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Extraction of *Bacillus* endospores from water, apple juice concentrate, raw milk and lettuce rinse solutions using tangential flow filtration

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ABSTRACT

As part of investigative sampling there is a need to rapidly concentrate the target from large sample volumes to enable downstream detection using suitable diagnostic platforms. To this end the following describes a method based on tangential flow filtration (0.2 μm pore size) for concentrating *Bacillus* endospores from sample matrices (water, raw whole milk, apple juice concentrate and lettuce rinse samples). *Bacillus* endospores were selected as a model system as a representative select agent of significance. From optimization studies it was found that the recovery of endospores was in the order of 2.5% and could not be enhanced through increasing the flow rate or transmembrane pressure (TMP). However, recoveries could be increased to >80% (concentration factor 6.74–8.86) by backwashing the system with 10% w/v Tween 80 solution to detach spores adsorbed onto the internal surface of the filtration unit. The recovery of endospores from raw milk and apple juice concentrate was problematic due to the presence of solids or high viscosity. However, by performing an initial dilution (1:10) prior to passing through the Tangential Flow Filtration (TFF) system it was possible to achieve over 80% endospore recovery with no significant decrease in filter performance (Concentration Factor of 6–10). The recovery of endospores from lettuce surfaces was facilitated by rinsing with 4% w/v Tween 80 solution. When used in conjunction with tangential flow filtration system it was possible to recover endospores derived from lettuce rinse solutions at levels in the order of 300 cfu/ml. The results of the study illustrate that TFF is a useful approach for concentrating microbial targets from a diverse range of food matrices.

1. Introduction

Microbiological testing has historically formed the foundation of food safety despite the inherent error when attempting to detect low levels of contamination (Hoofar, 2011). In broad terms microbial testing can be sub-divided into verification and investigative activities (Hutchison, Walters, Mead, Howell, & Allen, 2006). For verification testing there are defined sample sizes and criteria applied that have been devised based on risk analysis. Investigative sampling is more problematic as the sample size is not defined and the aim is typically to rapidly detect a biohazard to enable containment (Burnett, Henchal, Schmaljohn, & Bavari, 2005).

An example of investigative sampling is the screening of food samples for select agents such as *Bacillus anthracis* as part of a biodefense program (Petsko, 2008). Although the toxicity of *B. anthracis* is lower than other select agents the stability of the endospores in food systems, in addition to resistance against thermal inactivation, make the bacterium of major concern (Driks, 2009). Indeed, there have been several high profile incidents on the deliberate distribution of *B. anthracis* in acts of bioterrorism (Imperiale & Casadevall, 2011). Although there is a range of diagnostic platforms available to detect endospores based on immune- and genetic techniques there is a need to increase the concentration of the target to be compatible with such techniques (Isabel et al., 2012). Standard microbiological based tests typically involve an enrichment step that increases the levels of the target. The time for the enrichment step can be reduced by using techniques such as immunomagnetic separation (IMS) but the technique is limited to sample volumes in the order of 1 ml or 250 ml that ultimately reduces the sensitivity of the downstream diagnostic platform (Fisher et al., 2009). Yet, with any culture based techniques, analysis time is extended due to the need of an incubation step and containment facilities are required for cultivating pathogens.

There are several options for culture-free techniques (for example, gradient density centrifugation) to concentrate the target to a sufficient level that is compatible with downstream detection platforms (Fukushima, Katsube, Hata, Kishi, & Fujiwara, 2007;

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Nicholson & Law, 1999). Although sensitive, techniques such as centrifugation are expensive and restricted to low sample volumes. In comparison, filtration based methods do not have a restriction of volumes and use relatively simple equipment that is compatible with field-testing (McEgan, Fu, & Warriner, 2009).

The following reports on the application of Tangential Flow Filtration (TFF) to concentrate *Bacillus* endospores from a range of model liquid matrices of increasing complexity (water, apple juice concentrate and raw milk). The matrices were selected on the basis of susceptibility of natural or intentional contamination on the primary production source and also represent a challenge to filtration due to viscosity, in addition to solids content.

TFF is a cross-flow system that enables high filtration rates of large volumes of sample without excessive pore blocking as experienced with dead-end filtration techniques (van Reis & Zydney, 2001). TFF has been used extensively in the biotechnology industry to recover proteins or metabolic products from fermentations but increasingly as a sample preparation technique for concentrating microbes prior to detection (Naja, Volesky, & Schnell, 2006; van Reis & Zydney, 2001). For example, Fu et al. (2005) developed a TFF sampling system for testing spent irrigation water derived from alfalfa sprout production. Here, the spent irrigation water sample collected 48 h into the sprouting period was circulated within a TFF system achieving 100-fold concentration of inoculated *Salmonella* or *Escherichia coli* O157:H7 within 2 h McEgan et al. (2009) reported on an integrated concentration and detection system based on using TFF linked to a flow through ELISA sensor. Under optimized conditions it was possible to detect *Salmonella* in 10 L spent irrigation water at levels of 2.43 log CFU/ml within 4 h. TFF is also increasingly used for concentrating microbes from water testing where the target (i.e. enteric virus) cannot be increased by culturing, in addition to avoiding bias introduced by enrichment techniques (Gibson & Schwab, 2011a, 2011b; Knappett et al., 2011; Rhodes, Hamilton, See, & Wymer, 2011).

