MICROBIAL DISRUPTION IN HIGH FREQUENCY CAVITATING ULTRASOUND FIELDS.

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Abstract

Cell disruption in a novel 266 kHz system (0.6 ml) that has batch and flow-through capabilities was compared with that achieved with a 20 kHz (2.4 ml) generator. Batch suspensions of *Saccharomyces cerevisiae* were exposed to cavitation for times up to 5 min. Protein release (an assay of cell disruption) was measured using the Lowry method. The rate of protein release was similar for both systems. Temperature increases for similar cell disruption efficiencies were significantly lower at 266 kHz compared to 20 kHz. For 1 min batch sonications the temperature increase was 2.75 times lower at 266 kHz (8˚C increase) than at 20 kHz (22˚C increase). In a parallel study, detection of surface antigen released from Bacillus spores was measured by the enzyme linked immunosorbent assay (ELISA) technique. Samples sonicated in the 266 kHz system showed a 15-fold increase in assay sensitivity compared to unsonicated spores. The tolerable temperature increase in the 266 kHz flow system suggests that it has potential for increasing bacterial antigen detection in the environment.

Introduction

There is a requirement for rapid, sensitive detection of pathogenic bacteria both in the laboratory and the field. Assay sensitivity could be improved by cell disruption prior to the assay. Cell disruption releases protein and cell wall fragments that should give improvements in the detection of smaller fragments, and an increase in concentration of detectable particles.

Disruption of microorganisms by ultrasonic cavitation [1] has been exploited extensively for many years. Almost all commercially available devices have applicators that are resonant at 20 kHz. The half-wavelength sonication probes are about 15 cm long so that, when coupled to the driving transducers the systems are physically large and are not convenient for portable use in sampling environmental sites. Cell disruption devices that operate at higher frequencies e.g. 40 kHz [2] or 1 MHz [3] provide more compact systems. Conventional 20 kHz sonication devices require the placement of an ultrasonic probe directly into the liquid so that there is some potential [2; 4] for aerosol formation that could be hazardous when treating samples of pathogens.

Sonication is a rapid, reagentless method of cell disruption. However, rapid in-line disruption remains a considerable technical challenge, especially for bacterial spores. If an in-line device can be developed and integrated with detection systems, it should result in substantial improvements in sensitivity of immunoassay-based techniques.

Cell disruption has been investigated here in a novel closed sonication system developed around a tubular piezoelectric transducer. Electrical impedance measurements and simulations by a one-dimensional (1-D) transfer matrix approach [5] identified regions where cavitation activity was strong. The disruption of a model microbe *Saccharomyces cerevisiae* provided useful information on the mechanical behaviour of the cavitating system. The sensitivity of an immunoassay for the detection of Bacillus *subtilis* var. *niger* (BG) spores increased 15 fold compared with that of unsonicated spores following a 30 s batch exposure in the 266 kHz system. Since the accompanying temperature increase was modest, the approach has potential for in-line applications.

Materials and methods.

Cell disruption systems

(i) A 26 mm long tubular ceramic transducer (PZT4D, Vernitron, Southampton, UK) of outer diameter 63.8 mm and wall thickness 6.52 mm (298 kHz radial resonance of the transducer) was fitted with a 50.5 mm outer diameter, 3.8 mm inner diameter steel cylinder (Fig. 1). The ceramic/steel bond was formed by silver-loaded conductive epoxy resin (Circuitworks ®). The epoxy-coated steel was held in a lathe and the transducer gently pushed on. A wave generator (Agilent model HP 33120A) provided an input voltage to an amplifier (Model 240L, ENI Rochester, NY) whose output was applied to the transducer.

(ii) A 20 kHz ultrasonic cell disrupter (MSE Ltd., London, UK) with a 9.54 mm diameter titanium probe was driven at its maximum amplitude (60 watts). Batch samples of 2.4 ml in a glass beaker over ice were exposed to cavitation in this system.

(iii) A 20 kHz minisonicator (Cepheid, CA, US) with a 6 mm diameter titanium probe was driven at 100% amplitude for increasing time periods. Batch samples of 100 µl of spores with 0.5mg of 106 µm and finer

acid washed glass beads (Sigma) in smartcycler tubes (Cepheid, CA, US) were exposed to cavitation in this system.

