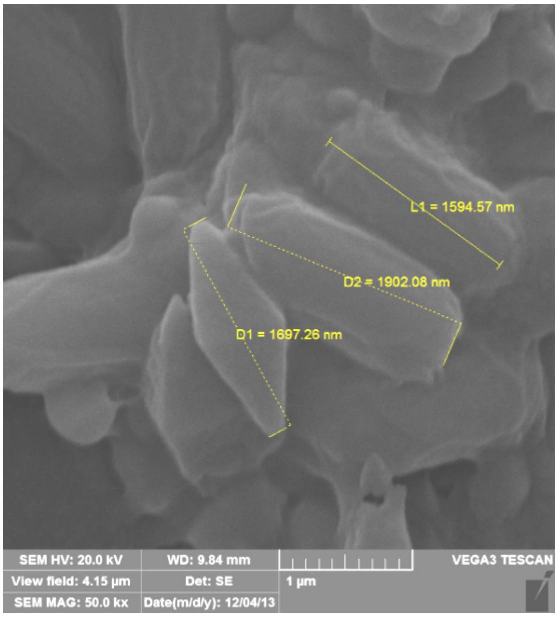


**Figure 5:** SEM image of the spore and crystal in the Bt fermentation broth applied in this work.

The results of the spore count and bioassay of the flux, when compared with the supernatant resulting from centrifugation (10,000 rpm for 20 minutes) on the second instar larvae of the flour moth *Ephestia kuehniella,* showed an acceptable performance for the two polymeric membranes. Three methods of settling, centrifugation, and ultrafiltration were used for the separation of Bt spores and crystals. Results showed that the biomass and CFU in the method of microfiltration were much greater, and the mortality of the recovered Bt spores and crystal on *Aedes aegypti* larvae was higher when using the microfiltration method than with the other two methods (Prabakaran and Hoti, 2008).

In addition to the MF process, the UF separation process has been used for downstream processing of the Bt fermentation broth (Adjalle *et al*., 2007). Hence, further experiments were carried out using two polymeric UF membranes – PES-6 and PES-10. Figure 6 shows the AFM images as well as the variation in the permeate flux versus filtration time for the applied UF membranes, when 80 psi pressure and 120 LPH flow rate are applied as the operating conditions. It is observed that PES-10 achieves a higher permeate flux compared to PES-6; however, the overall flux decline is more severe, especially in the first seven minutes of filtration. Although, a sharp flux decline was observed for both membranes, a slight constant permeation was observed after seven minutes of filtration. The spore–crystal rejection was also measured as 99.9% for both UF membranes.

On the basis of the present results, it can be concluded that both MF and UF processes can effectively be used for downstream processing of Bt-based biopesticides. However, it must be noted that the UF process needs higher operating pressures and the membrane cost is also higher than that for the MF process. On the other hand, it is indicated in the literature that, as the pore size gets smaller, the fouling problem becomes weaker (Zulaikha *et al*., 2014); comparison of Figures 2 and 6 supports this hypothesis. It is observed that, after 20 minutes of filtration, none of the MF experiments reach a steady-state flux; however, in the case of the UF experiment, after only seven minutes a relatively constant permeation flux can be observed. Generally speaking, the final decision for selecting a proper membrane for downstream processing of Bt fermentation broth depends on the average size of the spores and crystals, and also on the reusability of the broth for further fermentation steps.

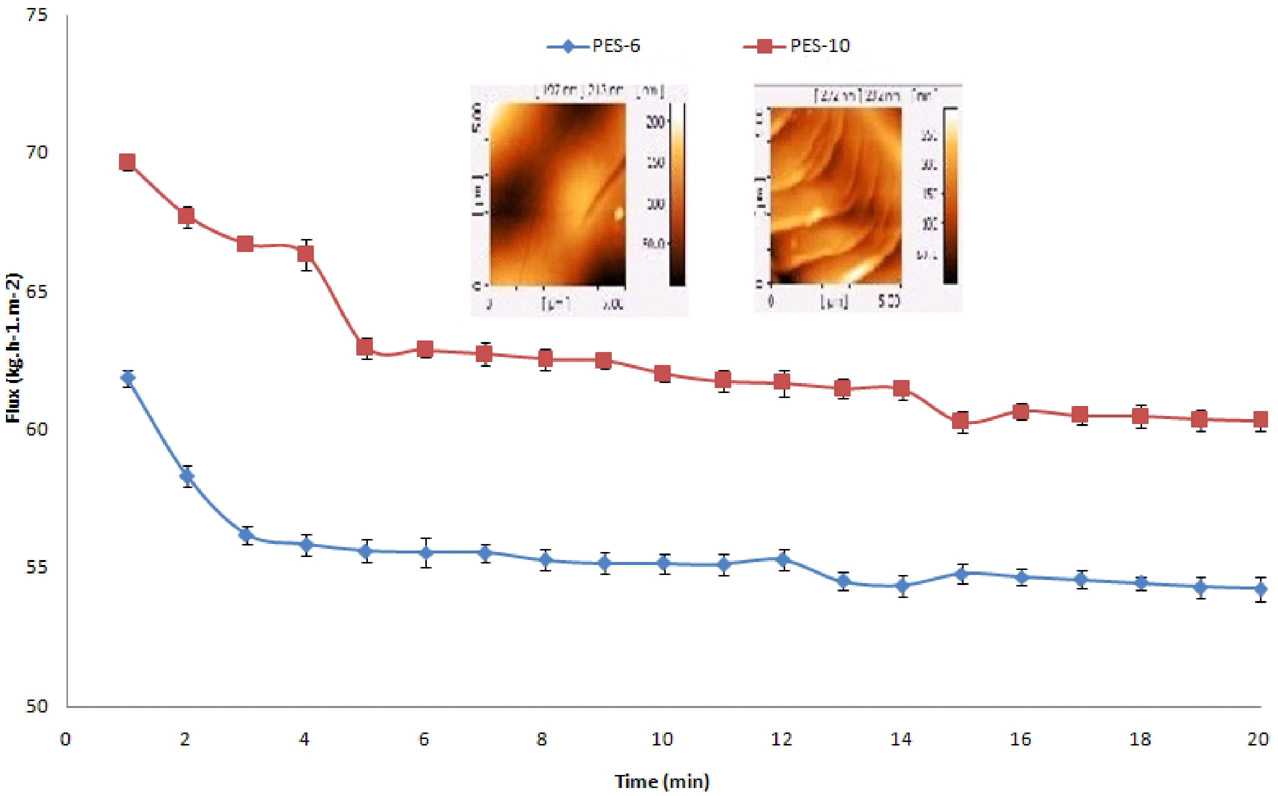


**Figure 6:** AFM images and flux versus filtration time for UF membranes under 80 psi and 120 LPH pressure and flow rate, respectively.

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