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Physical Methods of Separation and Concentration of Microbes in Food: An Aid for Rapid Detection

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Abstract: Without question, the major block to the ability to detect food borne pathogens in real or near real-time is the continued reliance on cultural enrichment. Indeed, decreasing or eliminating the need for enrichment would allow food microbiologists the opportunity to truly harness the power of the emerging molecular detection methods. It should be clear from the preceding discussion that further research into effective matrix preparation, specifically targeting bacterial concentration and purification methods will be necessary if we are to move forward toward achievement of this goal.

Key words: Real-time, reliance, microbiologists, matrix, concentration, India

INTRODUCTION

The identification of pathogenic organisms in foods has become increasingly important. While many methods of detection are available, food microbiologists must often choose between enumeration and identification without the option of both. Most rapid methods developments have sought to shorten detection time by replacing the selective and differential plating steps with methods such as DNA hybridization, agglutination and enzyme immunoassay (Dziezak, 1987). These approaches have shortened the time to detection but because these methods detect at best 103-104 cfu of the target pathogen, lengthy cultural enrichment steps are still necessary. Recent innovations such as the Polymerase Chain Reaction (PCR) and Nucleic Acid Sequence-Based Amplification (NASBA) offer several potential advantages like replacement of cultural enrichment and higher specificity for the rapid and reliable detection of microbial pathogens in foods (Bej and Mahbubani, 1994). Despite these advantages, most applications of nucleic acid amplification for the detection of pathogenic microorganisms in food samples remain in developmental stages with significant methodological hurdles.

It has been suggested that the uses of many rapid detection technologies could be expanded if the bacteria were separated, concentrated and purified from the sample matrix before detection (Swaminathan and Feng, 1994; Wilson, 1997; De Boer and Beumer, 1999). This approach would offer such advantages as facilitating the detection of multiple bacterial strains; removal of matrix-associated reaction inhibitors and provision of adequate sample size reductions to allow for the use of representative food sample sizes and/or smaller media volumes (Jaykus, 2001).

Furthermore, bacterial concentration could aid in improving sampling techniques needed to detect low levels of pathogens or sporadic contamination which may perhaps reduce the need for cultural enrichment prior to detection. Although, methods such as centrifugation, filtration and immunomagnetic separation have been reported for bacterial concentration in food systems, none of these is ideal and in many cases a technique optimized for one matrix or microorganism is not readily adaptable to others. In short, the separation and sub-sequent concentration of bacterial cells from a food sample during sample preparation continues to be a stumbling block in the advancement of molecular methods for the detection of pathogens. The purpose of this review is to provide a detailed understanding of the science, possibilities and limitations of separating and concentrating bacterial cells from the food matrix in an effort to further improve on the ability to harness molecular methods for the rapid detection of food borne pathogens.

Process of pathogen separation, concentration and purification: The ultimate goal of emerging methods for pathogen detection is to significantly reduce detection

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time. Given the hurdles to the practical use of some of the newer molecular detection methods, the technique chosen for pathogen separation and concentration must adequately address three issues that plague food microbiologists. These issues include:

- Separation of pathogens from sample particulates
- Removal of inhibitory compounds associated with the matrix
- Provision of sample size reduction with recovery of virtually all of the pathogens, preferably without disrupting bacterial cell viability

Separation can be defined as the removal of a select population from a complex mixture while concentration is defined as a sample preparation process that seeks to reduce sample volume while simultaneously recovering all of the initial bacterial population of interest. When applied to food microbiology, the analyst wishes to separate the bacterial population from the sample matrix in so doing, the food components are discarded and the remaining bacterial cells may or may not be concentrated in the process.

Separation and concentration of bacterial pathogens from foods is perhaps more complex than concentration of viral and protozoal pathogens because most bacterial cells are more fragile than these other pathogen types. A critical need is to develop bacterial concentration methods that do not destroy the bacteria yet are effective in sample preparation.