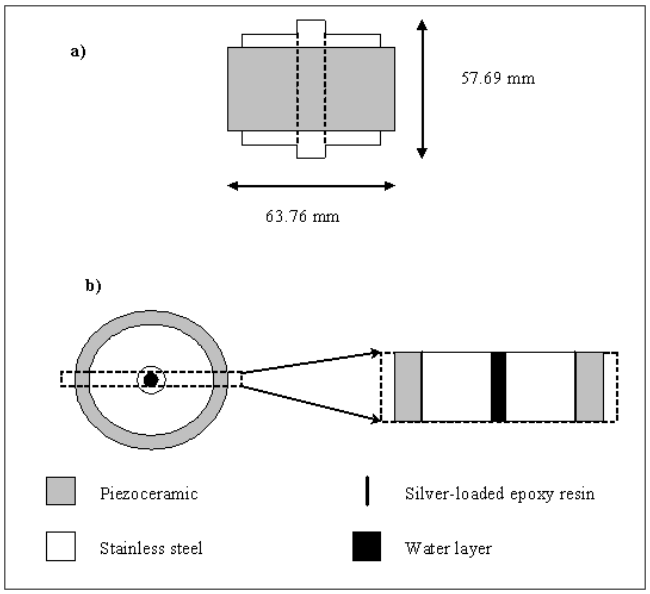

Figure 1. Schematic diagram of the cylindrical transducer cell disrupter (a) vertical plane and (b) horizontal plane.

Selection of operating frequency for tubular system.

The frequency of excitation of the transducer was swept gradually, under computer control from 200 to 500 kHz. Cavitation activity in the cell suspension was observed by stereo microscopy.

The electric admittance of the sonication system was measured using an Agilent Technologies Network Analyser (HP 8753E). The instrument was calibrated up to the acoustic filter's housing connector to ensure signal integrity and to eliminate any influence of the screened RF connecting cable.

A one-dimensional (1-D) transfer matrix [5; 6; 7] approach and an equivalent-circuit transducer model coupled with acoustic impedance transfer relationships [8] have been successfully employed to simulate plane acoustic wave propagation in planar multilayer systems. No treatment is available to simulate wave propagation in the tubular multilayer system employed here. We have, by default, applied the I-D matrix transfer model, without modification, to the tubular system. A cross-section of the layers through the system is shown in (Fig. 1b). The symmetry of the system about the central axis led to consideration of the resonance of a multilayered transducer, glue, steel, water, steel, glue and transducer system (Fig. 1b). The speed of sound, density, elastic stiffness constant and effective acoustic quality factors for the different layers were given by Hawkes et al., 2002. A number of different voltage amplitudes were applied to the amplifier input at the selected frequency. Batch samples of 0.6 ml were sonicated.

Cell disruption experiments

(i) Suspension cultures of the yeast strain *Saccharomyces cerevisiae* D1 were grown in yeast extract peptone dextrose (YEPD) media (10 g yeast extract, 20 g bacteriological peptone, 20 g glucose, 0.1 g adenine, and 0.1 g uracil in 1 l deionised water) in an orbital shaker (Mk X incubator shaker, LH engineering C$^{o.}$ Ltd.) at 150 rpm and 25°C for 48 h. Cells were washed twice by centrifugation at 3000 rpm for 5 min and resuspended in distilled water.

Yeast disruption was quantified by the Lowry modified Folin-Ciocalteu assay of released protein in the sample supernatant. Microscopic observation at x20 and x40 magnifications were employed to determine the presence of disrupted cell walls or cell debris.

(ii) A stock of washed BG spores were suspended in sterile distilled water at 10^{10} cfu/ml and 10^{11} cfu/ml.

An immunoassay-based technique of a direct ELISA was performed on samples utilising a polyclonal rabbit antibody raised against whole BG spores (supplied by Dstl, CBD Porton Down). Protein release was also determined for spore samples, as previously described.

Direct ELISA assay protocol

Samples of BG spores were coated onto Immulon 2, 96 well microtitre plates. The spores were diluted in carbonate bicarbonate buffer, pH 9.5 (Sigma) and left to incubate overnight at 4ºC. The plates were washed 3 times with PBS Tween 20 0.05% and once with PBS, then blocked in 1% milk powder PBS Tween 20 0.05% for 2 h at room temperature. Polyclonal rabbit α-BG antibody was then added at a final concentration of 10μg/ml to the appropriate wells and left to incubate for 1 h. The plates were washed as previously described, and a α-Rabbit HRP conjugate (Sigma) was added to the appropriate wells. This was left to incubate for 1 h, the plate washed 4 times with PBS Tween 20 0.05% and once with PBS. ABTS development buffer was added and the plates left to incubate until sufficient colour had developed. Absorbance was then measured at 414nm or at 405nm, depending on filter availability .

