Methods

Minimum Biofilm Eradication Concentration Assay

Three microorganisms, methicillin resistant *Staphylococcus aureus, Candida albicans,* and *Escherichia coli* were re-streaked from subcultures on Mueller-Hinton (MH) agar plates to obtain isolated colonies. Isolated colonies were suspended in sterile phosphate-buffered saline (PBS) to the optical density of the McFarland standard ($OD_{621\ nm} = 0.11 \pm 0.02$). This yielded suspensions containing approximately 1.5×10^8 CFU/mL, which were then diluted to ~ 1×10^5 CFU/mL in MH broth and used for inoculation of the biofilm growth plate (*Innovotech* MBEC). Plates were inoculated with 150 µL of the appropriate suspension in each well, and incubated on a shaker for 24 – 30 hours at 37 ℃ and 110 rpm. To reduce evaporation of media in the biofilm growth wells, beakers of water were included to maintain humidity in the incubator. After incubation, biofilm growth was assessed by breaking off two growth check (GC) pegs for each organism. Staining of the pegs with crystal violet followed by light microscopy was used to visually verify biofilm formation. An inoculum for serial dilution and spot plating quantification of the biofilm growth was prepared by rinsing the broken peg in PBS for 10 seconds, and then sonicating a microcentrifuge tube containing 200 µL of MH broth and the peg at 40 kHz for 30 minutes to dislodge the biofilm cells into suspension. The biofilm challenge plate was prepared with two-fold serial dilutions of the tested AMP in MH broth, with three replicates of each concentration per microorganism. There were also at least three growth control wells and one positive control well per microorganism, alongside three sterile wells per plate. The optical density at 621 nm of each well in the challenge plate was recorded, and then the biofilm peg lid was incubated in the challenge plate for 16 hours under the same conditions as previous. After incubation of the challenge plate, the peg lid was immediately transferred to a plate containing recovery media, which consisted of MH broth supplemented with surfactants (2% saponin, 1% Tween-80 w/v%) and 2.5 v/v% of a universal neutralizer 5% L-histidine, 5% L-cysteine, 10% reduced glutathione w/v%). The challenge plate (without peg lid) and recovery plate (with peg lid) were allowed to equilibrate to room temperature. The optical density at 621 nm of each well in the challenge plate was then recorded for determination of growth inhibition by comparison to the initial optical density. The recovery plate was then sonicated at 30 kHz for 30 mins to dislodge the biofilm cells into suspension. The viable cell concentration of each well of the recovery plate can then be determined by serial dilution and spot plating. The cell concentration of peptide treated wells can be compared to growth control wells for determination of antibiofilm activity.