Electronic Supplementary Information (ESI)

Environmentally Friendly Dual Active Organic Salts for Microbial Inhibition

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Content

1. General

All chemicals were purchased from Sigma and were >98% pure. They were used without further purification.

NMR spectra were recorded in d_6-DMSO (Cambridge Isotope Laboratories) on a Bruker Avance 400 (9.4 Tesla magnet) with a 5 mm broadband autotunable probe with Z-gradients and BACS 60 tube autosampler. Each resonance is reported according to the following convention: chemical shift (δ) measured in parts per million (ppm) from the reference signal tetramethylsilane, multiplicity, observed coupling constants (*J* Hz), number of hydrogen atoms and assignment. Multiplicities are reported as a singlet (s), doublet (d), quartet (q), and a multiplet (m).

Electrospray Mass Spectrometry (ESI) was carried out on the Micromass Platform II API QMS Electrospray Mass Spectrometer with a cone voltage of 25 V or 35 V, using methanol as the mobile phase. Analyses were conducted in both positive (ESI^+) and negative (ESI^-) modes.

All melting points were recorded using a Gallenkamp Variable Heat melting point apparatus.

2. Synthesis and characterisation of the reported compounds

Cetylpyridinium Sulfathiazole

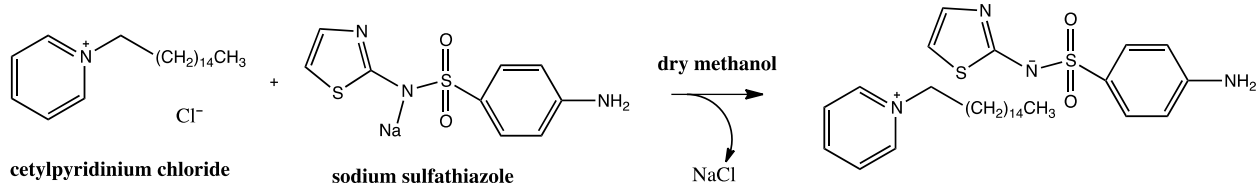

Figure S1: Synthesis of cetylpyridinium sulfathiazole (PS)

Cetylpyridinium chloride monohydrate (17.90 mmol, 6.41g) and sodium sulfathiazole (17.90 mmol, 4.96 g) were dissolved in dry methanol and stirred for three hours at room temperature. A deep yellow solution formed instantaneously. The solution was filtered through microfilters (0.20 μm). The methanol was removed to yield a liquid/solid residue. More dry methanol was added to the residue and a small amount of white precipitate was formed. The mixture was placed in the freezer overnight and then filtered to remove the white precipitate. The yellow crystalline compound was washed with distilled water to remove residual sodium chloride. The sodium chloride content (monitored with silver nitrate) was reduced to <100 ppm. Melting point: 95°C

^{1}H NMR (400 MHz, d_6-DMSO): δ (ppm) 0.82-0.85 (t, J 6.8, 3H, CH_3), 1.22-1.26 (m, 26H, 13 x CH_2), 1.87-1.91 (m, 2H, CH_2), 4.56-4.59 (m, 2H, CH_2), 5.37 (s. 2H, NH_2), 6.35-6.36 (d, J 4.0, 1H, CH), 6.43-6.47 (m, 2H, 2 x 1H), 6.86-6.87 (d, J 4.0, 1H, CH), 7.36-7.38 (m, 2H, 2 x CH), 8.10-8.14 (m, 2H, 2 x CH), 8.55-8.58 (m, 1H, CH), 9.06-9.07 (m, 2H, 2 x CH).

MS (ESI): ES^+ *m/z* 304.3 (Cetylpyridinium$^+$, 100 %), ES- *m/z* 254.0 (Sulfathiazole$^-$, 100%), 156.1 (Sulfathiazole$^-$ – $C_3H_3N_2S^-$, 15%).

Cetrimonium Nalidixate

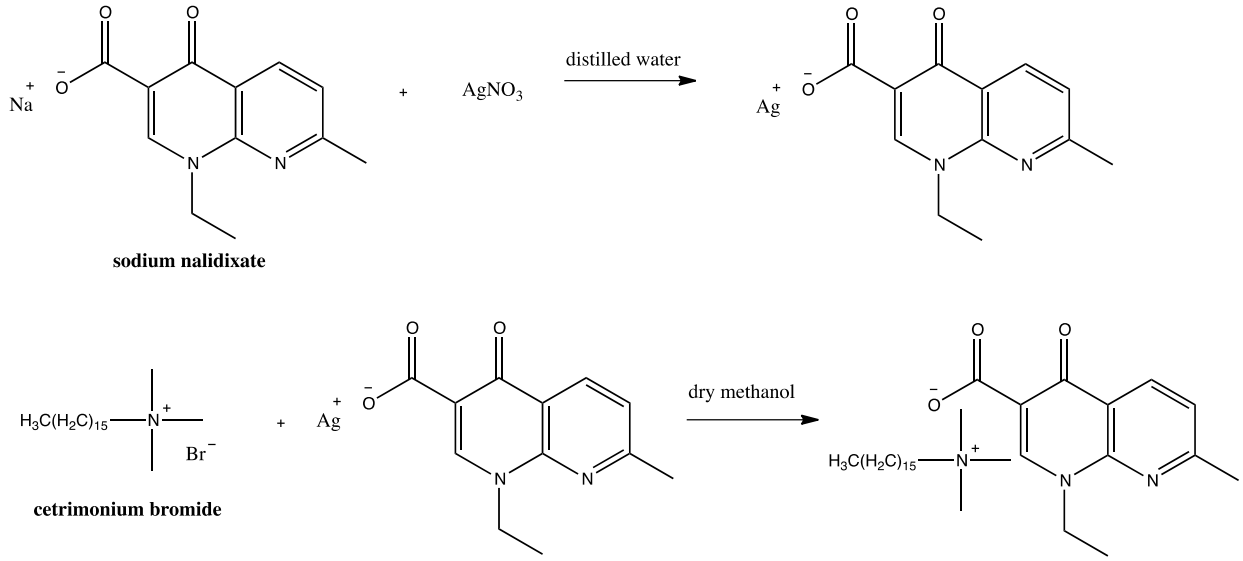

Figure S2. Synthesis of Cetrimonium nalidixate (CN)

Sodium nalidixate (59.00 mmol, 15.01 g) and silver nitrate (59.00 mmol, 10.03 g) were dissolved in water and added together. A white precipitate formed instantaneously. The mixture was protected from light and stirred at room temperature for three hours. The white precipitate was filtered off and washed thoroughly with distilled water. The solid was dried on a Shlenk line for 24 hours. Proton NMR confirmed the structure.

Cetrimonium bromide (30.00 mmol, 10.77 g) was dissolved in methanol and silver nalidixate (30.00 mmol, 10.021 g) was added to the solution. The mixture was protected from light and stirred at room temperature for four hours. The mixture was filtered through filter aid (pharmaceutical grade) and the filtrate re-filtered through microfilters (0.20 μm). Methanol was removed and an off-white solid remained. The off-white solid was dissolved in acetonitrile and diethylether was added to recrystallize the compound. The solution was placed in the freezer overnight. A white solid precipitated. This precipitate was filtered and dried. Melting point: 172 °C

^{1}H NMR (400 MHz, d_6-DMSO): δ (ppm) 0.78-0.93 (t, J 6.8, 3H, CH_3), 1.23 (s, 26H, 13 x CH_2), 1.30-1.33 (t, J 7.2, 3H, CH_3), 2.58 (s, 3H, CH_3), 3.07 (s, 9H, 3xCH_3), 3.26-3.30 (m, 2H, CH_2), 4.32-4.37 (q, J 7.2, 2H, CH_2), 7.20-7.22 (d, J 8.0, 1H, CH), 8.21-8.22 (d, J 4.0, 1H, CH), 8.36-8.38 (d, J 8.0, 1H, CH).

Cetrimonium Lactate

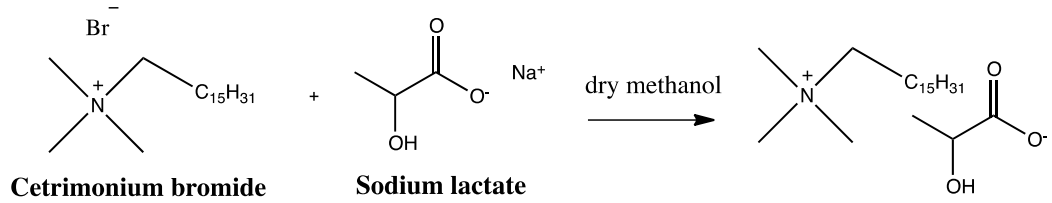

Figure S3. Synthesis of cetrimonium lactate (CL)

Pre-dried cetrimonium bromide (13.00 mmol, 4.88 g) and sodium lactate (13.00 mmol, 1.50 g) were added together into a pre-dried 3-necked round bottom flask connected to a condenser. Freshly distilled methanol (dried over sodium metal) was added and the cloudy solution was re-fluxed at 75 °C for 48 hours. The mixture was slightly cloudy. It was filtered

through microfilters (0.20 μm) and the methanol was subsequently removed resulting in a white solid residue. However it was apparent that a portion of the sodium bromide byproduct could not be removed successfully despite numerous attempts with different solvent combinations. NMR confirmed the presence of one mole of cetrimonium cation to one mole of lactate anion. Melting point > 180°C.

^{1}H NMR (400 MHz, d_6-DMSO): δ (ppm) 0.83-0.87 (m, 3H, CH_3), 1.06-1.08 (d, J 6.8, 3H, CH_3), 1.23-1.29 (m, 26H, 13 x CH_2), 1.63-1.69 (m, 2H, CH_2), 3.03 (s, 9H, 3 x CH_3), 3.22-3.27(m, 2H, CH_2), 3.49-3.54 (m, 1H, CH).

Ethambutol Hydrochloride Nalidixate

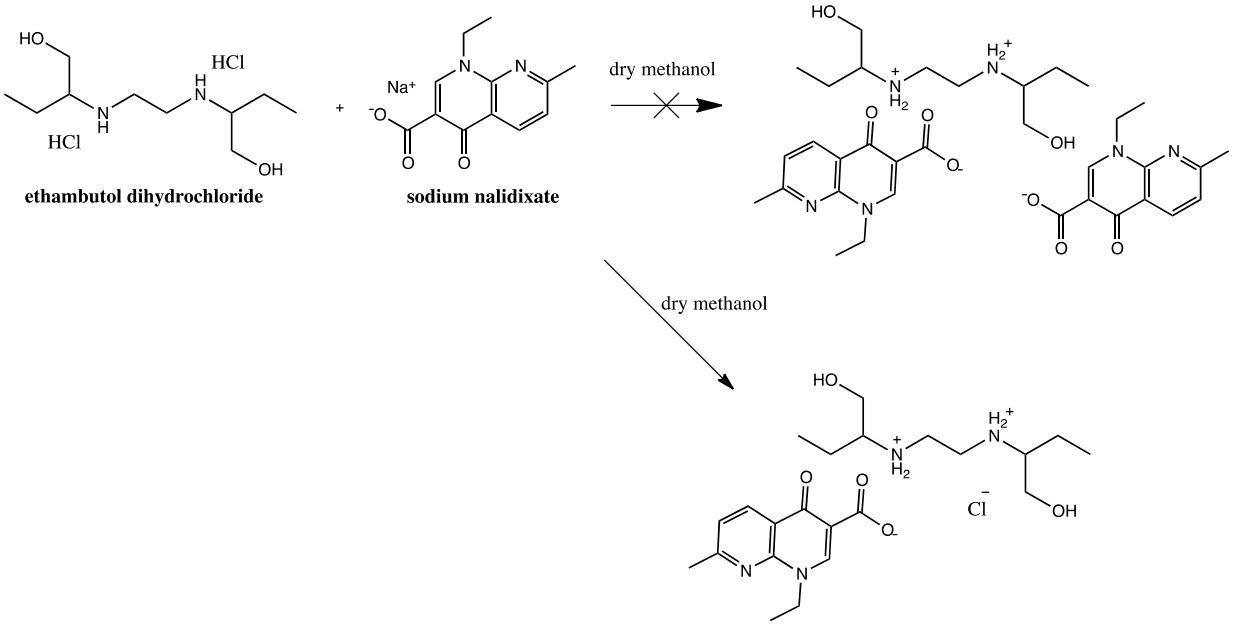

Figure S4. Synthesis of ethambutol hydrochloride nalidixate (EN)

Pre-dried ethambutol dihydrochloride (9.00 mmol, 2.51 g) and sodium nalidixate (18.00 mmol, 4.61g) were added together into a pre-dried 3-necked round bottom flask connected to a condenser. Freshly distilled methanol (dried over sodium metal) was added and the cloudy solution was re-fluxed at 75 ^{o}C for 48 hours. The mixture was filtered under N_2, and the filtered solid was subjected to NMR and MS studies which confirmed it to be sodium nalidixate. When the methanol was removed from the filtrate resulting in a solid residue, the NMR studies of this solid confirmed it to be the one to one mol ratio compound Ethambutol Hydrochloride Nalidixate with a small excess of the cation. Melting point 83°C.