Results

The frequency dependence of the admittance of the water-loaded 266 kHz multilayer system is shown in Fig. 2 together with a simulation using the (1-D) transfer matrix approach. The extent of cavitation activity was monitored over the frequency range. High activity was seen in an approximately 4 kHz band centred at 266 kHz. High activity was again seen at approximately 297 kHz, while a less violent activity was seen at 314 kHz. The frequency

dependence of disruption (monitored by microscopy) of a dilute suspension of human erythrocytes in buffer was in order 266 ≥ 297 > 314 kHz. The Mehrschicht plot identified a resonance at around 266 kHz while the admittance plot showed a small peak close by and larger peaks at the higher frequencies. It may be that the system was less disruptive at the higher frequencies because matching to the amplifier was less efficient for the low impedances at those frequencies. Subsequent experiments with yeast or spores were carried out at 266 kHz.

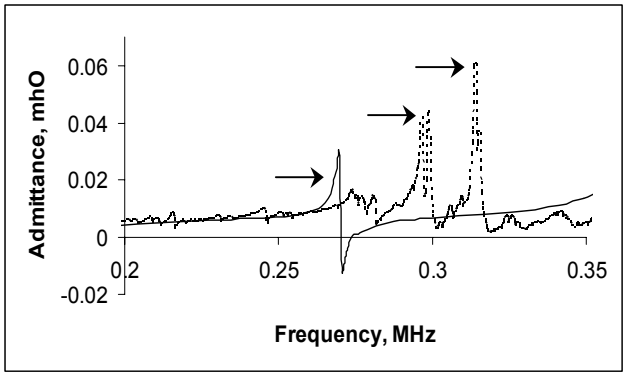

Figure 2. Frequency dependence of admittance measurements (---), and a plane wave spectrum simulated by the transfer matrix multilayer resonator model (—). The arrows identify the frequencies at which cavitation activity was strong.

Yeast cell disruption at an amplifier input voltage of $0.8V_{p\text{-}p}$ is shown in Fig. 3. The assay of protein release showed a time dependence of a rapid rise in the first 30 s and then a slower increase to a plateau level as exposure time increased. Microscopic observation at x40 magnification showed cell debris and broken cells after 90 s at 20 kHz, and 60 s at 266 kHz. Yeast cell disruption of 0.6 ml samples in the 266 kHz tubular system was comparable to that achieved in 2.4 ml samples in the 20 kHz ultrasonic cell disruptor. The measured temperature increase in the 266 kHz system was 13 K in 3 min.

ELISA assays of diluted suspensions of a sonicated 10^9 spore/ml suspension are shown in Fig. 4. Assay sensitivity was highest after 30 s sonication. Some denaturation may be occurring with longer exposure times as absorbance was lower. Drawing a horizontal line at an optical density of 0.1 (where a clear ELISA result is detected) suggests that the suspension sonicated at 266 kHz demonstrated an approximate 15-fold increase in sensitivity than a non-sonicated suspension. The improvement with the 266 kHz sonication system is at least as good as the 20 kHz cell disruptor and could be 50% higher. The 20 kHz minisonicator demonstrated an approximate 20-fold increase in sensitivity when optimised sonications of 30 s with cooling periods were performed (Fig. 5).

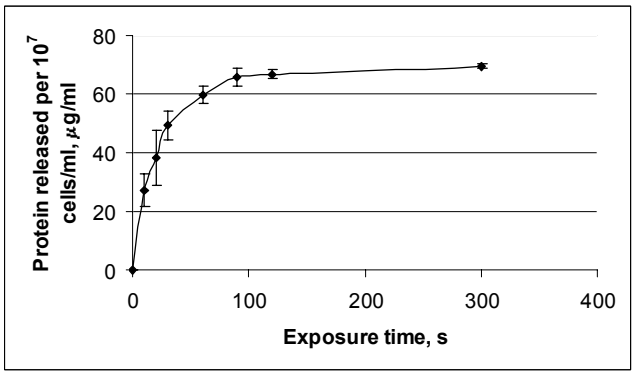

Figure 3. Yeast cell disruption in the 266 kHz system shown as protein release per 10^7 cells/ml, in µg/ml.

