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# Tandem shock waves to enhance genetic transformation of *Aspergillus niger*

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ABSTRACT

Filamentous fungi are used in several industries and in academia to produce antibiotics, metabolites, proteins and pharmaceutical compounds. The development of valuable strains usually requires the insertion of recombinant deoxyribonucleic acid; however, the protocols to transfer DNA to fungal cells are highly inefficient. Recently, underwater shock waves were successfully used to genetically transform filamentous fungi. The purpose of this research was to demonstrate that the efficiency of transformation can be improved significantly by enhancing acoustic cavitation using tandem (dual-pulse) shock waves. Results revealed that tandem pressure pulses, generated at a delay of 300 μs, increased the transformation efficiency of *Aspergillus niger* up to 84% in comparison with conventional (single-pulse) shock waves. This methodology may also be useful to obtain new strains required in basic research and biotechnology.


## 1. Introduction

Filamentous fungi form multi-cellular structures known as mycelia. These are important organisms because they produce proteins, metabolites and pharmaceutical compounds that have an increasing demand worldwide. For example, the global production of citric acid, an essential component of a great number of foods and beverages, is based on fermentation of the fungus *Aspergillus niger* (*A. niger*). This industry reaches 1.75 million tons annually with an annual growth of up to 4.0% [1]. Species of the genera *Aspergillus* generate large amounts of important enzymes [2–4]. Because of this, the secretion system of *A. niger* has been studied in detail to generate strains that produce high yields of extracellular proteins; however, most of these strains were generated by mutagenic agents like ultraviolet light or chemicals [5,6], which commonly cause unwanted changes in the sequences of deoxyribonucleic acid (DNA) and in many cases the nature of the mutation is not known.

For the fungi to produce the desired compounds, foreign DNA must be inserted into their genome. Unfortunately, gene delivery into filamentous fungi is problematic [7,8]. The complex structure of the fungal cell wall makes these organisms recalcitrant to genetic transformation. Because of this, novel and efficient methods for transformation of filamentous fungi are needed.

Some procedures to transform filamentous fungi use enzymes to degrade the cell wall. These protocols are expensive, laborious and time-consuming. The standard method for genetic transformation of *A. niger* is the production of protoplasts (cells that had their cell wall removed) with addition of polyethylenglycol, a compound that allows uptake of exogenous DNA. Unfortunately, the resultant protoplasts are short-lived and generally have genetic instability [9]. The use of electric fields (electroporation) or small accelerated tungsten particles (biolistics) usually generates a low number of transformants and low reproducibility [7,8,10]. Other commonly used biological methods such as *Agrobacterium*-mediated transformation (AMT) have shown low efficiency to transform *A. niger* [10].

Shock waves, as used in extracorporeal shock wave lithotripsy and orthopedics [11], have been reported to deliver genes into human and animal cells in vitro and in vivo [12–16]. Shock wave-mediated bacterial transformation has also been achieved [17,18]. Most of the pressure profiles used were generated by electrohydraulic, electromagnetic or piezoelectric shock wave sources [11] and consist of a compression pulse of up to 150 MPa followed by a decompression pulse, with a tensile peak of up to −25 MPa. The phase duration of the positive and negative pulses are

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0.5–3 μs and 2–20 μs, respectively, and the energy spectrum is between about 100 kHz and 1 MHz. Acoustic cavitation is believed to be the most important phenomenon involved in shock wave-mediated transfer of exogenous DNA into cell nuclei [19].

During in vitro experiments with cell suspensions, all microbubbles inside the test vial are compressed by the positive pressure pulse of the shock wave. The compression is followed by an inertial expansion. When a bubble reaches its maximum radius, the kinetic energy is converted to potential energy. This leads to a violent collapse after about 150–800 μs, producing a secondary shock wave and a high speed (up to 400 m/s) microjet of fluid along the wave propagation direction [20], which has been reported to act as micro syringe [21]. Each microjet may also strike adjacent cavitation bubbles, causing them to collapse faster. The energy of a single cavitation event is low; however, its concentration into a small volume causes a high energy density.

Recently, our research group reported that it is possible to transform filamentous fungi by underwater shock waves [22]. Transformation was achieved in four species of filamentous fungi. Efficiency was higher compared with current methods; however, most of the DNA was damaged by the shock waves. Because of this, the number of transformants per microgram of DNA was low compared to the standard methodologies.

The usefulness of tandem shock waves in medicine and biology has been reported by several authors [23–31]. It is known that the energy of a bubble collapse can be increased significantly if a second shock wave arrives during or shortly before the microbubbles, generated by the previous shock wave, start to collapse (Fig. 1). By adjusting the delay between both shock waves, it is possible to enhance microjet and secondary shock wave emission. Normally, this delay is between about 100 and 900 μs. Tandem shock waves may be generated at the same rate as standard single-pulse shock waves.