^{1}H NMR (400 MHz, d_6-DMSO): δ (ppm) 0.83-0.86 (t, J 7.2, 6H, 2xCH_3), 1.36-1.42 (m, (6.54)H, 2xCH_2, CH_3), 3xCH_2), 2.51 (s, 3H, CH_3), 3.16-3.17 (m, 2H, 2xCH), 3.17-3.18 (m, 2H, CH_2), 3.27-3.31 (m, 2H, CH_2), 3.34-3.56 (m, 4H, 2xCH_2), 4.60-4.63 (q, J 6.8, 2H, CH_2), 7.54-7.56 (d, J 8.4, (0.85)H, CH), 8.58-8.60 (d, J 8.4, (0.85)H, CH), 9.09 (s, (0.84)H, CH).

Ethambutol Hydrochloride Lactate

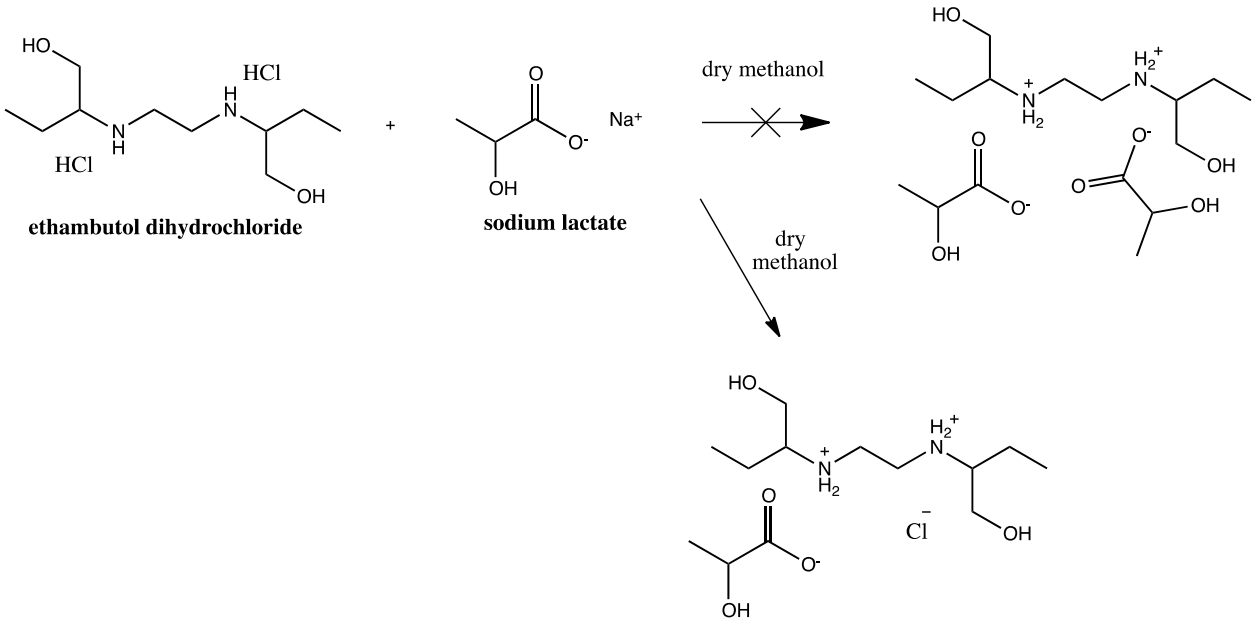

Figure S5. Synthesis of ethambutol hydrochloride lactate (EL)

Pre-dried ethambutol dihydrochloride (47.45 mmol, 13.16 g) and sodium lactate (94.90 mmol, 10.64 g) were added together into a pre-dried 3-necked round bottom flask connected to a condenser. Freshly distilled methanol (dried over sodium metal) was added and the cloudy solution was re-fluxed at 75 ^{o}C for 48 hours. Excess sodium lactate was removed. Methanol was removed from the filtrate resulting in a liquid/solid residue from which the liquid was decanted. The NMR studies of the liquid confirmed a one to one ratio of the ethambutol cation and lactate anion. Liquid at room temperature.

^{1}H NMR (400 MHz, d_6-DMSO): δ (ppm) 0.79-0.93 (m, 6H, 2 x CH_3), 1.08-1.19 (d, J 6.8, 6H, 2 x CH_3), 1.48-1.60 (m, 4H, 2 x CH_2), 2.81-2.84 (m, 2H, 2 x CH), 3.12-3.21 (m, 4H, 2 x CH_2), 3.44-3.49 (m, 2H, 2 x CH), 3.59-3.63 (m, 2H, 2 x CH), 3.79-3.85 (q, J 6.8, 2H, 2 x CH).

3. LD_{50} values of starting materials

Table 1. LD_{50} values of the starting materials from Material Safety Data Sheets

Starting material	LD50
Ethambutol dihydrochloride	LD50 Oral - rat - 6,800 mg/kg LD50 Intraperitoneal - mouse - 1,000 mg/kg Remarks: Peripheral Nerve and Sensation:Local anesthetic. Behavioral:Tremor. Behavioral:Convulsions or effect on seizure threshold. LD50 Subcutaneous - mouse - 1,800 mg/kg
Sodium nalidixate	ORAL (LD50): Acute: 2040 mg/kg [Rat]. 572 mg/kg [Mouse].
Cetrimonium bromide	Oral rat LD50: 410 mg/Kg
Cetylpyridinium chloride	LD50 Oral - rat - 200 mg/kg
Sodium sulfathiazole	LD50 Oral - mouse - 3,800 mg/kg LD50 Intravenous - mouse - 708 mg/kg LD50 Intraperitoneal - mouse - 1,320 mg/kg LD50 Parenteral - mouse - 1,950 mg/kg LD50 Subcutaneous - mouse - 1,434 mg/kg

4. Bacterial Preparation

The bacterial strains used in this study are those identified in the literature as being associated with microbial biofilms on stainless steel in the marine environment. *Citrobacter freundii* (ATCC® 8090™), *Enterobacter cloacae* (ATCC® 961™), *Vibrio alginolyticus* (ATCC® 33787™), and *Desulfovibrio desulfuricans* (ATCC® 27774™), were purchased from the ATCC (Cryosite Distribution Pty Ltd, Granville, NSW, Australia) as pure freeze-dried stock strains. The strains were recovered as instructed by the supplier and genomic information is also available for all of these candidates. Frozen stocks of each strain were stored in 20% sterile glycerol in Mueller-Hinton Broth (MHB) (Oxoid, Thermo Scientific, Melbourne, VIC, Australia) at -80°C until use. Each bacterial strain was diluted from fresh Mueller-Hinton agar plate stocks to 0.5 on the McFarland Standard (OD_{600nm} = 0.1 - 0.15) and cultured aerobically overnight in MHB, or in Modified Barr's Medium for the *D. desulfuricans,* under anaerobic conditions (<1% O_2, 9-13% CO_2) for 4 days. On the day of each experiment, bacterial strains were first diluted from broth cultures in the appropriate medium, then cultured at 37 °C with shaking at 210 rpm until bacterial growth reached mid-logarithmic phase. These strains were then diluted to OD_{600nm} = 0.1 - 0.15 in MHB and used as detailed below. Modified Baar's Medium for Sulfate Reducers was made as directed on the ATCC datasheet for the *Desulfovibrio desulfuricans* (ATCC® 27774™).

5. High throughput methodology and Analysis

This high-throughput method for testing the novel organic salts for antimicrobial action is based on the 'Methods for the Dilution Antimicrobial Susceptility Tests for Bacteria that grow Aerobically; Approved Standard-Eighth Edition'. Clinical and Laboratory Standards Institute. 2009;29(2):M07-A8. (Wikler, M.A.). Briefly, into a 96 well plate, 100 µL of the Mueller-Hinton broth was added to each well (200 µL for blank, column 1) except column 2 where 200 µL of organic salt was added. To titrate, 100 µL of the organic salt was transferred to column 3 and pipetted up and down slowly three times to mix, 100 µL was removed from column 3 and transferred to column 4. This process was repeated until column 11 and after mixing 100 µL was removed and discarded. A ½ dilution is achieved. Column 12 contained 100 µL of MHB only, and becomes the 'no treatment' control for bacterial growth. In column 2 to 12, 100 µL of bacterial culture (as described above) was added to each well. Gentamicin (Invitrogen-Gibco, Newcastle, NSW, Australia), a well known bacteriosidal antibiotic was used as a

positive control for microbial growth inhibition in this assay system. The plates were incubated at 25 °C for 16 hours (overnight), or 72 hours for *D. desulfuricans*, followed by analysis using a Perkin Elmer Microplate Reader to obtain the Optical Density (OD_{600nm}), processed using fusion 4.0 software.

Analysis of the OD_{600nm} data was as follows: the data was firstly transformed by subtracting the average background 'noise' from MHB alone (Column 1, 8 x blank wells) from the raw data. This transformed data was then normalised to provide the percentage survival by dividing each of the sample results by the average from the 'no treatment' control Column 12 (said to be 100% bacterial survival) on each individual plate and then multiplying by 100.

6. Survival Curves

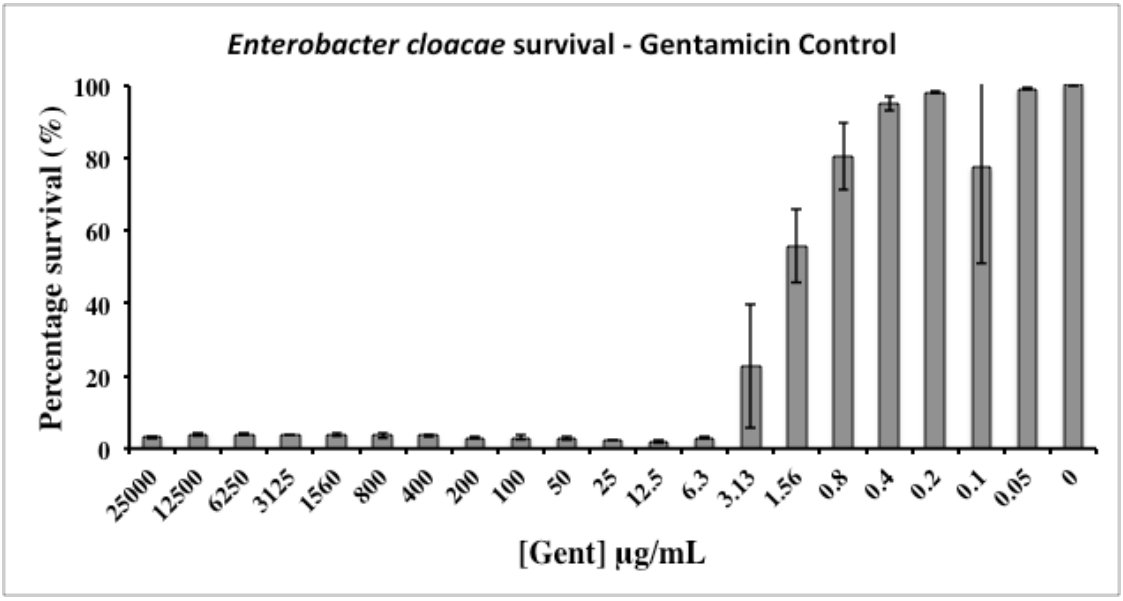

Figure S6. Percentage survival of *Enterobacter cloacae* in the presence of gentamicin control (Gent)