To date, the majority of work describing TFF for concentrating microbial targets has used relatively simple matrices such as water. With more complex matrices such as apple juice concentrate and raw milk there is the potential problems caused by fouling of filters by polymeric materials (Govindasamy-Lucey, Jaeggi, Martinelli, Johnson, & Lucey, 2011; Marella, Muthukumarappan, & Metzger, 2011; Tomasula et al., 2011). Here, the deposition of polymers and other constituents on the filter surface leads to decreased filtration rates due to pore clogging that ultimately limits concentration efficacy. Therefore, the objective of the following study was to evaluate the recovery of *Bacillus* endospores from different liquid sample matrices using TFF.

2. Experimental section

2.1. Bacillus endospore propagation and enumeration

Bacillus subtilis PS 346 was donated by P. Setlow (University of Connecticut) and used as a surrogate for *B. anthracis*. Endospore crops were prepared by cultivating the bacterium in 500 ml baffled flasks containing 50 ml 2× SG medium (Goldrick & Setlow, 1983) supplemented with an equal concentration D-glucose and D-ribose (5.7 mM total carbohydrate concentration) (Warriner & Waites, 1999). Batch cultures were incubated in a rotary shaker, operating at 250 rpm and maintained at 37 °C. Sporulation was followed using a phase contrast microscope and when at least 95% endospores were present (typically 3–4 days) the culture was harvested and subsequently washed by repeated centrifugation/resuspension in sterile distilled water at 4 °C. After the tenth wash the endospore pellet was resuspended in sterile distilled water and stored at 4 °C until required.

Bacillus endospores were enumerated by initially applying a heat activation step whereby the samples were heated at 70 °C for 10 min. A serial dilution of the sample was then prepared in sterile water and aliquots (0.1 ml) plated onto Tryptic Soy Agar (TSA). The plates were incubated at 30 °C for 48 h prior to enumerating colonies.

2.2. Tangential flow filtration

The general TFF system, developed by Fu et al. (2005), was adopted. The hollow fibre polysulfone membrane was constructed from SPE-PES fibres with a total working area of 1.30 m^2 and 0.2 μm pore size (Spectrum Labs, CA, USA). Other components of the TFF system were a 10 L capacity holding chamber, peristaltic pump (Masterflex Economy Pump, Cole–Parmer, Vernon Hills, Illinois, USA), pressure gauges at the in- and outlet of the TFF filter (Fig. 1). The sample to be concentrated was placed in the 10 L holding chamber and re-circulated in a closed loop system through the TFF filtration unit. The flow rate was adjusted by the speed of the pump with the inlet and outlet pressure being controlled by tightening screw clamps (Thermo-Fisher) on the retentate side of the TFF.

The transmembrane pressure and flux rate were calculated using the following equations:-

$$\text{Transmembrane pressure} = [(\text{feed pressure} + \text{retentate pressure})/2] - \text{filtrate pressure}$$

2.3. Sample preparation and endospore recovery

Endospores were inoculated into the test matrix (5–10 L) to a level of 2–6 log cfu/ml. The test matrices were municipal water, apple juice concentrate (21°Brix; obtained from Imperial Juice, Toronto, Canada) and raw milk (obtained from University dairy herd and used on the day of collection). A sample was withdrawn from the feed solution and endospore counts enumerated as described in Section 2.1. The feed solution was then passed through the TFF system at a defined flow rate until the system dead-volume had been attained. The filtration rate was determined by measuring the flow of filtrate leaving the TFF over a 60 s period. Samples (100 ml) of the filtrate were passed through a microporous filter membrane that was subsequently overlaid onto the surface of a TSA plate that was incubated at 37 °C for 24 h. Recovery of colonies from the filtrate was

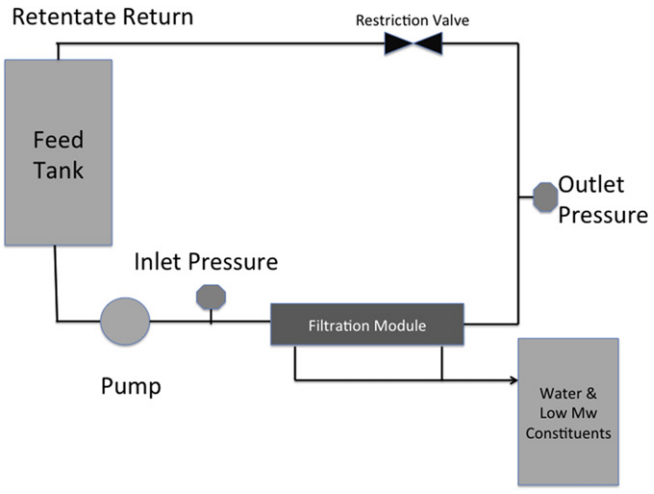

Fig. 1. Tangential flow filtrations used in the study. The set-up used extraction of *Bacillus* endospores. Samples re-circulated in closed loop system. Pressure measured with in-outlet pressure gauges.