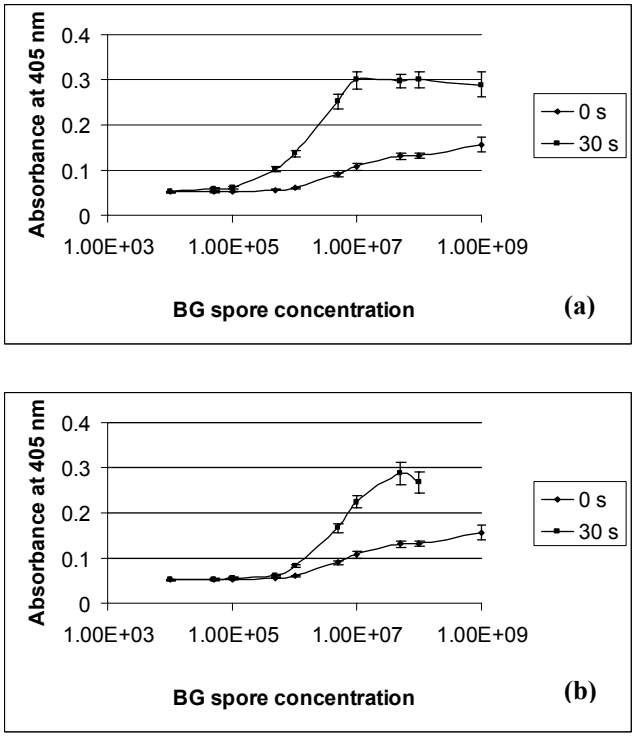

Figure 4. ELISA assay absorbance (A^{405}) as a function of dilution of a 10^9 spores/ml sample exposed in (a) the 266 kHz chamber and (b) the 20 kHz ultrasonic cell disruptor, for times of 0 s and 30 s.

Protein release was also measured from sonicated spore samples in the 266 kHz tubular system and the 20 kHz minisonicator. The pattern of release over time was similar, but the minisonicator released a significantly higher amount of protein. Although the amount of protein being released from the samples is steadily increasing over time, the immunoassays both show a decline in antigenicity of the samples with increased sonication time suggesting that the antigen is being denatured somehow. It is likely that heat (particularly in the minisonicator), the sonication process itself, or release of other spore components such as proteases are contributing factors to this observed decline in antigenicity.

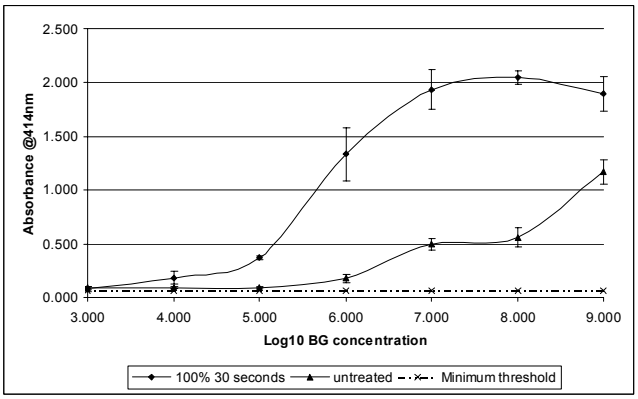

Figure 5. Detection of sonicated BG spores by ELISA (A^{414}). Spores were sonicated using the Cepheid system at 100% amplitude for times of 0 s and 30 s.

Discussion.

The concentric tubular transducer system provides high sound pressures in the volume close to the tube axis. This focusing is achieved without reaching the temperatures (range 50 – 95°C) reported when a segment of a tubular transducer of thickness resonance 1 MHz was employed to sonicate a spore suspension in a narrow plastic tube [3]. These high temperatures may be tolerable when the purpose of the exposure is to extract DNA for a PCR analysis that in any case involves thermal cycling. They are not tolerable when extraction of a protein antigen is required.

Optimal enhancement of antigen detection (15 fold) was achieved in 0.6 ml spore samples that had been exposed to ultrasound for 30 s in the 266 kHz tubular system (Fig. 4). The temperature rise occurring in the chamber was about 13 K in 3 min. These results suggest that flow rates of the order of 1 ml/min can be achieved in this closed disruption system without excessive temperature rises.

Optimal enhancement in the 20 kHz minisonicator achieved comparable cell disruption to that of the 266 kHz tubular system. However, the optimisation of the sonication protocol in the minisonicator (sonicate for 5 s then hold on ice for 1 min) for a total of 120 s sonication would require a total time of 25 min in an in-line system. This would not be an acceptable time for rapid disruption. The optimal sonication conditions in the 266 kHz tubular system do not have the same restrictions on time due to a lower temperature increase during sonication and no need for intermittant cooling periods. This tubular system has the potential for development to a flow through, in-line system that can sonicate large sample volumes, and rapidly disrupt bacterial cells for increased detection sensitivity at low cell concentrations.

Summary

The compact 266 kHz tubular system achieved comparable cell disruption to existing commercial 20 kHz devices. The modest temperature rises seen in this system allow sensitive immunoassay detection of bacterial spores, and scope for development of a compact in-line detection system.

Acknowledgements

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