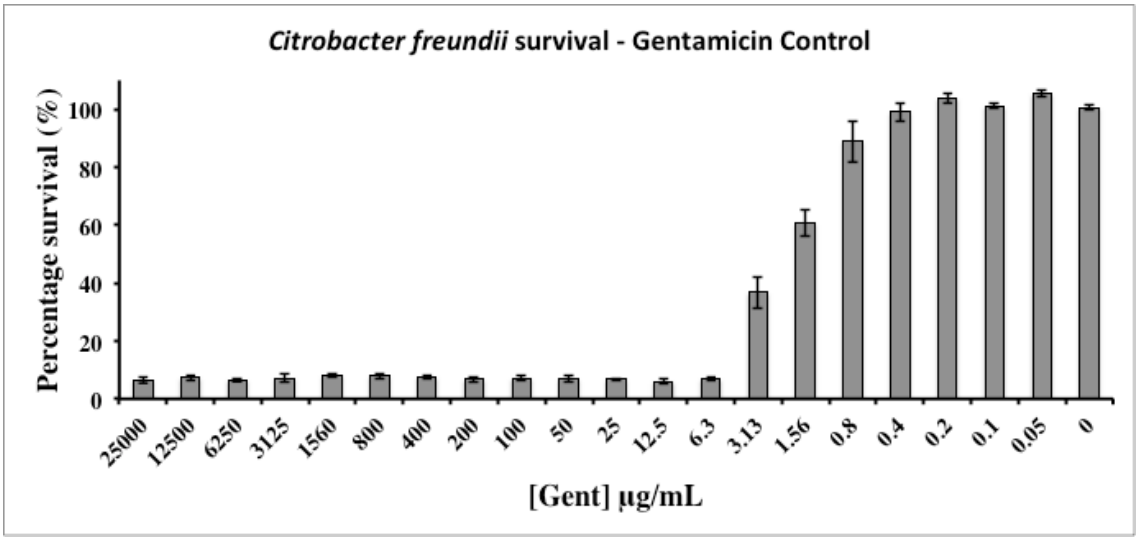

Figure S7. Percentage survival of *Citrobacter freundii* in the presence of gentamicin control (Gent)

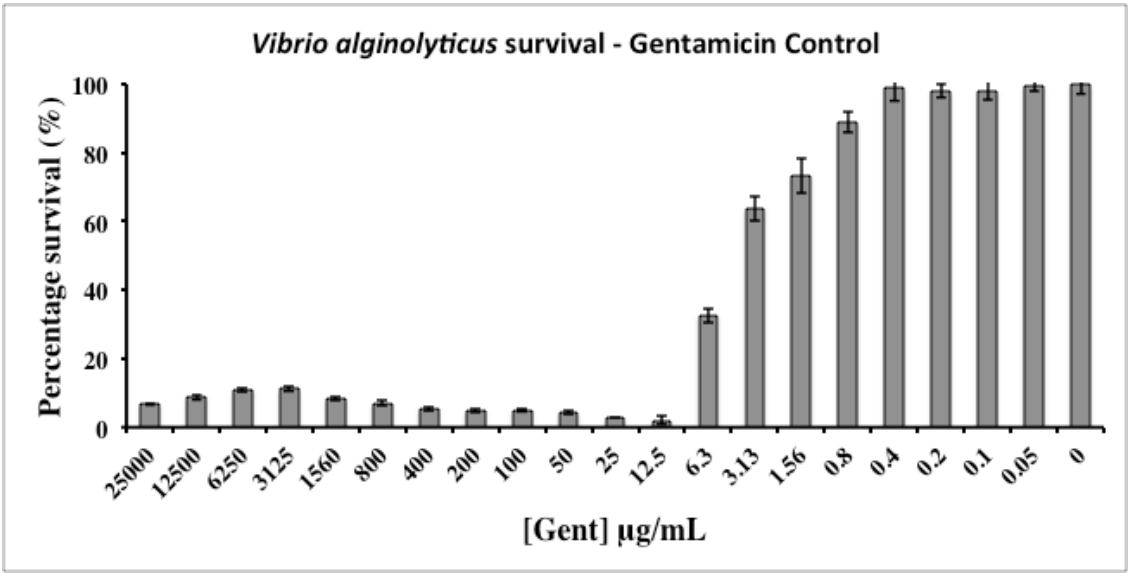

Figure S8. Percentage survival of *Vibrio alginolyticus* in the presence of gentamicin control (Gent)

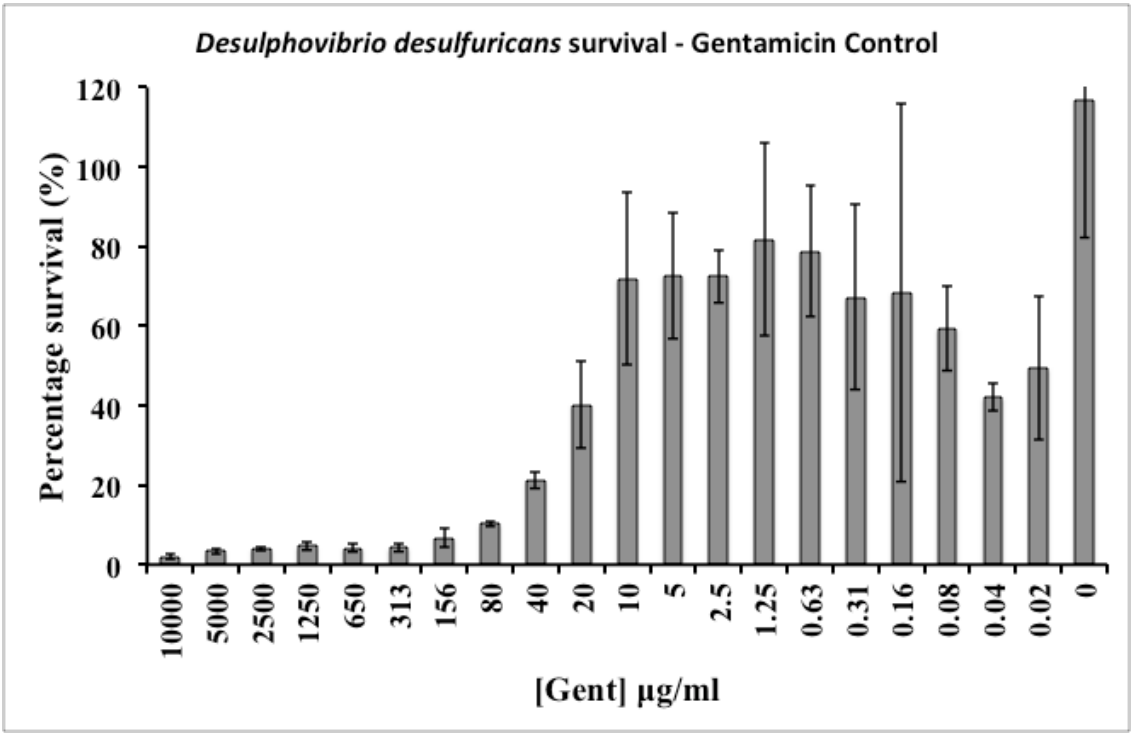

Figure S9. Percentage survival of *Desulphovibrio desulfuricans* in the presence of gentamicin control (Gent)

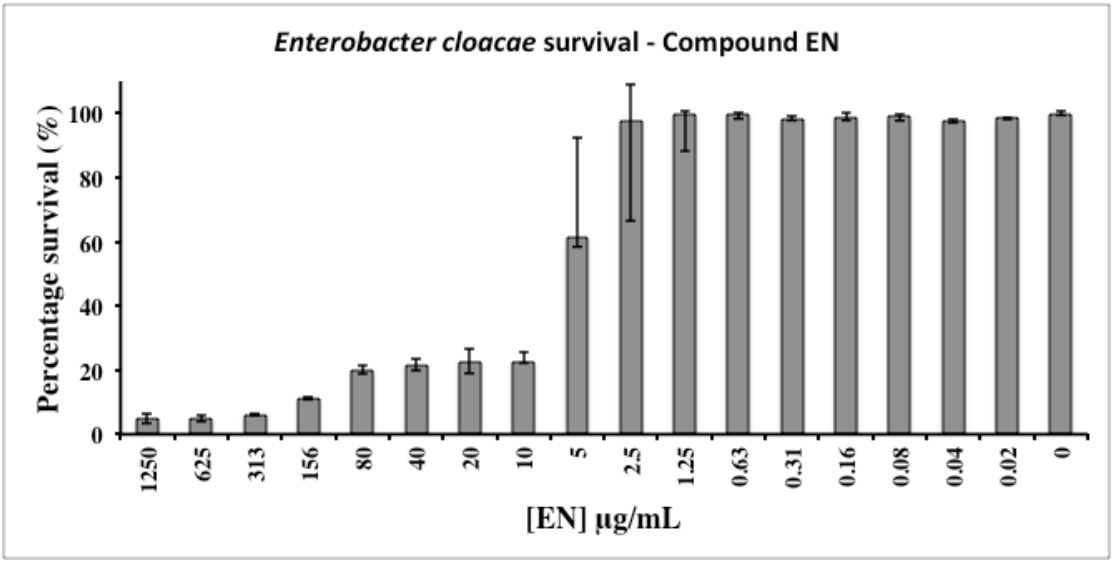

Figure S10. Percentage survival of *Enterobacter cloacae* in the presence of ethambutol hydrochloride nalidixate (EN)

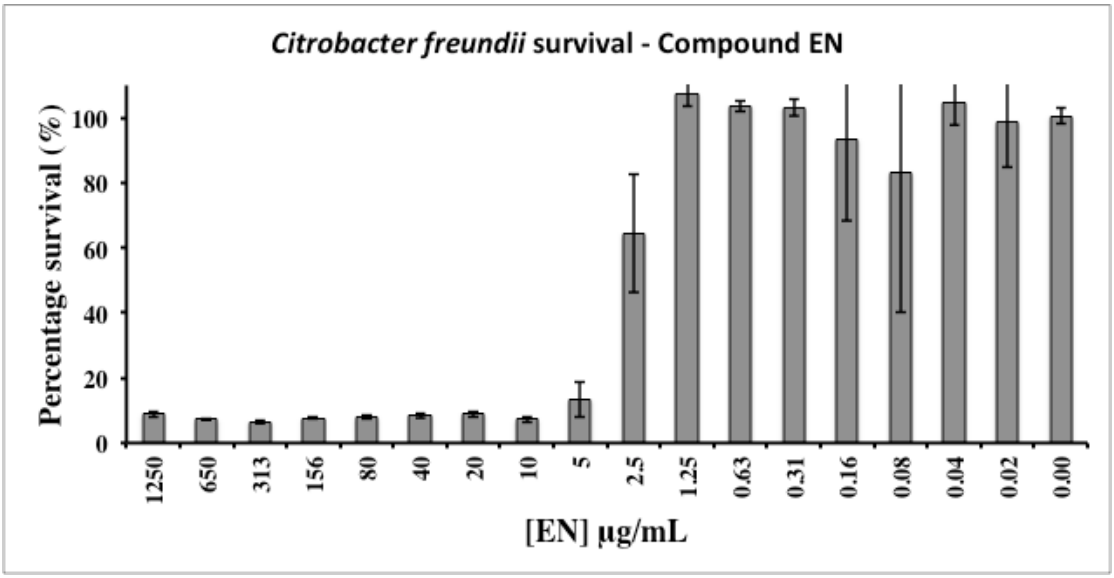

Figure S11. Percentage survival of *Citrobacter freundii* in the presence of ethambutol hydrochloride nalidixate (EN)

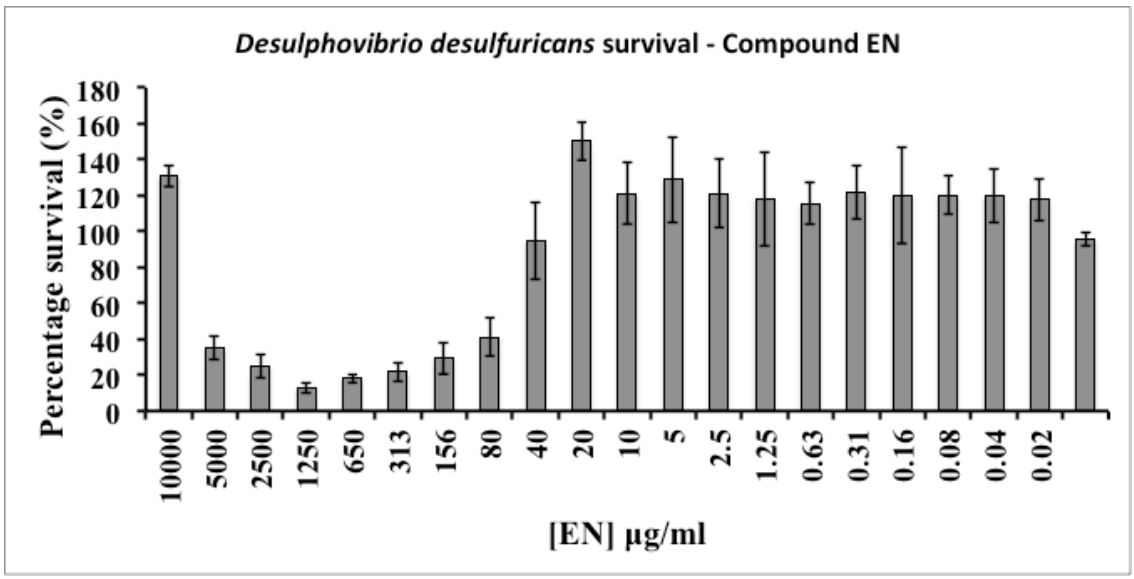

Figure S12. Percentage survival of *Desulphovibrio desulfuricans* in the presence of ethambutol hydrochloride nalidixate (EN)

