

# Evacuation of yeast and fungal cells using the Sponge-Like Protocol: A new approach for fungal control

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Dear Editor,

A protocol named Sponge-Like Protocol was introduced in 2013 for evacuating *Escherichia coli* cells (Amara *et al.*, 2013a, 2013b, 2014a).

The first major challenge was to determine the best compounds that are able to induce pores in the bacterial cell wall and degrade their genetic material. Four main compounds were collected. They are sodium dodecyl sulphate (SDS), NaOH, CaCO<sub>3</sub>, and H<sub>2</sub>O<sub>2</sub>. SDS, NaOH, and H<sub>2</sub>O<sub>2</sub> are active compounds that affect cell macromolecules and cell wall. CaCO<sub>3</sub> facilitates the process of transferring these compounds into the cells.

The second major challenge was how to calculate the correct concentrations that induce evacuation without damaging bacterial cell wall or their 3D structure. The minimum inhibitory concentration (MIC) and the minimum growth concentration (MGC) of each of the used chemical compounds were calculated. For optimizing the evacuation process, successive washing using 0.5% NaCl and 60% ethanol has been conducted to remove the cytoplasmic content released due to evacuation. Gentle centrifugation process and shaking were used to enhance the evacuation process. From 12 experiments that represent the Plackett–Burman experimental randomization design using *E. coli* BL21, only a single colony survived. In fact, *E. coli* BL21 was selected because it contains a plasmid that carries the lysozyme gene. In the first design, such success was not expected, and lysozyme was expected to be complementary to the protocol. Thus, the whole cells from the experiment that showed a single surviving colony were subjected to a killing mechanism via the activation of the lysozyme gene (Amara *et al.*, 2013a). Then, the protocol was applied to another *E. coli* strain, *E. coli* JM109 (Amara *et al.*, 2014a).

This strain was so sensitive to the protocol, and MIC/MGC values determined were different from those of *E. coli* BL21. Since Plackett–Burman design is a complicated statistical randomization design, the protocol was simplified and reduced to a reduced design that still involves the best two experiments obtained from the original protocol (Amara *et al.*, 2013b). The prepared ghost cells from each experiment were examined using a spectrophotometer at 260 and 280 nm to determine the concentration of released genetic material or protein as an indication for the quality of the evacuation process. Agarose gel was used to investigate the existence of DNA, which is an indicator of the release of cytoplasmic content. The cells' quality was investigated using both light and electron microscopes. It becomes clear that the protocol succeeded in evacuating *E. coli* BL21 and JM109 using the MIC/MGC combination and successive steps that avoid the interaction of the different compounds with each other and involving washing and centrifugation steps that enhance the cells' evacuation.

The third major challenge was to investigate the quality of the surface antigens. For that purpose, a pathogenic microbe, *Salmonella typhimurium* ATCC 14028, was used. The prepared ghost cells showed positive interaction against *S. typhimurium* antibody obtained from the market. Also, the serum obtained from immunized rats showed positive results against *S. typhimurium* ATCC 14028 in the hemagglutination test (Amara *et al.*, 2014b). It becomes clear that the surface antigens of ghost cells prepared using the Sponge-Like Protocol are able to show antigenicity against the specific antibody and to induce a humoral immune response in the rats (Amara *et al.*, 2014b).

The fourth major challenge was to conduct a full immunization map for both humoral and cell-mediated immunization. *Klebsiella pneumoniae* was used for this purpose, and the results demonstrated that the prepared ghost cells showed immunization for humoral and cell-mediated immune response (Menisy *et al.*, 2017). Later, the same results were obtained using *Acinetobacter baumannii* (Sheweita *et al.*, 2019). Other experiments were conducted using immunization with different ghost cells and then inducing immunocompromisation

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in rats, followed by immunization with a virulent pathogen. The immunocompromised rats have defense ability against the virulent pathogen in the challenge test.

The fifth major challenge was to apply this protocol to Gram-positive microbes. The same concept was used, but instead of using chemical compounds, a white egg that contains the lysozyme was used against a Gram-positive microbe that lives in harsh environments. Lysozyme and proteinase K have been used to evacuate *Bacillus stearothermophilus* (spore-forming bacteria) (Amara, 2016). The protocol succeeded in evacuating *B. stearothermophilus*. Another report has used the original protocol to prepare ghost cells from *Listeria monocytogenes* (Wu *et al.*, 2017).

The sixth major challenge was to apply the protocol or part of it to evacuate microbes outside the prokaryotic kingdom. The first was Newcastle disease virus (Lasota strain) (El-Baky and Amara, 2014), and the second was Pestikal Lasota viruses (Amara, 2020). The virus, which has an acellular structure, was treated only by H<sub>2</sub>O<sub>2</sub> at concentrations used in *E. coli* evacuation. The study proved successful evacuation of the virus from its RNA (El-Baky and Amara, 2014). The yeast cell represented by *Saccharomyces cerevisiae* was also evacuated (Amara, 2015b), and the evacuated cells were further processed to be used as a drug delivery system for the gossypol acetic acid (Amara, 2015a). The main change in protocol was using NaHCO<sub>3</sub> instead of CaCO<sub>3</sub>. NaHCO<sub>3</sub> is more suitable for eukaryotic microbial cell wall. The evacuation of *S. cerevisiae* encourages the application of the protocol on filamentous fungi.

Recently, the protocol has shown success to evacuate fungal cells of *Aspergillus flavus* and *Aspergillus niger* (El-Baky *et al.*, 2021, 2018a, 2018b), in addition to the spores of oyster mushrooms, which were evacuated as well (Haddad *et al.*, 2019). The randomization of the conditions highlights the effect of each of the used chemicals at either MIC or MGC value. SDS is responsible for the destabilization of cell wall/plasma membrane and pore formation in the fungal cell wall, while H<sub>2</sub>O<sub>2</sub> is responsible for DNA degradation. The protocol was investigated to control the growth of both *A. flavus* and *A. niger* on jojoba tissue culture as a preliminary trial to control fungal growth on plants (El-Baky *et al.*, 2021). This was the first trial on plant using this protocol. The Sponge-Like Protocol has succeeded in killing the fungal cells that have been sprayed on the jojoba tissue culture as well as on the surface of growth media (compared with the control plants).

This protocol can be applied to control human fungal pathogens and diseases associated with fungal spores and protect *in vitro* tissue culture of plants from fungal infections or even may be developed to formulate chemical sprays to treat plants in field to control different plant fungal diseases.

Other scientific groups have found the protocol interesting and used it to evacuate or to kill some targeted microbes (Vinod *et al.*, 2014; Wu *et al.*, 2017). Others considered it one of the suggested protocols to develop vaccine trials (Batah and Ahmad, 2020) and to be used in drug delivery (Alanazi *et al.*, 2020).

We want to draw the attention of the scientific community to the application of the Sponge-Like Protocol to evacuate and control fungal pathogens.

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