Electronic Supplementary Material (ESI) for RSC Advances

A facile method for estimating viable bacterial cells in solution based on "subtractive- aggregation" of gold nanoparticle

Manab Deb Adhikari,[a] Biswa Ranjan Panda,[b] Umakanth Vudumula,[a]
Arun Chattopadhyay,*[b,c] and Aiyagari Ramesh*[a]

[a] *Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781039, India. Fax: +91 361 2582249; Tel: +91 361 2582205; E-mail: aramesh@iitg.ernet.in*

[b] *Department of Chemistry, Indian Institute of Technology Guwahati, Guwahati 781039, India. Fax: + 91 361 2582349; Tel: +91 3612582304; E-mail: arun@iitg.ernet.in*

[c] *Centre for Nanotechnology, Indian Institute of Technology Guwahati, Guwahati 781039, India.*

Electronic Supplementary Information

Control experiments

These comprised a series of experiments to study the effect of the order of addition of the three interacting components namely bacterial cells, PLL and Au NPs. All these experiments were conducted with bacterial cultures of *E. coli* MTCC 433 and *L. monocytogenes* Scott A.

In control experiment I, bacterial cells (in 0.2 mL PBS) in the range of 10^2, 10^4 and 10^6 CFU were taken separately in tubes to which 0.2 mL of PLL solution was added to achieve a final PLL concentration of 3.5 x 10^{-5} % w/v in 0.4 mL. To this 1.5 mL of citrate-stabilized Au NP solution was added drop wise. The samples were mixed and incubated at room temperature under shaking condition for 30 min. Control samples were included devoid of either bacterial cells or PLL. UV-vis extinction spectrum of all the samples was recorded and the area under each extinction spectra was calculated.

In control experiment II, 0.2 mL of PLL solution was taken and added separately to 1.5 mL of citrate-stabilized Au NP solution. The mixtures were incubated at room temperature for 30 min following which 10^2, 10^4 and 10^6 CFU bacterial cells (in 0.2 mL PBS) were added to the tubes separately and incubated at room temperature under mild shaking condition for 30 min. In the control samples, 0.2 mL of PBS only (devoid of bacterial cells) was added to the tubes having pre-incubated PLL solution and citrate stabilized Au NP

solution. UV-vis extinction spectrum of all the samples was recorded and the area under each extinction spectra was determined.

In control experiment III, bacterial cells of varying concentrations (10^2, 10^4 and 10^6 CFU in 0.2 mL PBS) were taken in separate tubes to which 1.5 mL Au NP solution was added. In the control sample, only 0.2 mL PBS was added to 1.5 mL Au NP solution. All the samples were incubated for 30 min at room temperature under mild shaking condition. Subsequently, the samples were centrifuged at 8,000 rpm for 3 min. The supernatant from all the samples was recovered (nearly 1.7 mL) to which 0.2 mL of PLL was added. The samples were incubated at room temperature for 30 min and UV-vis extinction spectrum was recorded. Bacterial cell pellet recovered after centrifugation was also resuspended in 0.2 mL PBS to which 0.2 mL of PLL was added to achieve a final concentration of 3.5×10^{-5} % w/v in 0.4 mL. To this 1.5 mL Au NP solution was added. The samples were incubated at room temperature for 30 min and UV-vis extinction spectrum was recorded. Area under each extinction spectra was determined for all samples.

Propidium iodide (PI) staining of heat-killed bacterial cells

Aliquots of bacterial cells (10^2, 10^4 and 10^6 CFU) were taken in separate tubes in phosphate buffer saline (PBS) and subjected to autoclaving at 121^oC and $15lb/in^2$ for 20 min. A 1.5 mM stock solution of propidium iodide (PI) of relative molecular weight Mr 668 (Sigma-Aldrich, USA) was prepared in sterile MilliQ grade water. Following heat treatment, bacterial cells were washed with sterile MilliQ grade water and PI was added to the cells at a final concentration of 30 µM. After 10 min of incubation in a circulating water bath incubator (Amersham, USA) set at 37°C, samples were centrifuged and washed in sterile MilliQ grade water to remove excess dye and the cells were resuspended in sterile MilliQ grade water. A 10 µl aliquot of the PI stained sample was spotted on a clean glass slide, air dried and observed under fluorescence microscope (Axioskop2MAT, Carl Zeiss, Oberkochen, Germany) with green light excitation at 495-570 nm and long pass filter above 617 nm.