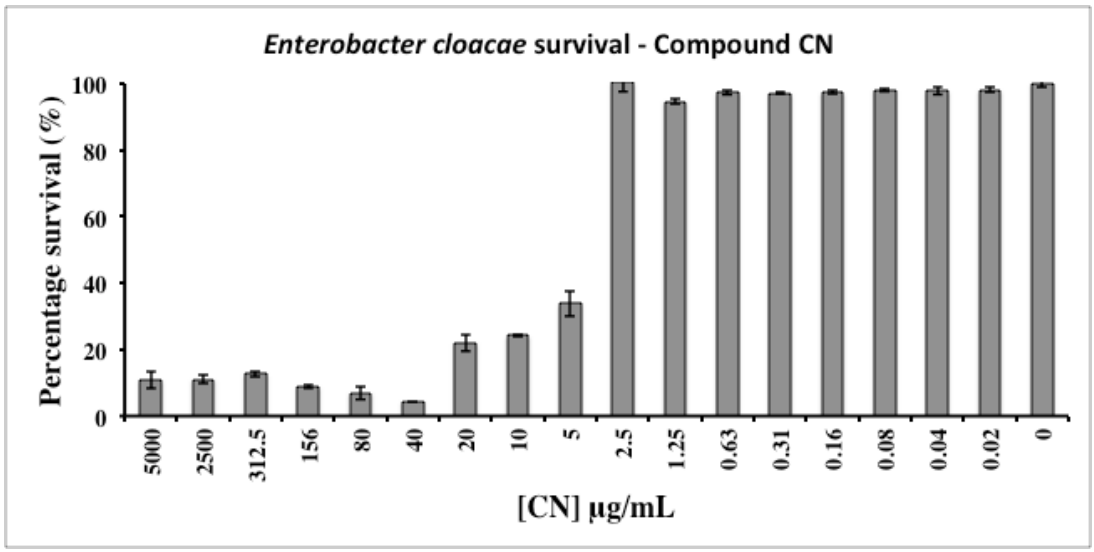

Figure S13. Percentage survival of *Enterobacter cloacae* in the presence of cetrimonium nalidixate (CN)

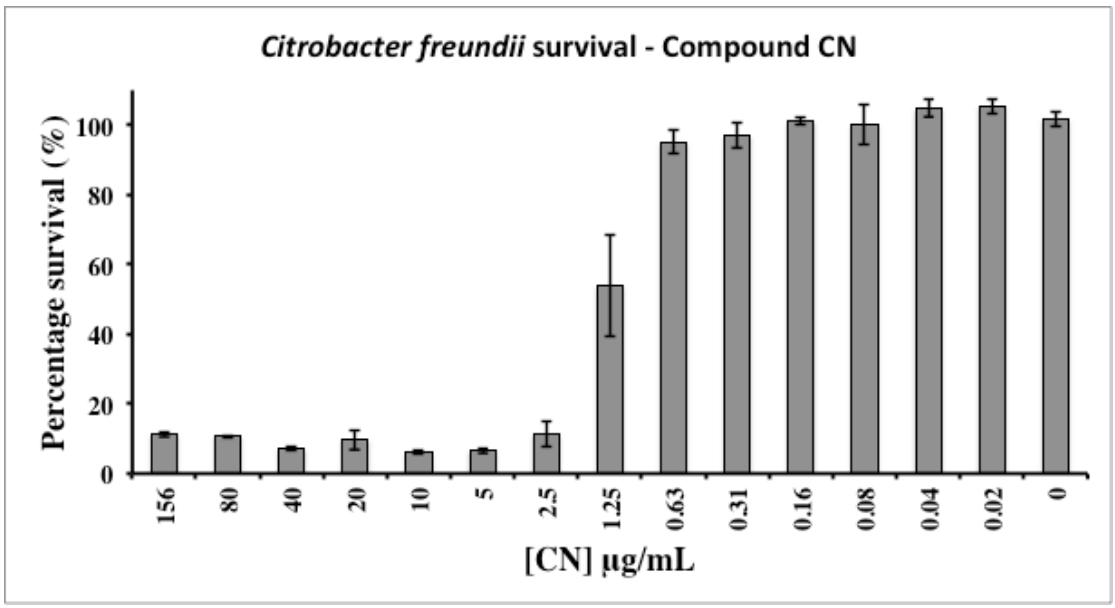

Figure S14. Percentage survival of *Citrobacter freundii* in the presence of cetrimonium nalidixate (CN)

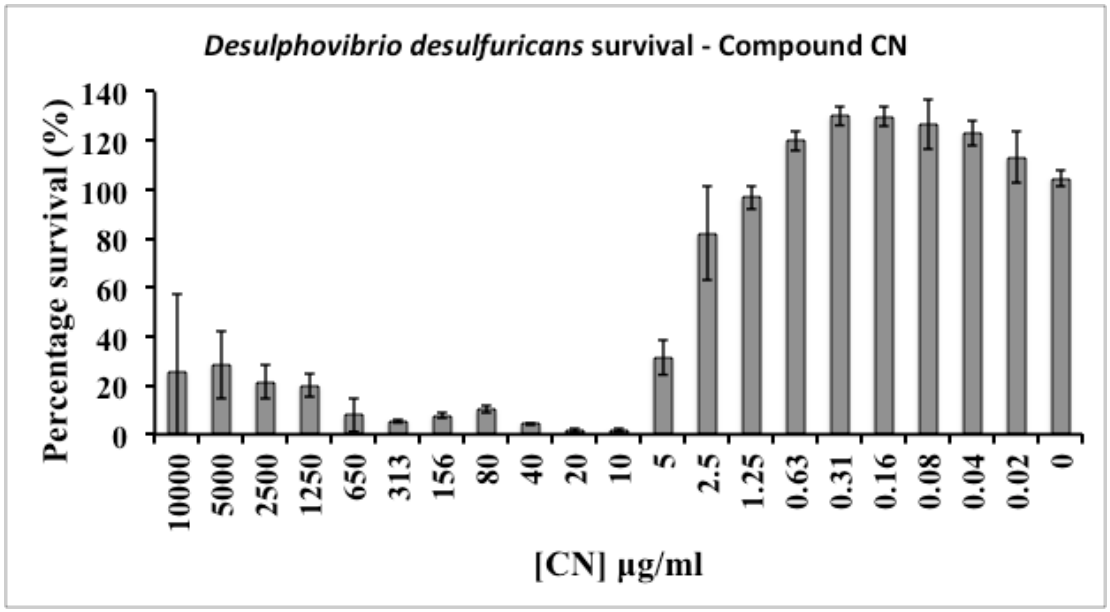

Figure S15. Percentage survival of *Desulphovibrio desulfuricans* in the presence of cetrimonium nalidixate (CN)

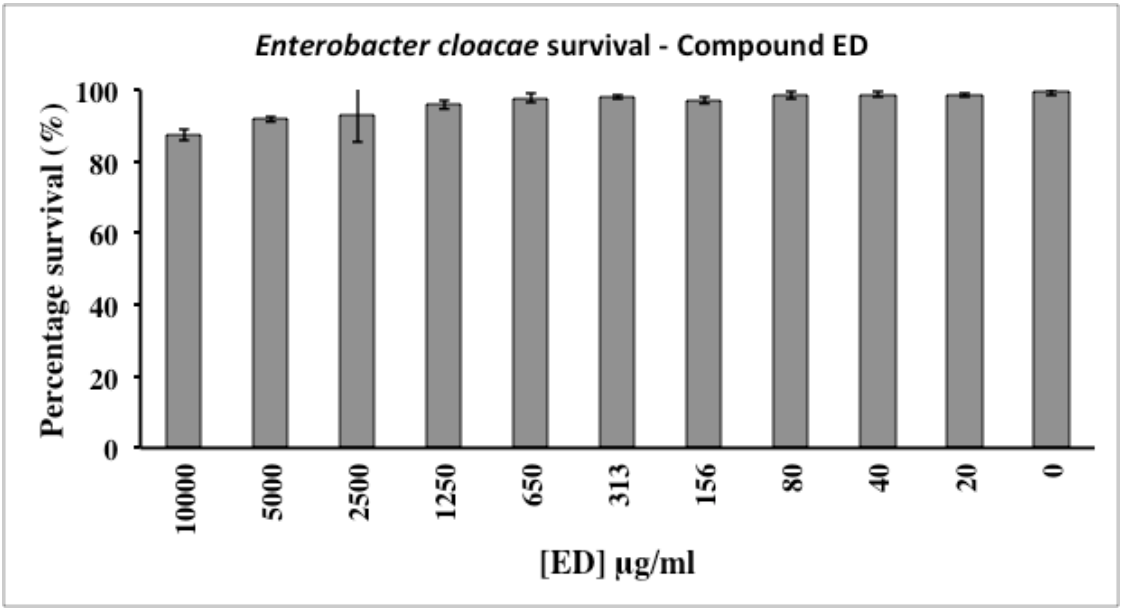

Figure S16. Percentage survival of *Enterobacter cloacae* in the presence of ethambutol dihydrochloride (ED)

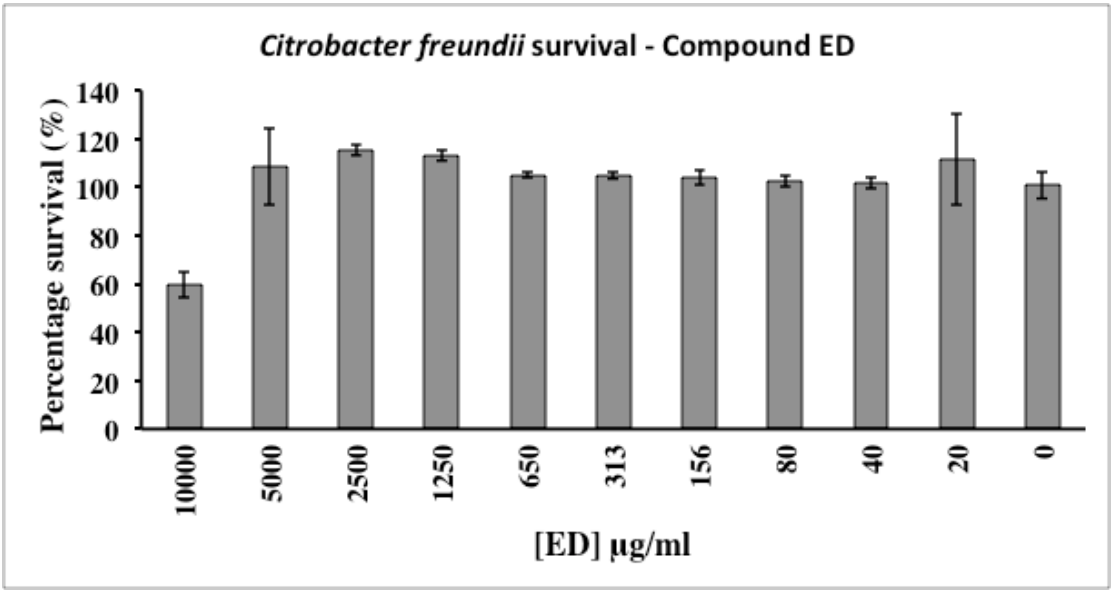

Figure S17. Percentage survival of *Citrobacter freundii* in the presence of ethambutol dihydrochloride (ED)

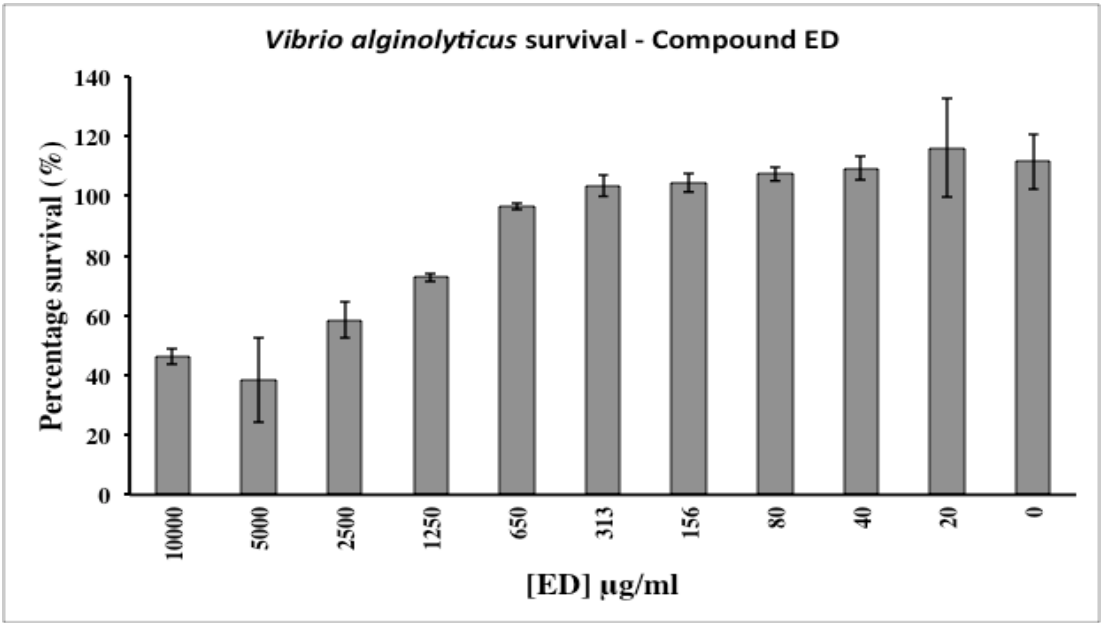

Figure S18. Percentage survival of *Vibrio alginolyticus* in the presence of ethambutol dihydrochloride (ED)

