

Sonobioreactors: using ultrasound for enhanced microbial productivity

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Enhanced metabolic productivity of microbial, plant and animal cells in bioreactors can greatly improve the economics of biotechnology processes. Ultrasound is one method of intensifying the performance of live biocatalysts. Ultrasonication is generally associated with damage to cells but evidence is emerging for beneficial effects of controlled sonication on conversions catalyzed by live cells. This review focuses on the productivity enhancing effects of ultrasound on live biological systems and the design considerations for sonobioreactors required for ultrasound-enhanced biocatalysis.

Many methods have been applied to enhancing bioreactor productivity [1]. Use of ultrasound in specifically designed sonobioreactors can potentially substantially increase the productivity of a biological process. However, little work has been reported on the effects of ultrasound on live microbial and other cellular systems in bioreactors and design and operation of ultrasound enhanced sonobioreactors have been investigated even less. This article is concerned primarily with ultrasonic enhancement of the performance of live microbial and other cells in sonobioreactors.

Ultrasound, or sound of frequency >20 kHz (Box 1), is inaudible to the human ear. Irradiation with ultrasound is widely used in medical imaging, sonochemical processing [2], ultrasonic cleaning of surfaces and as the basis for underwater sonar ranging. Ultrasound is potentially useful in many food-processing applications [3–6]. Medical imaging applications use megahertz range (1–10 MHz), low power, diagnostic ultrasound. High energy or ‘power ultrasound’ in the 20–100 kHz frequency range is used in many sonochemical processes. High power ultrasound treatment in aqueous media has been used to reduce hatch times of fish eggs and germination times of seeds [3] (which can be achieved under dry conditions). There is now ultrasound equipment for processing large quantities (e.g. 600 kg h^{-1}) of dry seed at 20 kHz and a vibrational amplitude (Box 1) of between $1\text{ }\mu\text{m}$ and $40\text{ }\mu\text{m}$ [3].

Enhanced membrane permeation (a phonophoretic effect) of ultrasound on cells has been widely reported [7]. Ultrasound has been used successfully to induce transfer of genetic material into live animal [8] and plant cells [9]. At sufficiently high acoustic power inputs, ultrasound is known to rupture cells and ultrasonication is a

well-established laboratory technique of cell disruption [10]. A cell can be inactivated by ultrasound at intensities less than those needed to cause disruption [11]. Intense ultrasound is known to damage macromolecules such as enzymes [3,10,12], probably from unfolding and scrambling the native protein and breaking the chain into radicals or small peptides.

High-power ultrasound induces cavitation, generation of free radicals and other mechanical and chemical effects. During cavitation, microbubbles form at various nucleation sites in the fluid and grow during the rarefaction phase of the sound wave. Then, in the compression phase, the bubbles implode and collapsing bubbles release a violent shock wave that propagates through the medium [10,13]. Cavitation is associated only with power ultrasound and is used to explain the performance enhancing effects of ultrasound in nonbiological sonochemical systems. Cavitation causes intense local heating with temperature rising to $>4\text{ }000^{\circ}\text{C}$ and pressure in a collapsing cavitation bubble can reach $\sim 1\text{ }000\text{ atm}$. Local temperature in the vicinity of a forming or collapsing bubble can change extremely rapidly at $>110^{\circ}\text{C s}^{-1}$. Cavitation, often accompanied by emission of light, can break apart relatively robust small molecules and bioactive macromolecules, and thus life does not survive cavitation for long. Intermittent, power ultrasound of short duration can cause a productivity enhancing effect in live systems. Cavitation generates microstreaming and other actions in the fluid. Just as chemical additives can be used to dampen hydrodynamic turbulence in animal cell culture [14,15] similar additives can be used to modulate ultrasound effects such as extracellular microstreaming. In addition, a high viscosity suppresses cavitation.

The mechanism of cell disruption by ultrasound is probably linked with cavitation phenomena and the resulting shock wave, and not ultrasound-induced microeddies. Ultrasonic cell disruption generally results in very fine cell debris that is morphologically different from the coarser debris produced during other fluid shear-based processes [10] of disrupting cells.

In sonochemical processing, cavitation is desired. Effective insonation in a sonochemical process requires the energy input to exceed the cavitation threshold throughout the working volume of the fluid. By contrast, in a sonobioreactor the cavitation threshold energy is not exceeded in most of the reactor volume. Cavitation threshold values can vary widely depending on the fluid being

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Box 1. Ultrasound terminology

Ultrasound is sound of frequency >20 kHz. Wave frequency is the number of repetitions (or cycles) per second of a defined vibrational state at a fixed location in space. As with any longitudinal wave, ultrasound waves have a wavelength (λ) equaling the distance between two successive peaks or troughs. The wavelength of ultrasound in air at a mean sound velocity of $\sim 330 \text{ m s}^{-1}$, is $<0.015 \text{ m}$.