Methods of bacterial separation and concentration can be categorized as chemical, physical, physico-chemical or biological approaches, keeping in mind that many methods actually apply any number of these general principles in combination. Furthermore, separation and concentration schemes can be used singly or combination but in all cases the goal is to provide a sample of extremely small volume with high recovery of viable target bacteria and removal of inhibitory compounds. We will outline the principles of physical methods separation approaches and provide examples in which they have been applied to the concentration and purification of bacteria from the food matrix.

PHYSICAL METHODS

Principles of physical separation methods: Centrifugation is a separation method that uses rotation about a fixed axis to produce a centrifugal force. This force propels particles suspended in a liquid medium to sediment with the rate of sedimentation dependent upon a variety of physical factors. The settling of cells in a centrifugal field such that the rate of sedimentation depends on the particle diameter, particle density, solution density, volume, angle and speed of rotation. The sedimentation rate is zero when the particle density is the same as the liquid density.

Filtration is another physical method that can be used to separate microorganisms from a food sample. During filtration, a food product or a food product homogenate is passed through a filter and microorganisms are retained on the filter while the surrounding food sample filtrate is discarded. If desired, the organisms can theoretically be released from the filter using principles of elution. Filter type, pore shape and pore dimensions all contribute to the ability to elute microorganisms from the filter.

CENTRIFUGATION METHODS

Simple high speed centrifugation (<60,000×g): Many investigators have used fairly simple centrifugation methods to concentrate bacterial cells from microbiological media before extracting nucleic acids and detecting by DNA hybridization or PCR. For instance, Darby *et al.* (1970) centrifuged at 23,000×g to harvest Clostridium welchii cells from 20 L of growth medium and the same centrifugation speed was used to remove these cells from a food sample for sub-sequent isolation and analysis of nucleic acids and polysaccharides. In pure culture, Fliss *et al.* (1991) concentrated *Lactobacillus* and *Lactococcus* species in broth culture by centrifuging at 10,000×g before suspending in acetone and proceeding with lysis of the cells. These investigators were able to get excellent yields of high quality nucleic acids for sub-sequent PCR detection with a detection limit of 10 cfu mL^{-1}.

DIFFERENTIAL CENTRIFUGATION

Differential centrifugation is based on differences in the sedimentation rate of particles of differing sizes and densities. This method has been used to separate cells primarily based on cell size differences using stepwise increases in centrifugation speed (Catsimpoolas, 1976). At each step, the particles of higher density are separated from those that are less dense. The speed of centrifugation is increased until the target particle settles after which the final supernatant is removed and the pellet is resuspended for further assay. The main advantages of differential centrifugation is that it is rapid, easy and requires fairly low gravitational forces while its major disadvantage is that the centrifugal force required to pellet the bacterial cells is frequently sufficient to pellet food components that may inhibit detection methods applied downstream (Catsimpoolas, 1976).

The most common application of differential centrifugation is the use of low-speed centrifugation to

eliminate heavier particles in foods followed by a higher-speed centrifugation to sediment bacterial cells. Niederhauser *et al*. (1992) improved PCR detection limits for *L. monocytogenes* in meat homogenates by 1000 fold after spinning at 100×g to eliminate large food particles followed by a second centrifugation at 3000×g to collect the bacteria. They reported PCR detection limits of 103 cfu *L. monocytogenes*/g meat. Differential pelleting also can be used as a primary separation procedure in the analysis of multiple cell types in which case the resulting pellet and/or supernatant can be further fractionated using a second round of differential centrifugation, density gradient centrifugation or other fractionation technique (Catsimpoolas, 1976).

DENSITY GRADIENT CENTRIFUGATION

In general, differences in density between the cell and the surrounding medium play a minimal role in separation (Catsimpoolas, 1976). However, density gradient centrifugation techniques rely upon a suspending solution that decreases in density from the bottom (highest density) to the top (lowest density) of the tube. This method has been used to separate bacteria that are not adhered to particles. Basically, cells and particulates will migrate to the portion of the tube that is at equilibrium with its own density and form a band which can be removed for further analysis. Materials commonly used to generate density gradients include sucrose, Ficoll, iodinated media such as Metrzamide and Nycodenz and Percoll (colloidal suspension of polyvinylpyrrolidone-coated silica particles) (Lindqvist, 1997).