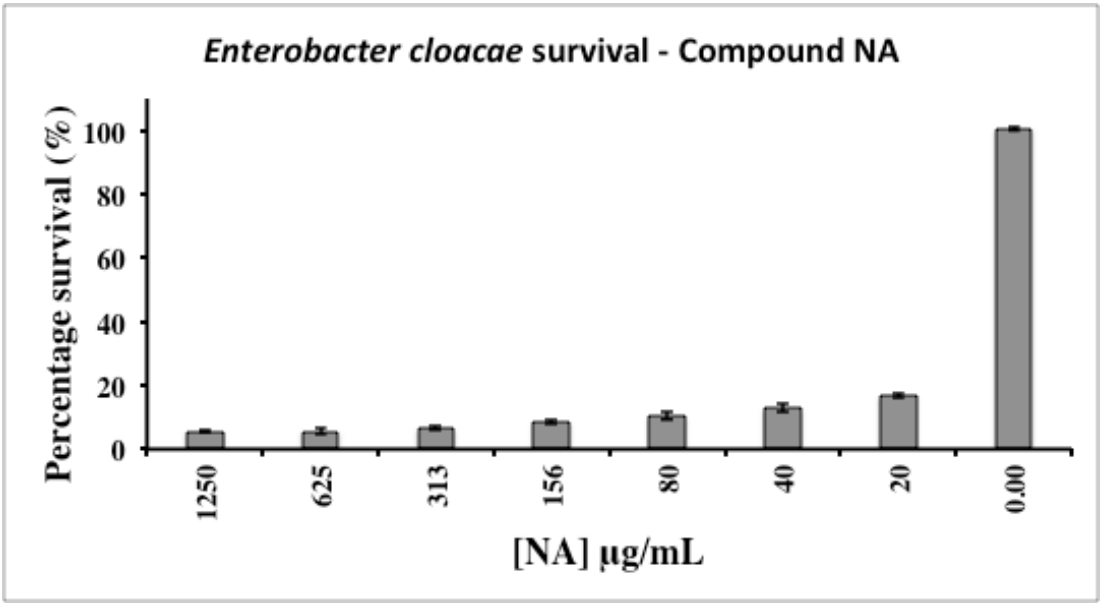

Figure S19. Percentage survival of *Enterobacter cloacae* in the presence of sodium nalidixate (NA)

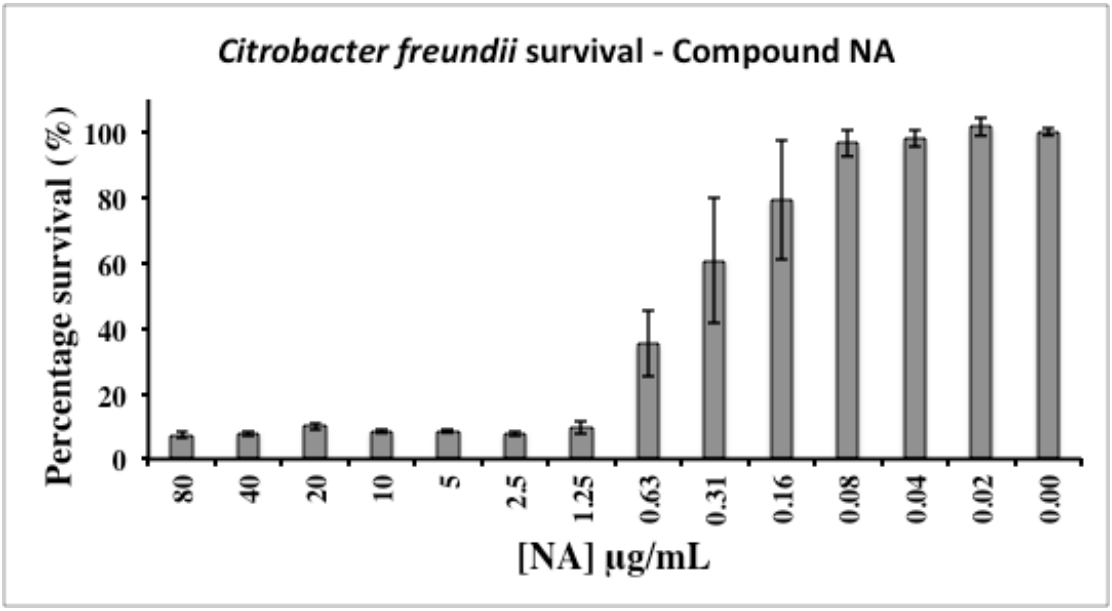

Figure S20. Percentage survival of *Citrobacter freundii* in the presence of sodium nalidixate (NA)

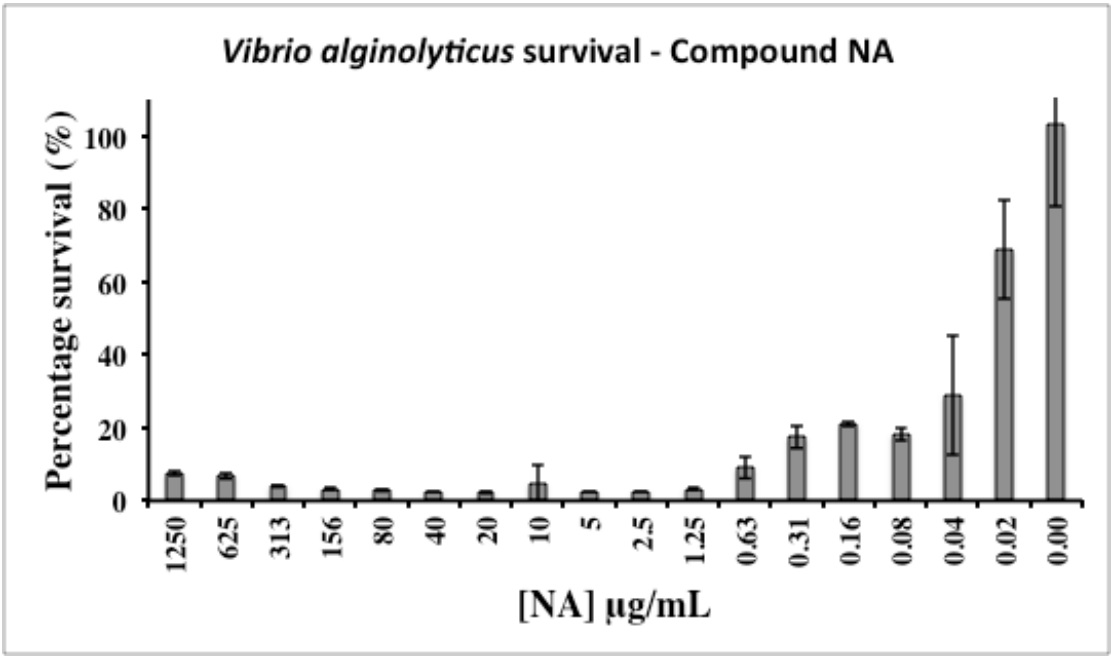

Figure S21. Percentage survival of *Vibrio alginolyticus* in the presence of sodium nalidixate (NA)

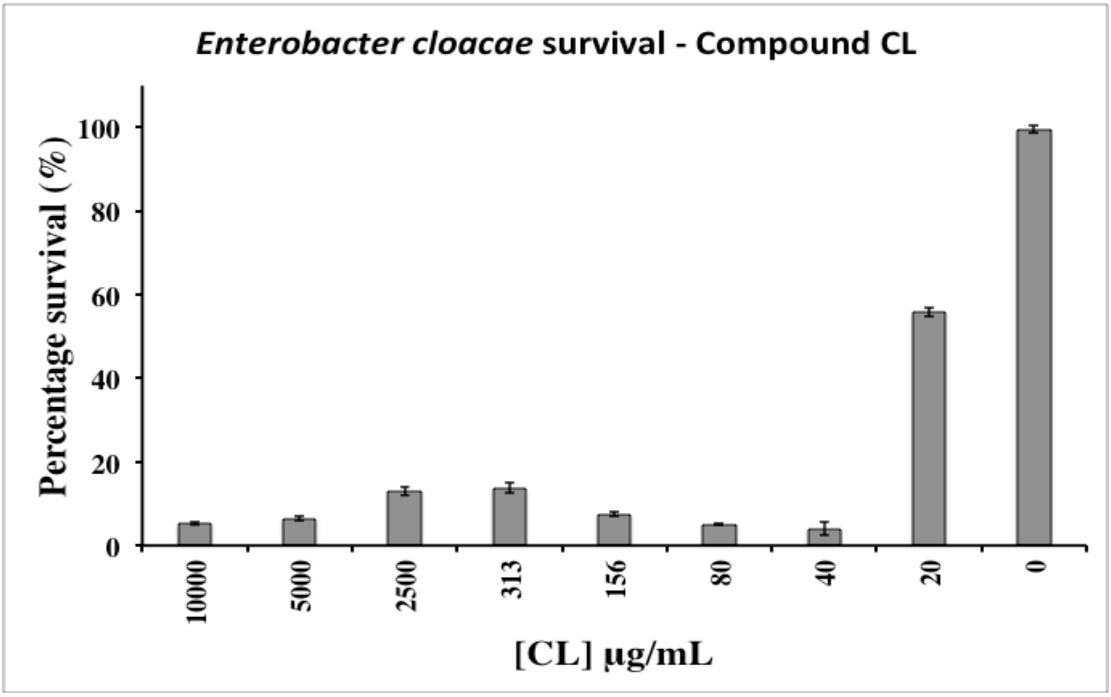

Figure S22. Percentage survival of *Enterobacter cloacae* in the presence of cetrimonium lactate (CL)

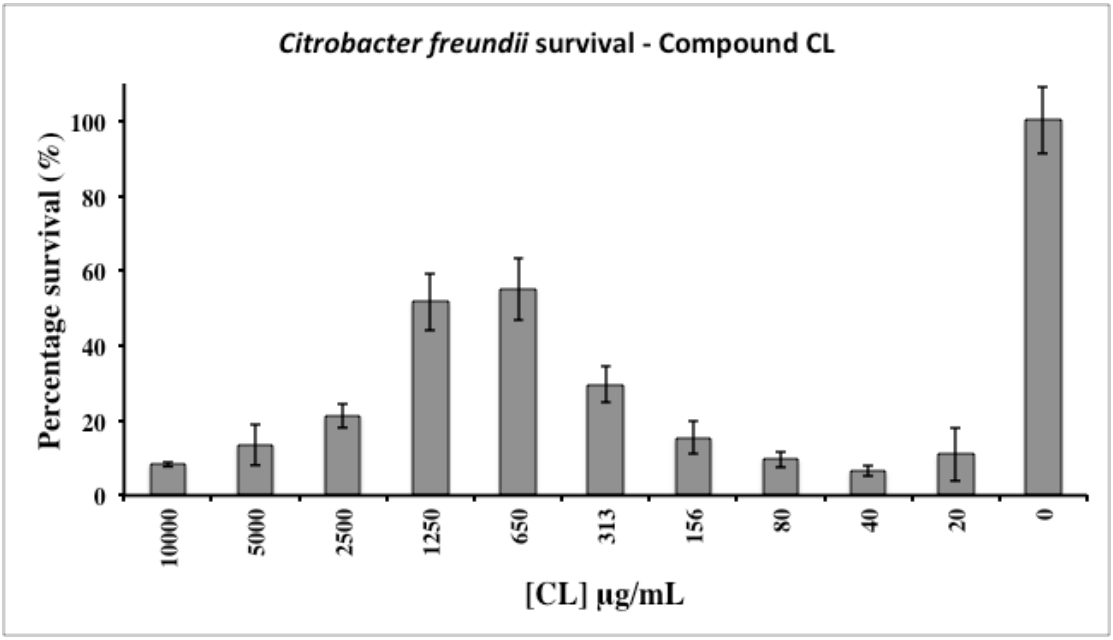

Figure S23. Percentage survival of *Citrobacter freundii* in the presence of cetrimonium lactate (CL)