The velocity c of the sound wave depends on the medium the wave is traveling through. For a liquid medium such as a fermentation broth, the wave velocity is related with the density (ρ_L) and the compression modulus (K) of the broth:

$$c = \sqrt{\frac{K}{\rho_L}}$$

The wavelength, frequency (f) and the velocity are related as follows:

$$\lambda = \frac{c}{f}$$

Adjacent peaks (or troughs) of a high frequency wave occur closer in time than the adjacent peaks of a low frequency wave (Fig. 1) traveling through the same medium. In other words, a high frequency wave has a short wavelength.

The energy imparted to the fluid by ultrasound depends on the intensity (I , W m^{-2}) of the sound, that is the energy passing through an area in unit time. The intensity is related to the velocity of the wave and its energy density, ω :

$$I = \omega \cdot c$$

The energy density is calculated as follows:

$$\omega (\text{J m}^{-3}) = \frac{1}{2} \frac{P}{\rho_L c^2}$$

P (Pa) is the pressure amplitude of the sound wave (pressure amplitude is the difference between maximum and minimum pressure at the wave peak and trough, respectively).

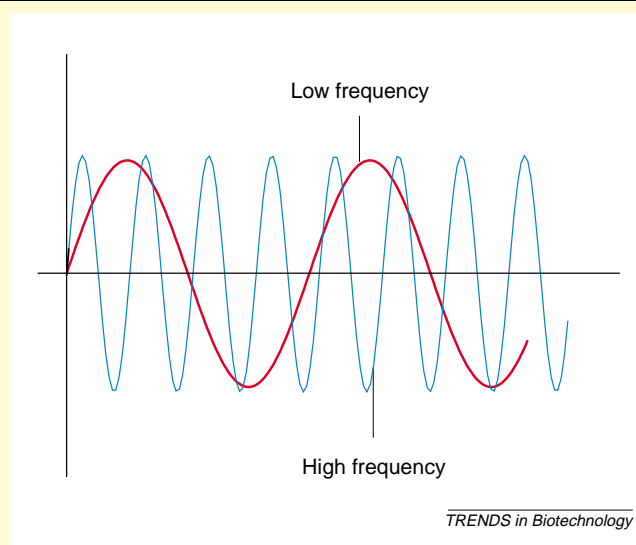


Fig. 1. A low and a high frequency wave.

An ultrasound wave can be a propagating wave or a nonpropagating standing wave. A standing wave is produced when a wave traveling in one direction encounters a wave of the same frequency traveling in the opposite direction. All points on a propagating wave undergo displacement. By contrast, certain fixed points or nodes on a standing wave undergo no displacement. Particles suspended in a medium insonated with standing wave ultrasound accumulate at the nodes.

sonicated. Typical cavitation threshold values are between 15 and 65 kW m^{-3} .

Sensitivity of cells and biocatalysts to ultrasound

Cells can differ significantly in their sensitivity to ultrasound. For example, a 5 min daily exposure to ultrasound (20 kHz, 50 W pulse) has enhanced growth rate and the final biomass yield of *Anabaena flos-aquae*, a cyanobacterium [16,17]. The same treatment reduced the growth rate of the microalga *Selenastrum capricornutum*. For both microorganisms, the ultrasonic treatment enhanced the protein content of the cells but levels of chlorophyll *a* and the uptake of ^{14}C -labelled bicarbonate were not affected [16]. A 46% increase in biomass yield of *A. flos-aquae* was attained relative to controls. Effects of this magnitude for only short periods of sonication during culture suggest substantial commercial potential for ultrasound enhanced bioprocesses.

Continuous and intermittent ultrasonication at 200 kHz and 17.2 kW m^{-2} power input has damaged *Lactobacillus delbrueckii* cells, causing them to leak intracellular β -galactosidase [18,19]. Cells can respond differently to propagating and standing wave (Box 1) ultrasounds of equal intensity [20]. Standing wave sound appears to be less damaging than propagating ultrasound. Animal cells are perhaps the most fragile of live biocatalysts [15,21] but they withstand standing wave ultrasound of considerable power. A power input of 220 kW m^{-3} did not affect viability, the glucose uptake rate and antibody production

of a hybridoma culture exposed to megahertz-range standing wave.