Cell viability must be considered when using density gradient centrifugation since it can be significantly impacted if the osmotic strength of the gradient is too high or too low in comparison to the osmotic strength of the cell. Recovery of cells from a gradient may also be negatively impacted if the gradient viscosity is too high or if repeated washings are necessary to remove the separated cells from the gradient material (Catsimpoolas, 1976). Many foods tend to have density gradients of their own which also interferes with bacterial separation from the food (Payne and Kroll, 1991). Foods containing a high concentration of fats tend to trap bacteria at the fat interface, preventing bacterial separation (Payne and Kroll, 1991).

COAGULATION AND FLOCCULATION

The efficiency of centrifugation can be improved if the particle diameter is increased and this can be accomplished by either coagulation or flocculation. Coagulation is facilitated by the removal of electrostatic charges (e.g., usually by pH change) which allows particles to adhere to one another, thereby facilitating sedimentation by lower centrifugation speeds. Flocculation is achieved by adding small amounts of high molecular weight, charged materials which bridge oppositely charged particles to produce a loose aggregate which may be readily removed by centrifugation or filtration.

These principles have been routinely applied when attempting to concentrate viruses and parasitic protozoa from food and environmental samples but they should likewise be applicable to bacterial concentration. While centrifugation methods have met with some success, these are far from optimal. It has been noted that concentration of matrix-associated PCR inhibitors is likely to occur when centrifugation is the only method of sample preparation (Jaykus *et al*., 1993). For this reason, centrifugation is usually applied in conjunction with other methods. Furthermore, foods that contain fat such as meat or dairy products may trap bacteria in the fat globules, preventing sedimentation during centrifugation. Highly viscous foods pose similar problems. Additionally, foods with large particles may entrap bacteria as they settle, even at low centrifugation speeds (Lantz *et al*., 1994). It is clear that the interplay between bacterial adsorption and desorption to the food matrix must be considered in conjunction with centrifugation in the development of effective bacterial concentration methods.

FILTRATION METHODS

Physical concentration methods such as filtration tend to be less selective and have the added advantage of removing food components that may interfere with sub-sequent pathogen detection. The commercially available Iso-Grid method (Neogen Corporation, Lansing, MI) for the detection and quantification of microorganisms is a dual filtration procedure (Payne and Kroll, 1991). The food sample or sample homogenate is first passed through a 5 μm pre-filter to remove gross food particulates. Microorganisms that are bound to food particles may be retained along with the food particle at this pre-filter stage which can sub-sequently result in an underestimate of bacterial load. The sample is then passed through a 0.45 μm filter that is hydrophobic and demarcated with a grid pattern. The filter is designed to minimize the spread of colonies and is divided into sections of a known area to facilitate counting after incubation on the surface of a solid agar plate (Payne and Kroll, 1991).

The Iso-Grid methods for *E. coli* O157:H7, Salmonella, yeast and mold, coliform/*E. coli* and total

aerobic plate count have received AOAC approval. Additional Iso-Grid methods are available for *Listeria* sp., L. monocytogenes, Staphylococcus aureus, fecal streptococci, total Gram-negative bacteria, *Vibrio parahaemolyticus*, *Yersinia enterocolitica* and *Pseudomonas* sp. Since, bacteria tend to have a net negative charge, electropositively charged filters can also be used to separate bacteria from food samples on the basis of charge. While bacterial adsorption to these filters is usually quite efficient, desorption rates are relatively poor (Payne and Kroll, 1991).

Filtration itself can be followed by detection techniques including staining and epifluorescent microscopy for direct enumeration or PCR for detection. Walls *et al*. (1990) used a combination of membrane filtration and epifluorescent microscopy to separate bacteria from a beef homogenate.