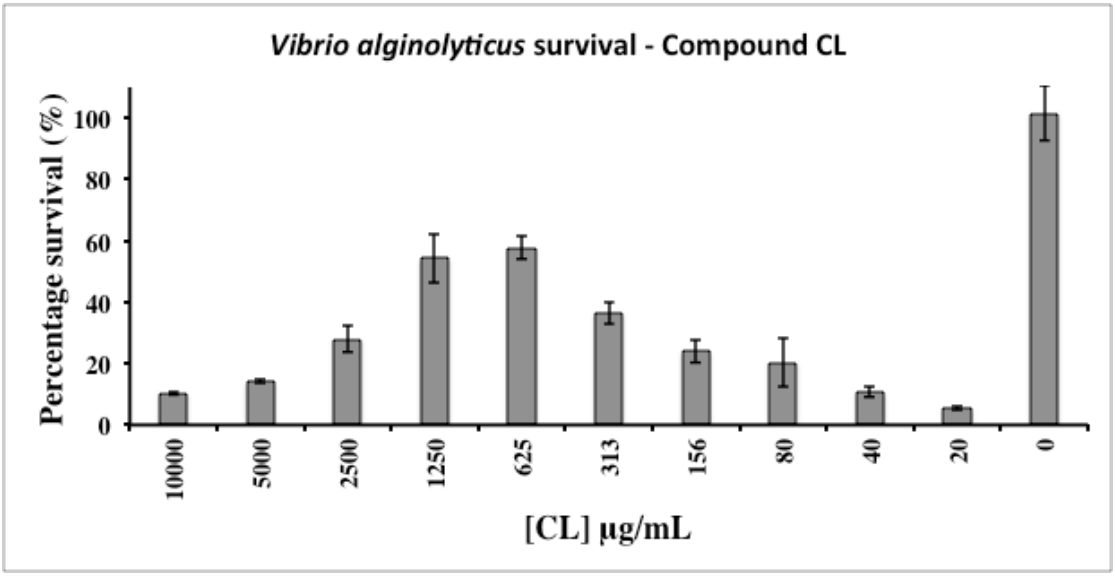

Figure S24. Percentage survival of *Vibrio alginolyticus* in the presence of cetrimonium lactate (CL)

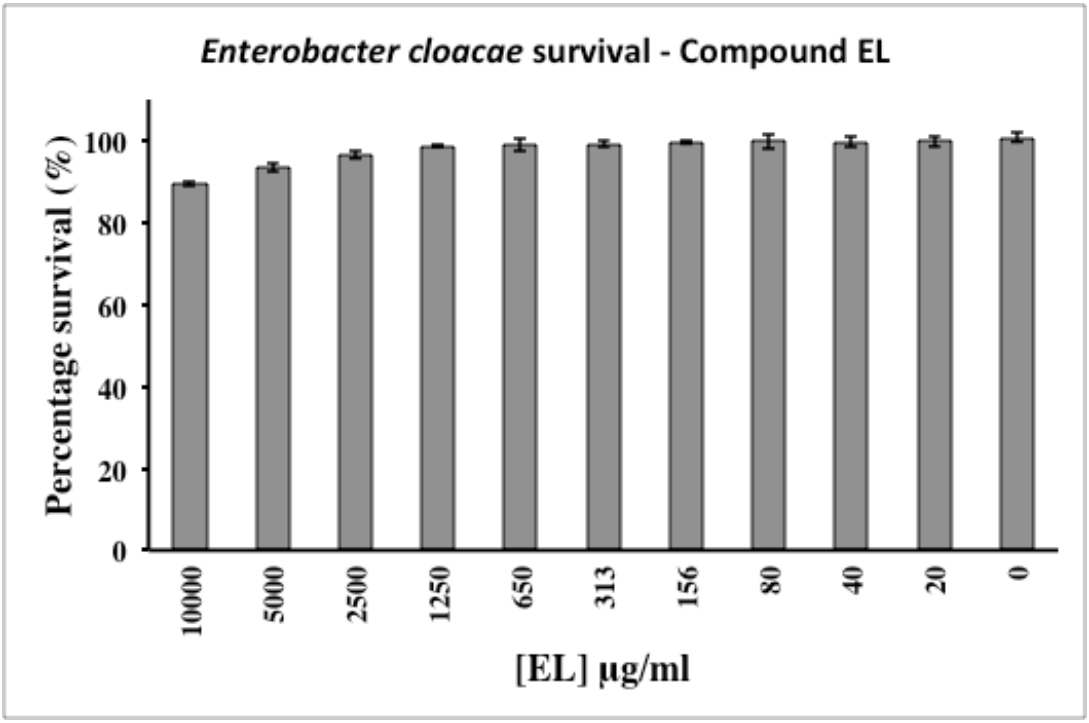

Figure S25. Percentage survival of *Enterobacter cloacae* in the presence of ethambutol hydrochloride lactate (EL)

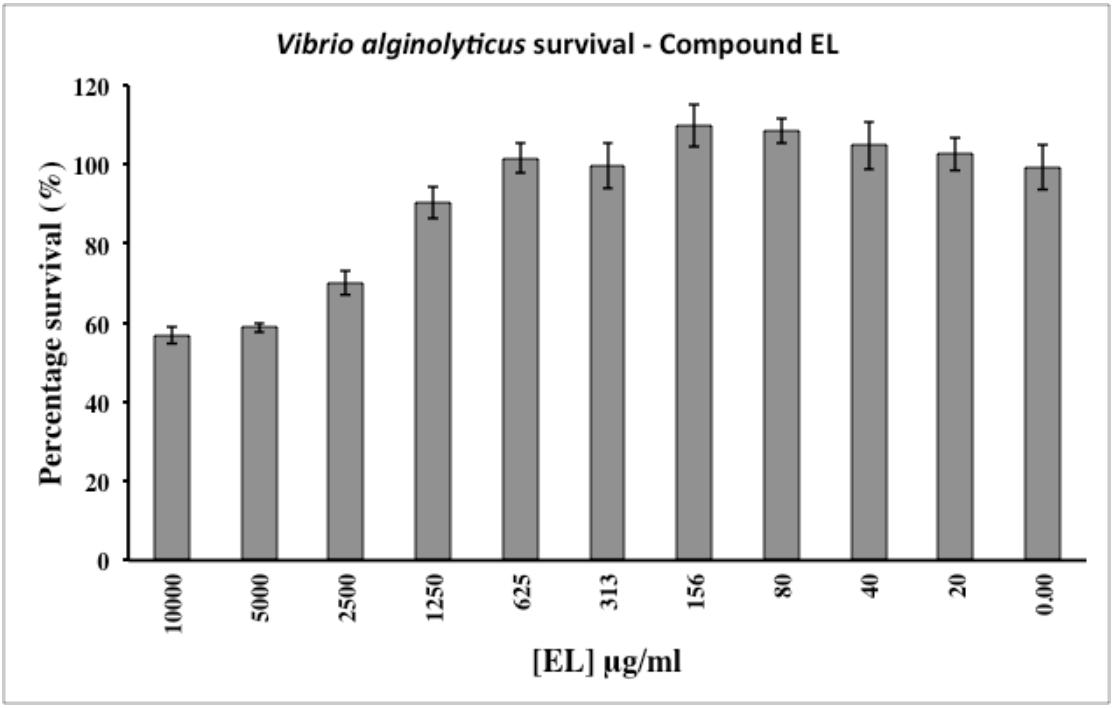

Figure S26. Percentage survival of *Vibrio alginolyticus* in the presence of ethambutol hydrochloride lactate (EL)

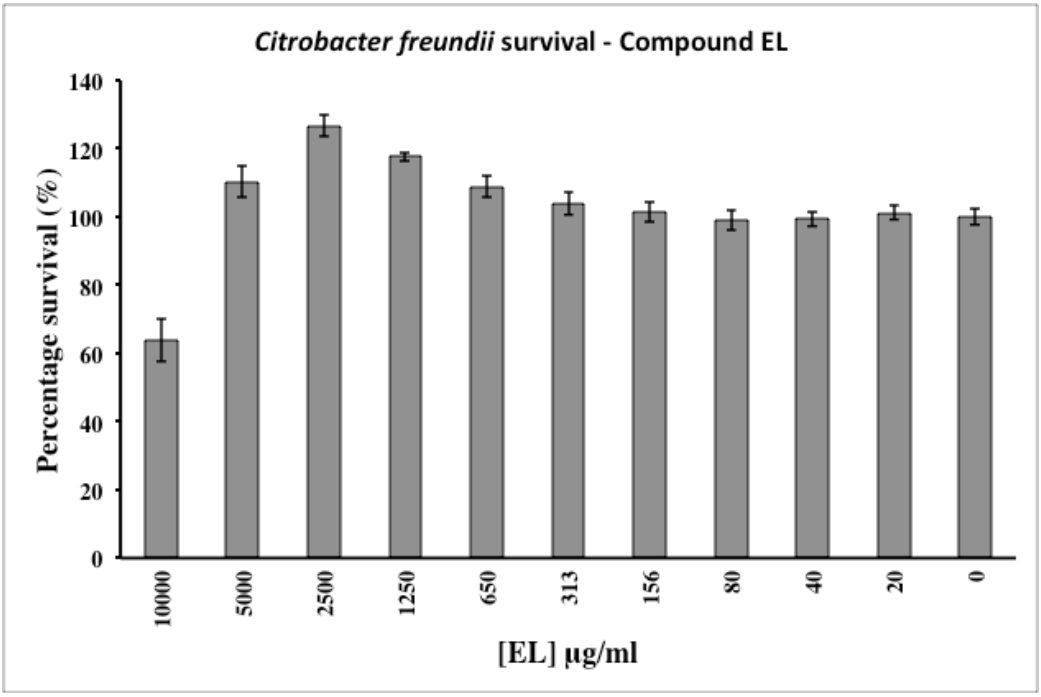

Figure S27. Percentage survival of *Citrobacter freundii* in the presence of ethambutol hydrochloride lactate (EL)

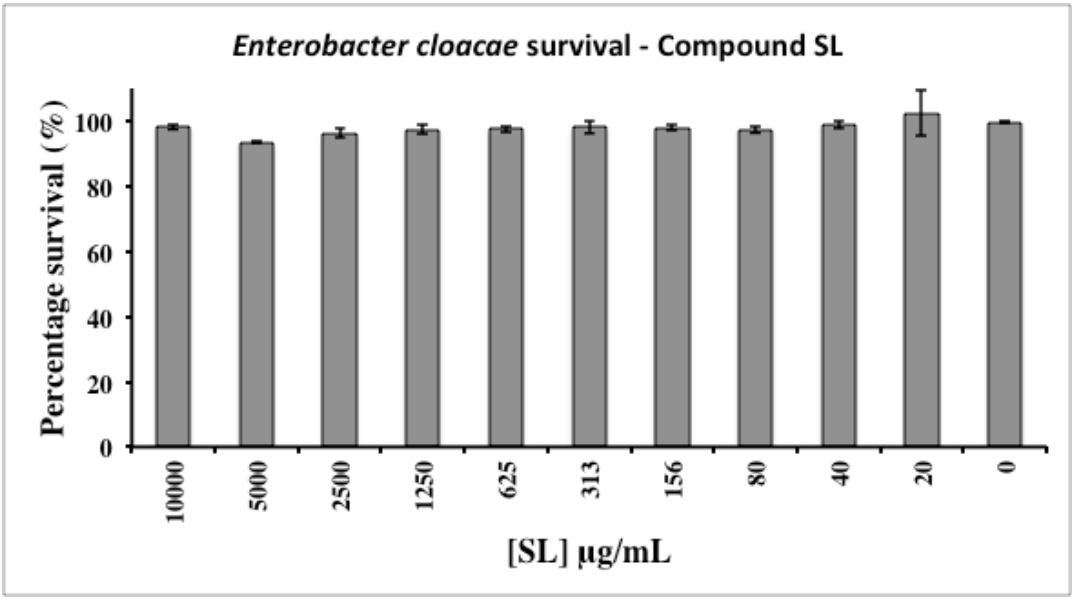

Figure S28. Percentage survival of *Enterobacter cloacae* in the presence of sodium lactate (SL)