For *Petunia hybrida* plant cell suspensions, viability loss was insignificant up to an energy density of 25 J m^{-3} during 40 min of continuous sonication in 2.43 MHz standing wave fields [22]. Higher energy densities produced a time-dependent loss in cell viability. Rapid loss of viability occurred at energy density of only 8.5 J m^{-3} in a propagating wave field (2.15 MHz) but not in a standing wave field of identical frequency and energy density [22].

The relatively less cell damage in standing wave fields is explained by the migration of the particles suspended in such a field, to positions of low acoustic pressure and mechanical stress. The particles then remain in zones of low stress. By contrast, in a propagating wave field, the cells remain uniformly distributed in the fluid and experience a greater mean acoustic pressure [22]. Cavitation, microstreaming and other effects of ultrasound are generally more intense in a propagating wave field.

Stimulation of *Panax ginseng* suspended cells with short duration (0.5–6.0 min) ultrasound (power $\leq 100 \text{ kW m}^{-3}$, 38.5 kHz) affected cross-membrane ion fluxes within 2 min after exposure [23]. Low doses of ultrasound ($600\text{--}800 \text{ kJ m}^{-3}$) were sufficient to induce these responses. Ultrasound stimulated the synthesis of secondary metabolites, without reducing the net biomass yield [23]. Similar enhancements in metabolite productivity by low-power ultrasound have been reported with *P. ginseng* [24] and *Lithospermum erythrorhizon* cells [25].

Whether sonication is continuous or intermittent also affects the survival behavior of cells in a sonic field. During production of ethanol by *S. cerevisiae*, intermittent sonication at 300 W m^{-3} and 25 kHz more than doubled the yield of ethanol but this effect did not occur in continuously sonicated culture [26]. Cell growth and ethanol production persisted at higher intensity sonication (12 kW m^{-3}) but at rates comparable with nonsonicated controls [26]. Continuous ultrasonication at low power (300 W m^{-2} , 43 kHz) reduced fermentation time by 50 to 64% in producing of wine, beer and sake using various strains of the yeast *S. cerevisiae* [27]. Ultrasound enhanced yeast growth and decreased the concentration of inhibitory dissolved carbon dioxide [27] possibly through improved degassing. Ultrasound has stimulated sterol synthesis in bakers' yeast [28].

In one study, intermittent ultrasound treatment for a cumulative period of 150 s using a 25 kHz tube resonator (constant 80 W effective output) caused a $\sim 76\%$ increase in the release of intracellular gentamicin during production by *Micromonospora echinospora* [29]. What effect ultrasonication might have had on biomass production or viability was not clear. The broth was sonicated by continuous recirculation between a stirred bioreactor and an externally located sonication chamber [29].

Many studies have documented enhanced rates of enzyme catalyzed reactions by ultrasonication [30], but ultrasonication has also been associated with damage to isolated enzymes [3,10,12,31]. Fungal enzymes, such as cellobiase and endoglucanase of *Trichoderma longibrachiatum* and *Aspergillus niger*, respectively, appear to withstand 150 W (36 kHz) ultrasound over a continuous exposure of 48 h in a 10 l stirred vessel [32]. In general, live cells are expected to be more sensitive to ultrasound than enzymes are.

Sonobioreactor performance effects of ultrasound

Mass transfer enhancement

Ultrasound enhances mass transfer and reaction rates in both multiphase reactors and homogeneous systems [33]. Under typically used regimens of operation of conventional bioreactors, small microbial cells suspended in the culture fluid are invariably surrounded by a stagnant film of liquid [34,35]. This film can impede mass transfer of nutrients and products. In other cases, solid–liquid and gas–liquid mass transfer of substrates and nutrients can be a rate controlling factor [35]. In a sonicated bioreactor, the pulsation of microbubbles of gas in the fluid generates microstreaming and other effects [36] that can thin the fluid boundary layer around cells positioned close to the bubbles [37], thus enhancing mass transfer.

Ultrasound has the potential for enhancing mass transfer within a cell. At certain intensities of ultrasound, intracellular microstreaming has been reported inside animal and plant cells [33]. Similarly, rotation of organelles and induced circulation within vacuoles of plant cells, have been associated with ultrasound [36]. A membrane permeation enhancing effect of ultrasound has been noted already. Extremely high frequency ultrasound ($>1\text{ MHz}$) has been used to repeatedly harvest vacuole-located secondary metabolites from *in vitro* grown plant cells [37,38].

Distinct from enhancing transport through the fluid boundary layer around cells, ultrasound can greatly increase the rates of gas–liquid oxygen transfer, removal of carbon dioxide and dissolution of suspended solids and drops. This increases the supply of low solubility substrates and, indirectly, enhances a culture's productivity.