Agar well diffusion assay for nisin activity

Nisin was procured from Sigma-Aldrich, USA. A stock solution of nisin (10000 IU/mL) was made in 0.75% NaCl (pH 5.3). The assay plate had a bottom layer of MRS medium (deMan-Rogosa and Sharpe) with agar (1.5% agar), which was overlaid with MRS soft agar (0.8% agar) seeded with 10^6 cells of freshly grown target strain of *Leuconostoc mesenteroides*

NRRL B640. Requisite number of holes of 6 mm diameter was punched in the assay plate. To each well, 50 µl of test sample (1000 IU/mL, 800 IU/mL, 400 IU/mL, 200 IU/mL, 100 IU/mL, 50 IU/mL nisin and 0.75% NaCl, pH 5.3) was added. The plates were pre-incubated at 4°C for 3 h to facilitate diffusion of the sample followed by incubation at 37°C for 24 h. Nisin activity of the samples was determined by observing the zone of inhibition produced around the wells.

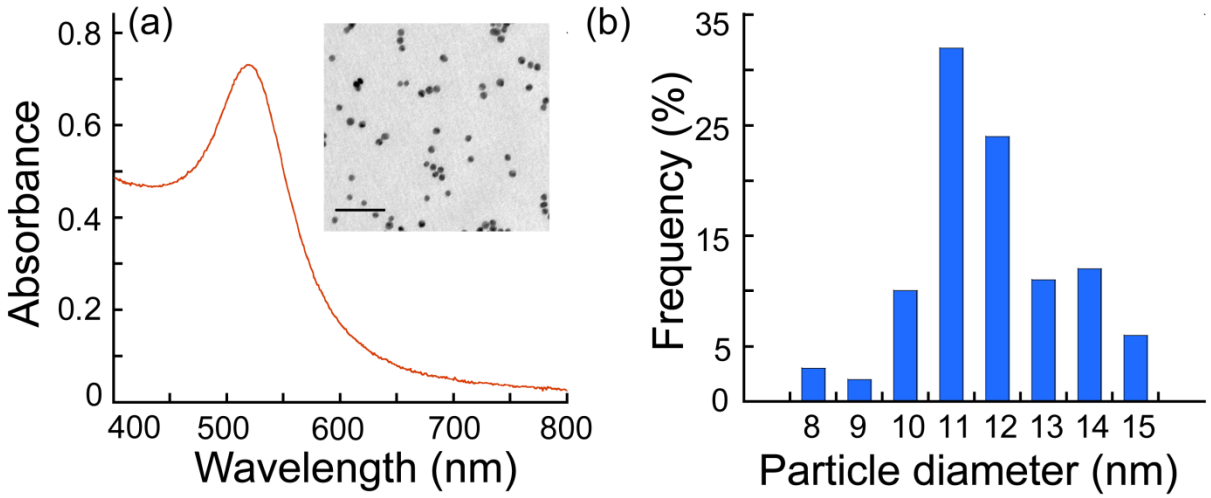

Fig. S1 (a) UV-vis extinction spectra of citrate-stabilized Au NP. Inset showing transmission electron micrograph of Au NP. The scale bar is 50 nm. **(b)** Particle size distribution of Au NP.