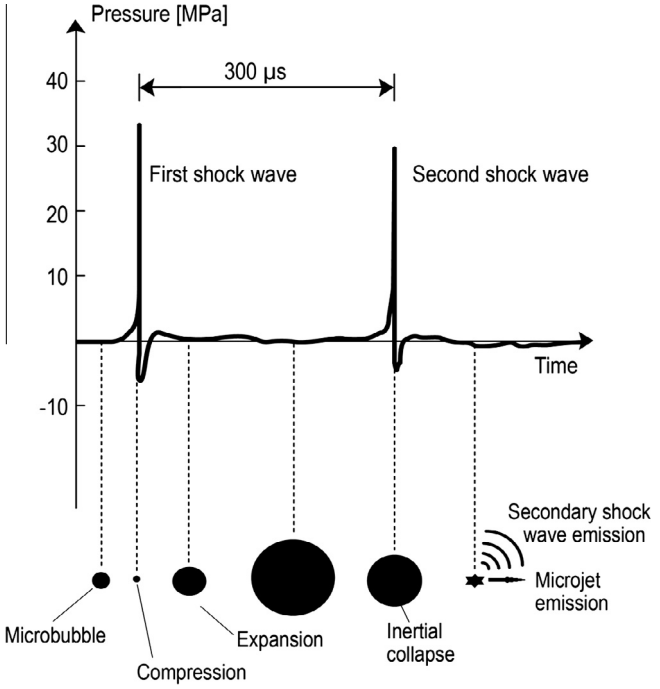


**Fig. 1.** Sketch of a tandem shock wave pressure-profile and dynamics of an air bubble in water exposed to the pressure variation shown above. Initially, the first positive pulse compresses the bubble. Once the shock wave has passed, its volume increases until an inertial collapse starts. The collapse can be intensified significantly if a second shock wave arrives shortly before or during bubble collapse. Because the bubble does not collapse symmetrically, a high-speed microjet of fluid and a secondary shock wave are emitted.

The objectives of this study were to show that acoustic cavitation is indeed responsible for shock wave-mediated genetic transformation and to demonstrate that the use of tandem shock waves can significantly increase the transformation efficiency in *A. niger*. The effect of tandem shock waves on the viability of this fungus was also analyzed by using a plasmid (DNA molecules that are separate from chromosomal DNA) conferring resistance to an antibiotic (Hygromycin) and containing the green fluorescent protein (GFP) reporter gene.

Asexual spores (conidia) of *A. niger* were used in this study, because conidia aggregates obtained in cell cultures can be separated easily into independent cells. Furthermore, conidia are mononucleated, which are genetically more stable, and single conidia can grow and produce a mycelium that is easily detected in culture plates.

## 2. Materials and methods

### 2.1. The research shock wave generator

A Piezolith 2501 shock wave source (Richard Wolf GmbH, Knittlingen, Germany) was used to generate either standard (single-pulse) or tandem (dual-pulse) shock waves. The equipment produces shock waves by high-voltage discharges applied to an array of piezoelectric crystals mounted on a hemispherical bowl-shaped backing. Each electric pulse causes the sudden expansion of all crystals, producing a pressure pulse. Shock waves arriving at the center of the sphere (F) result after superposition of the pressure pulses generated by each crystal. The concave side of the shock wave source, that is, the side where the crystals are mounted, is insulated by a polymer and placed inside a water tank (Fig. 2). To produce two shock waves at the short time delays needed for this experiment, it was necessary to duplicate the high voltage charge/discharge circuit of the standard arrangement. A special pulse generator was designed to trigger both high voltage systems. Two capacitors, one in each system, remain charged until their spark-gap is fired and the energy is discharged through the piezoelectric crystal array. The device produces two similar shock waves at an adjustable delay between 50 and 950 μs. It can be operated in manual or repetition mode (0.1–1 Hz). This research tandem shock wave generator is similar to a previously reported experimental tandem shock wave generator [25] and a tandem lithotripter [28] which were both based on a Piezolith 2300 (Richard Wolf GmbH, Knittlingen, Germany) shock wave source. Tap water at 23 °C was used to couple the shock waves into the sample fastened at the focus F. All experiments were performed using a discharge voltage of 5.5 ± 0.125 kV. The mean peak positive and negative pressures, recorded at F using a polyvinylidene difluoride needle hydrophone (Imotec GmbH, Würselen, Germany), were approximately 34 and −4.1 MPa, respectively. Associated errors correspond to standard deviations. In the tandem mode, because the bubbles generated by the first pressure pulse interfered with the following shock wave, the peak positive and peak negative pulses of the second shock wave were approximately 26% and 14% smaller, respectively, than the values of the first pressure profile.

### 2.2. Plasmid preparation

The plasmid pANGFPHPH was used in all experiments. It contained the strong promoter of glyceraldehyde 3-phosphate dehydrogenase (gpdA) of *Aspergillus nidulans*. This sequence provided high levels of expression of the Hygromycin resistance gene (hph). Fused to this gene the anthranilate synthetase terminator (trpC) allowed a correct expression of the resistance gene. It also contained a fragment of the *gpdA* promoter joined to the GFP