Ultrasound-enhanced diffusion of nutrients through gels has been used to explain improved dehydrogenation of hydrocortisone by gel-entrapped cells of *Arthrobacter simplex* [39]. Ultrasound did not affect the rate of biotransformation by freely suspended cells [39]. Because multiphase mass transfer can be a significant limitation in many bioprocessing situations [35], mass transfer enhancing effect of ultrasound has many potential applications.

A 20% increase in ethanol production was observed by intermittent ultrasonication during simultaneous saccharification and fermentation of cellulose pulp by a genetically modified bacterium *Klebsiella oxytoca* [32]. Mass transfer enhancement might have contributed to this effect. Continuous sonication under the same conditions had a bacteriostatic effect. A 36 kHz tube resonator generated the ultrasound at an effective power of 150 W in a 10 l working-volume conventional stirred bioreactor [32].

Ultrasonic irradiation at 20 kHz for 5 s every 10 min at a power output of 22 kW m^{-2} enhanced the rate of biotransformation of cholesterol to cholestenone by resting cells of *Rhodococcus erythropolis* [33]. This effect was explained in terms of an enhanced dissolution of the substrate by ultrasonication and the ultrasound-induced enhancement of mass transfer within and outside a cell [33]. In contrast to these observations with cells, ultrasound had no effect on cholesterol oxidation by the enzyme cholesterol oxidase [33].

Improved cell retention for high-density culture

Aggregation of animal cells and other particles in standing wave ultrasound (Box 1) has been discussed widely [40–49] and is being used to increase bioreactor productivity by improving cell retention in continuous culture. Cells are retained without relying on an invasive mechanism such as a spin filter [15]. Use of acoustic retention of animal cells has been claimed to enhance perfusion culture productivity by 70-fold and greater compared with conventional batch culture [49]. For a given degree of cell retention, the power demand of an acoustic separator increases as the culture perfusion rate is increased. Apparently because of differences in sizes and densities, viable cells are preferentially retained compared with nonviable cells.

In one study, standing ultrasound waves (2.2 MHz, 14 W electrical power input) did not damage *S. cerevisiae* cells suspended in water but caused damage when the suspension contained 12% (vol/vol) ethanol [20]. Under the same sonication power, propagating ultrasound waves damaged the yeast cells suspended in water. These results suggest that cell damage during sonication is at least partly linked with the degree of pressure impact on cells [20].

Ultrasound transmission and absorption in sonobioreactors

Sonobioreactors are derived essentially by modifying the many conventionally configured bioreactors [50] to provide

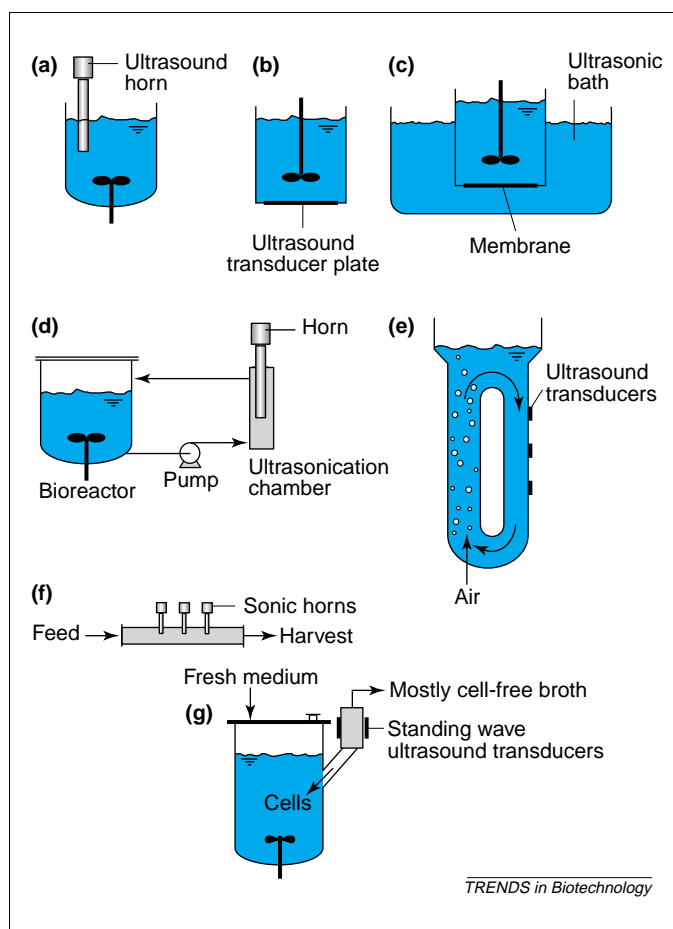


Fig. 1. Some current and future concepts of sonobioreactors. (a) Stirred bioreactor with an ultrasound horn; (b) stirred reactor with flat-plate ultrasound transducer; (c) ultrasound transmission from an ultrasonic bath through a membrane or plate sonocoupling; (d) external ultrasound irradiation of broth in a recycle bioreactor; (e) airlift sonobioreactor; (f) tubular flow sonobioreactors; (g) high-density cell culture with ultrasonic retention of cells using a standing wave. All devices except (g) can be used for continuous or intermittent sonication.