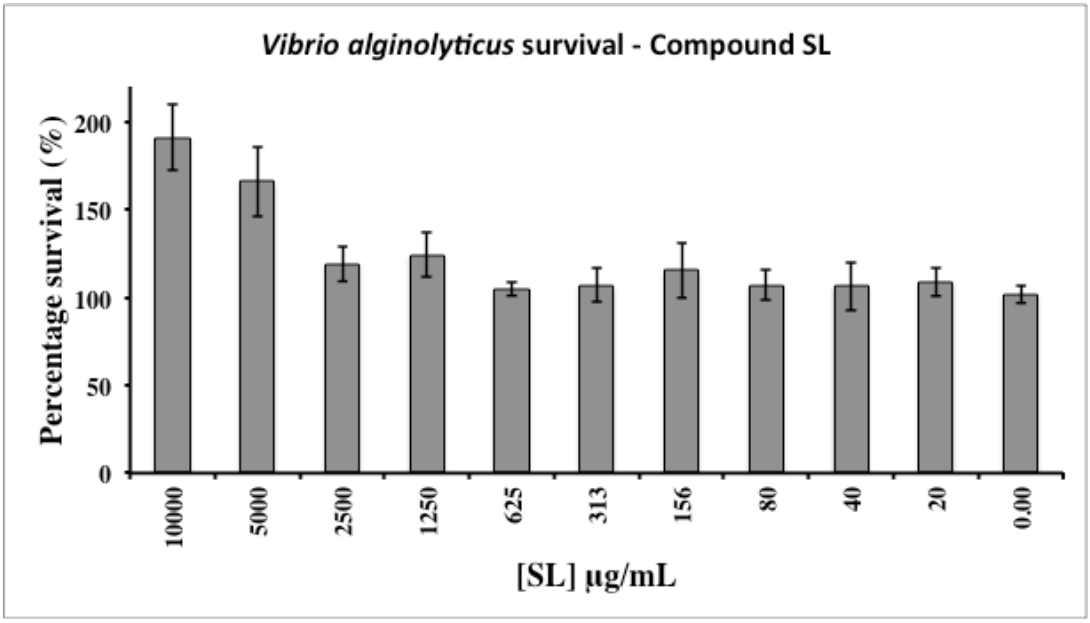

Figure S29. Percentage survival of *Vibrio alginolyticus* in the presence of sodium lactate (SL)

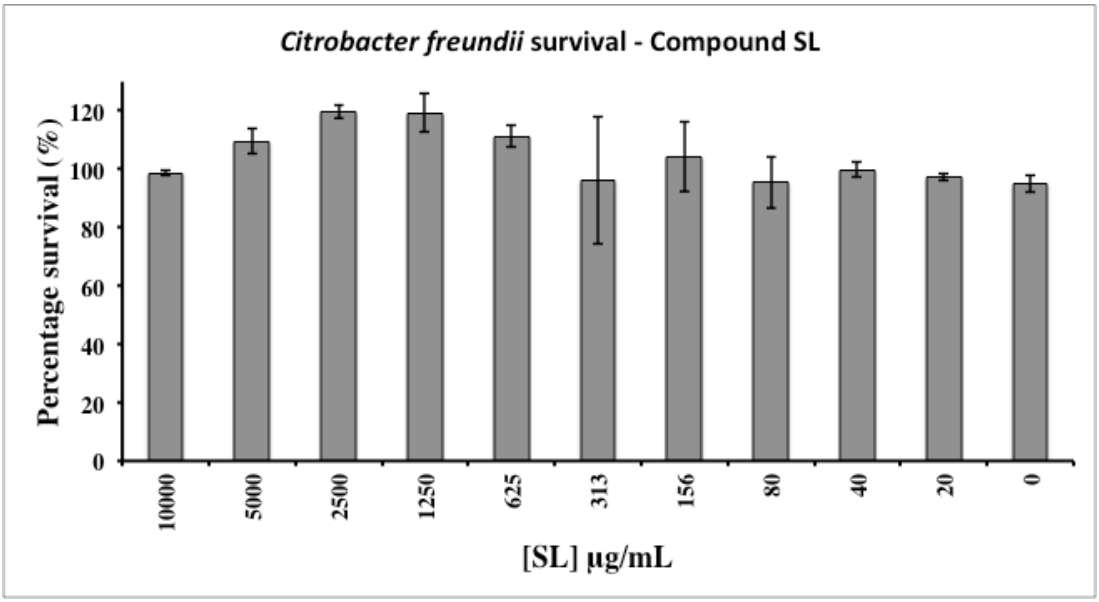

Figure S30. Percentage survival of *Citrobacter freundii* in the presence of sodium lactate (SL)

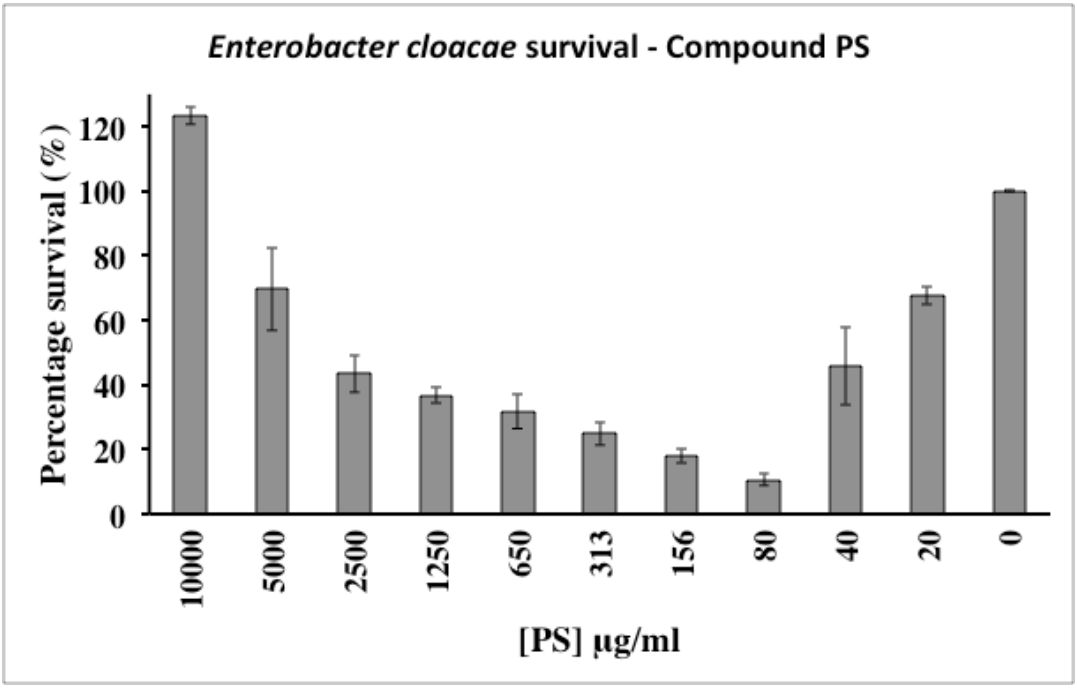

Figure S31. Percentage survival of *Enterobacter cloacae* in the presence of cetylpyridinium sulfathiazole (PS)

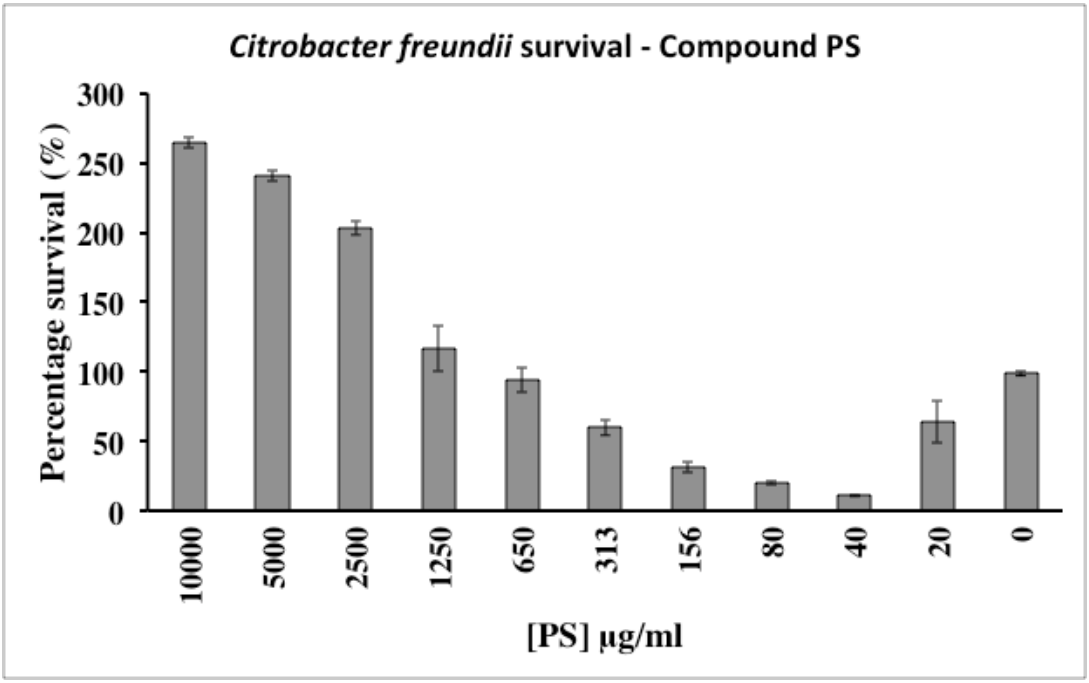

Figure S32. Percentage survival of *Citrobacter freundii* in the presence of cetylpyridinium sulfathiazole (PS)

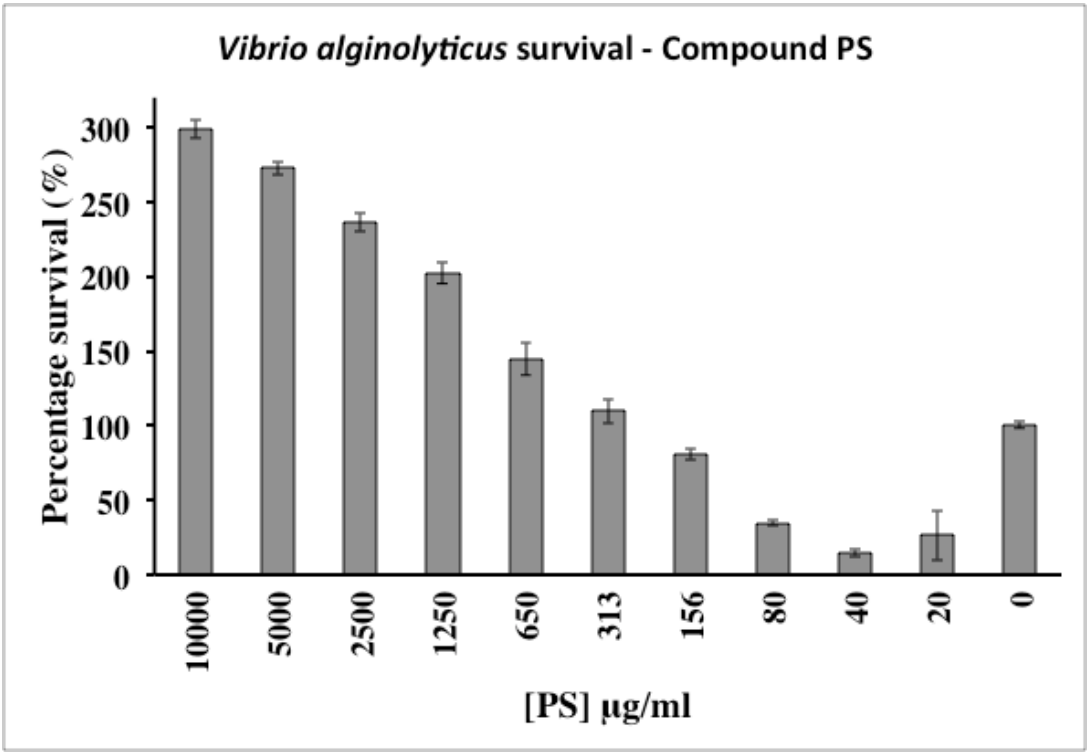

Figure S33. Percentage survival of *Vibrio alginolyticus* in the presence of cetylpyridinium sulfathiazole (PS)