Other researchers have combined filtration with other methods to process food samples in preparation for detection. Wang *et al*. (1992) used Whatman filter study #4 to remove gross particulate matter from meat and cheese samples followed by heating the filtrate to lyse the cells and release the nucleic acids for sub-sequent detection by PCR. This group was able to detect <10 cfu *L. monocytogenes*/g from artificially contaminated meat products without prior cultural enrichment but were unable to get detection from soft-ripened cheese samples processed in a similar manner.

When taken together, the use of filtration for bacterial concentration from food samples has been largely unsuccessful and is limited because large particles tend to clog the filters, compounds inhibitory to PCR may also be concentrated with the bacteria and some filters actually inhibit nucleic acid amplification (Oyofo and Rollins, 1993). In addition, filter pore size must be small enough for efficient trapping of the bacteria, creating limitations related to the type of filters and volumes of samples that can be processed (Sharpe, 1977). Furthermore, microorganisms may become attached to the upper surface of the filter or may become trapped within the filter pores (Thomas, 1988). Issues impacting the recovery of the microorganisms from the filter continue to present a challenge and it can generally be concluded that bacterial elution from filters is almost always incomplete. The act of filtration itself may also impair the ability of microorganisms to grow on solid media (Thomas, 1988).

FLOW CYTOMETRY

Flow cytometry is an optically-based method for analyzing individual cells in complex matrices and has occasionally been applied to food systems. Fluorescently-stained microorganisms pass through a beam of laser light and a signature pattern is achieved by the combination of both the adsorption and scattering of the light (Breeuwer *et al*., 1995; De Boer and Beumer, 1999). Overall, detection limits for this method were estimated to be as few as 10^2 yeast cells or approximately 10^2-10^3 bacterial cells mL^{-1}.

Detection can be completed in a few minutes and some believe that the method is suitable for detecting low numbers of specific micro organisms in fluids or rinses (De Boer and Beumer, 1999; Marie *et al*., 1999). The main drawbacks of flow cytometry are the high cost of the equipment and the need for specialized training of personnel. Additional methodological problems include interference by non-specific fluorescence or by particulate matter, less than optimal detection limits, difficulty in applying the method to solid or particulate food samples and the inability to differentiate between viable and dead cells unless specialized staining is used (Van der Zee and Huis in't Veld, 1997; Quintero-Betancourt *et al*., 2002). Destruction of cellular viability may also occur during sample processing. All told, the method is not very promising for routine use by food microbiologists.

ULTRASOUND

Ultrasound waves (sonication) have been used to promote detachment of microorganisms from surfaces such as filters, produce and seeds (Thomas, 1988; Kirk and Rowe, 1994; Scouten and Beuchat, 2002; Seymour *et al*., 2002). Ultrasound separations result in clumps of target cells that are removed from the system by physical manipulation, in this case via changes in the frequency (Coakley, 1997; Coakley *et al*., 2000).

Using a combination of filtration and ultrasound, Hawkes and Coakley (1995) were able to achieve a 5 fold reduction in sample volume and 99.9% recovery of yeast cells when a high innoculum was used. The use of ultrasound technology for separation and concentration of bacterial cells is limited by the need for high cell concentrations, low sample volume and limited data on recovery efficiency or the effect of ultrasound on bacterial cell viability.

CONCLUSION

The ideal method would be able to both concentrate pathogens and remove matrix-associated inhibitors; further, it would be universal (e.g., applicable to multiple food types and microorganisms), simple, rapid and inexpensive. This should be done in a manner that minimizes the chance for false positive results that might occur because of cross-reactivity with residual matrix components or because of the detection of dead target cells. Each of the techniques described in this review meet some but certainly not all of these criteria.

The need for combined and/or sequential methods is apparent; novel as yet unreported approaches are also needed. In the near future the possibility of near real-time detection of food borne pathogens is not a distant hope but a realistic and attainable goal.

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