ultrasound to a large volume of fluid. The absorption of sonic energy by a fluid depends on the prevailing pressure. An increase in ambient pressure up to a certain limit increases the conversion of sound energy to shock wave energy. This suggests that an ultrasound transducer should be positioned close to the base of a sonobioreactor vessel where the hydrostatic pressure is high.

Ultrasound is produced using magneto-restrictive or piezoelectric transducers, which convert the alternating current of an electric oscillator into mechanical waves that are transmitted to the suspension through a cylindrical rod-shaped probe or 'horn' (Fig. 1). The horn, usually made from titanium, vibrates with the same frequency as the oscillator and might take the shape of a flat plate. A simple probe-type ultrasound horn delivers power ultrasound to a distance of only 70–100 mm. For a given effect, the total input of the acoustic power required will increase with increasing volume of the fluid. For small bioreactor vessels, an ultrasound horn with the tip submerged in the fluid can be sufficient. Large volumes of fluids can be sonicated in continuous or recycle-flow sonobioreactors, some of which are shown in Fig. 1. Mechanically mixed bioreactors with a polymer membrane base can be

sonicated using an alloy transducer placed next to the membrane and outside the bioreactor vessel [19]. Immersing the bioreactor vessel in a water bath provides a means of transmitting energy to the fluid in the bioreactor and through the membrane base. Similar setups for use without a water bath have been described [26].

Most of the effects of sonication are linked with the energy imparted to the culture broth and cells in a sonobioreactor. Other relevant factors in assessing the effects of sonication include the spatial variation of the sonic energy dissipation rate in the bioreactor. The amplitude of the sound waves (Box 1) and the viscosity of the broth influence the outcome of sonication. The amplitude of the wave relates directly with the acoustic power delivered to the broth. Some processes are influenced in linear proportion to the acoustic power imparted to the fluid.

At present, a satisfactory comparison of the ultrasonication results of different groups is not readily possible. This is mainly because of the several different and not necessarily correct ways the various authors have reported the ultrasonic energy input to the fermentation broth. Different sonobioreactors are probably best compared in terms of equal specific absorption (W m^{-3}) of ultrasound energy. In some cases, only the value of the electrical power (W) of the ultrasound device has been reported [20] but this is certainly more than the power actually absorbed by the fermentation broth. In other instances, an absence of information on the quantity of the fluid sonicated, makes a specific power input calculation impossible. When intensity of ultrasound is reported as W m^{-2} (Box 1), the area of the transducer tip needs to be known to calculate the power delivered to a given volume of broth. Ideally, the power absorbed by the broth should be determined experimentally by calorimetry.

For calorimetric measurements, a microbial broth or other fluid in a thermally insulated sonobioreactor is sonicated for time t . The resulting rise in temperature (ΔT), the sonication time and the heat capacity (C_p) of the fluid can then be used to calculate the energy absorption rate, E , using the equation 1:

$$E(\text{W}) = \frac{mC_p\Delta T}{t}$$

In the above equation, m is the mass of the fluid. It is noteworthy that the specific power input calculated by the various methods is only an average value. The local power input is position dependent and probably varies greatly with location. This introduces additional difficulty in accurately estimating the activation and damaging thresholds of ultrasound energy for different cells.

Because the ultrasound energy absorbed eventually appears as heat, any ultrasonicated bioreactor will require more cooling than a comparable conventional bioreactor. Generally, heat generation is not a major limitation in sonobioreactors for live catalysts because only low inputs of ultrasound energy are sufficient to produce the desired biological effects.

Conclusion

Suitably controlled ultrasonication has shown beneficial effects on the metabolic performance of live systems. These effects appear to have multiple causes that remain to be clarified fully. Intensive work is now underway for deciphering the mechanisms responsible for ultrasonic enhancement of a culture's metabolism. As mechanisms of action and suitable sonication regimens are better identified, a rational design of high-performance sonobioreactors should become possible.

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