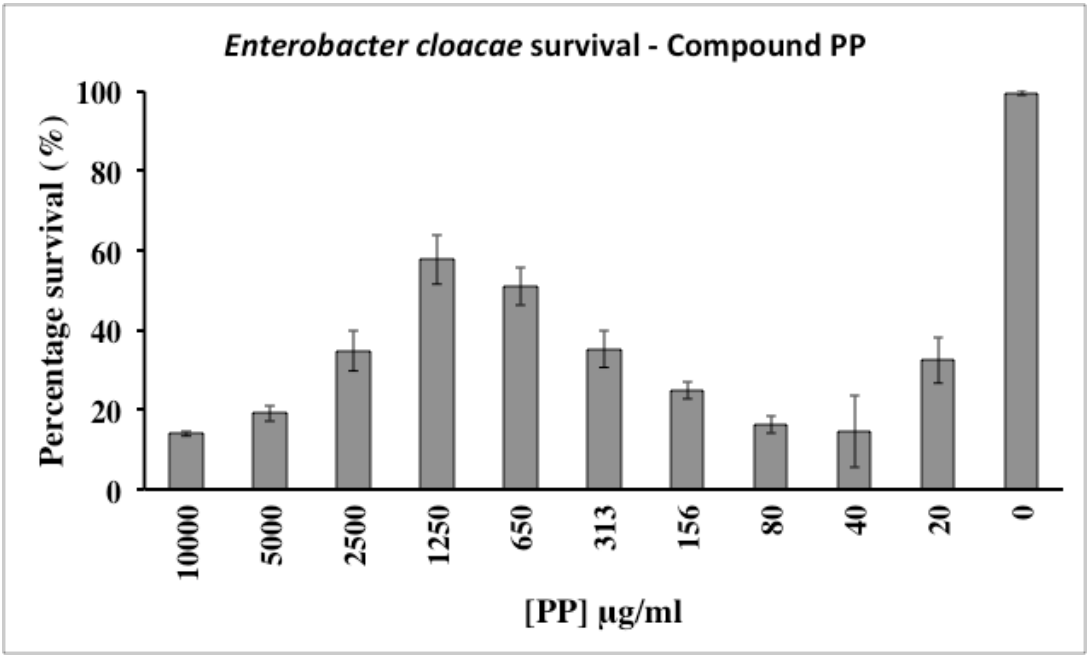

Figure S34. Percentage survival of *Enterobacter cloacae* in the presence of cetylpyridinium chloride monohydrate (PP)

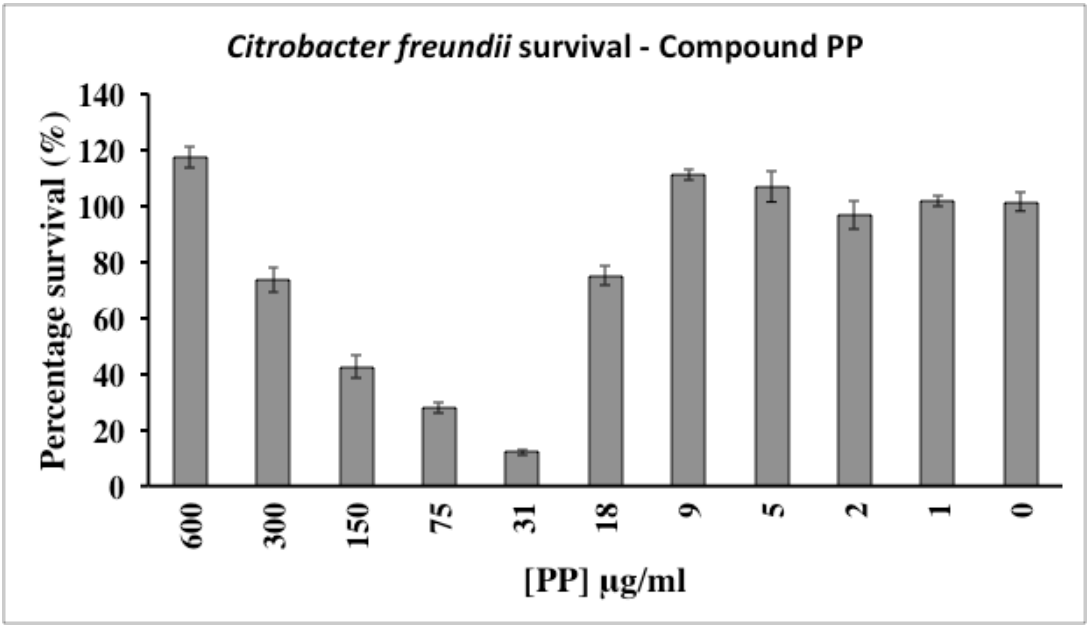

Figure S35. Percentage survival of *Citrobacter freundii* in the presence of cetylpyridinium chloride monohydrate (PP)

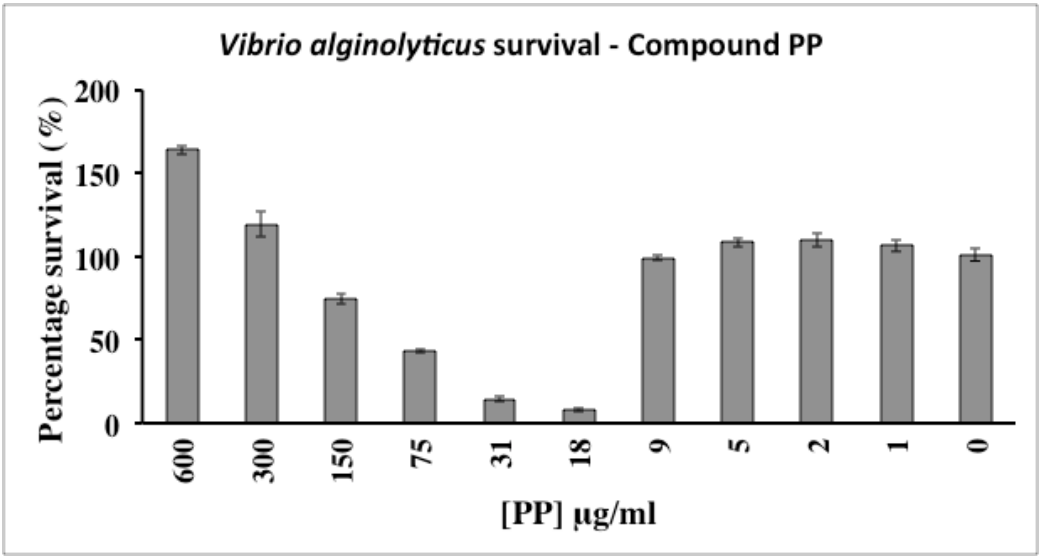

Figure S36. Percentage survival of *Vibrio alginolyticus* in the presence of cetylpyridinium chloride monohydrate (PP)

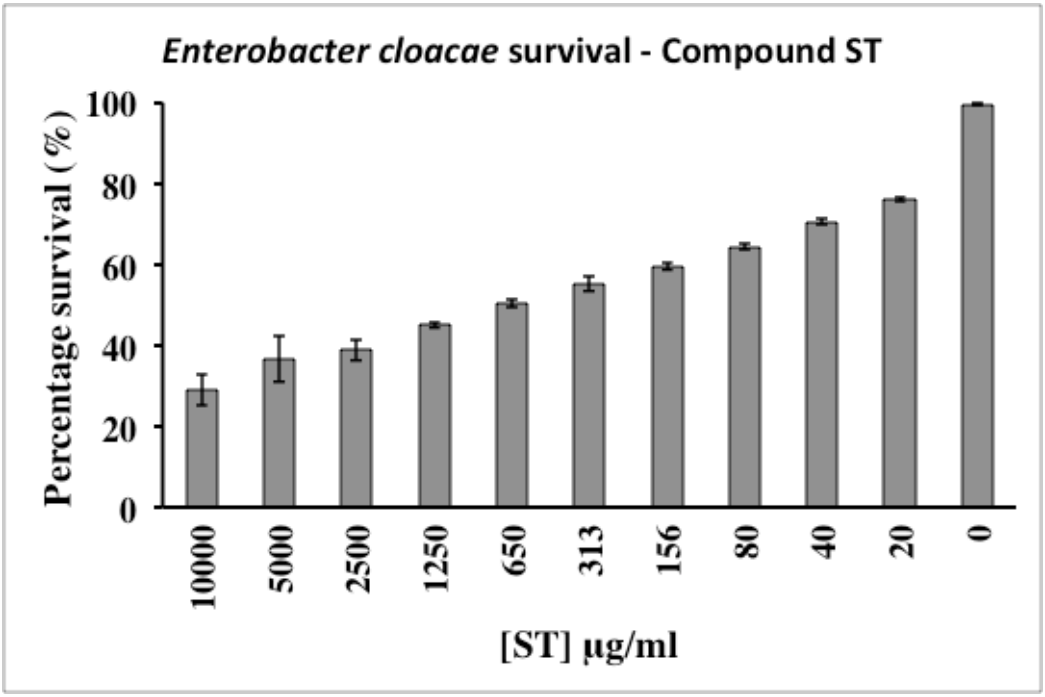

Figure S37. Percentage survival of *Enterobacter cloacae* in the presence of sodium sulfathiazole (ST)

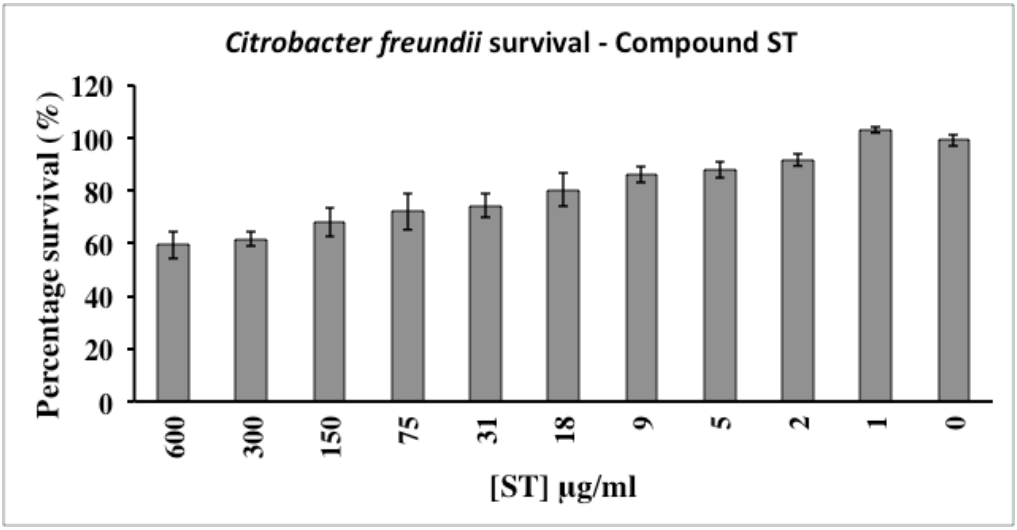

Figure S38. Percentage survival of *Citrobacter freundii* in the presence of sodium sulfathiazole (ST)

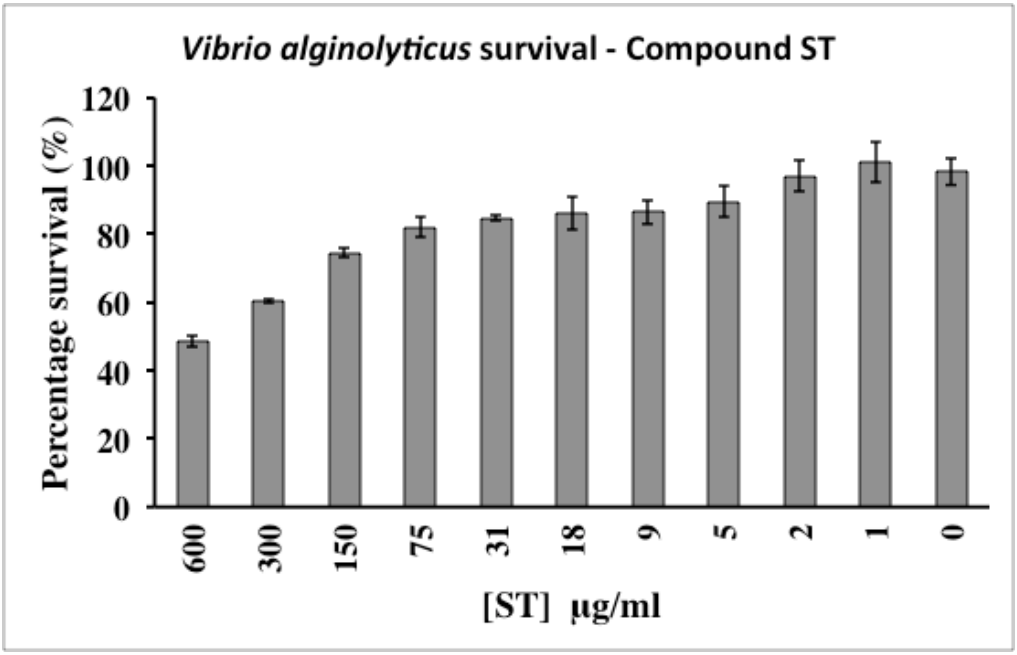

Figure S39. Percentage survival of *Vibrio alginolyticus* in the presence of sodium sulfathiazole (ST)