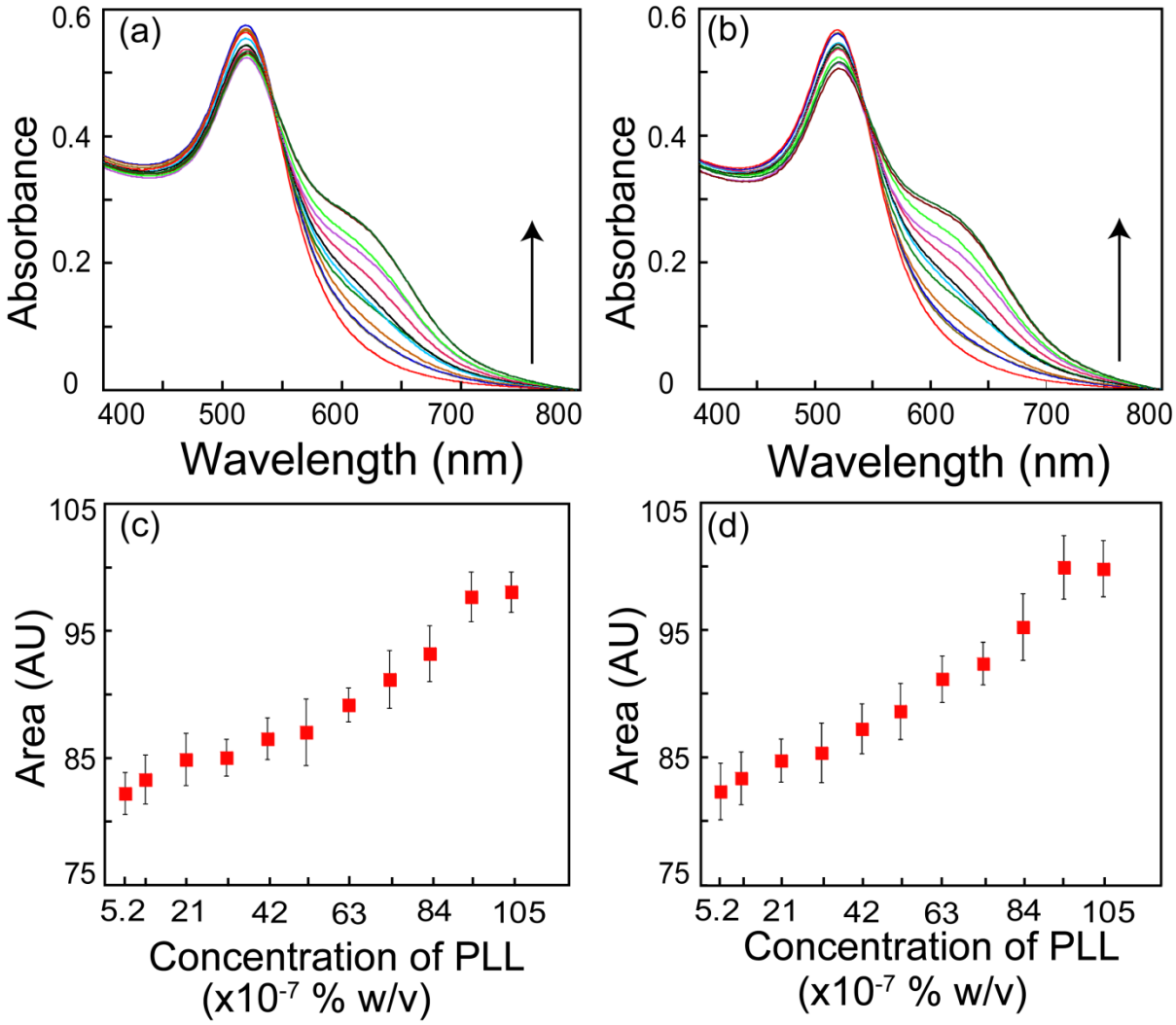

Fig. S2 UV-vis extinction spectrum of citrate-stabilized Au NPs with increasing concentrations of PLL (5.2 x 10^{-7}, 10.5 x 10^{-7}, 21 x 10^{-7}, 31.5 x 10^{-7}, 42 x 10^{-7}, 52 x 10^{-7}, 63 x 10^{-7}, 73.6 x 10^{-7}, 84 x 10^{-7}, 95 x 10^{-7} and 105 x 10^{-7} % w/v). Measurements were taken after **(a)** 15 min and **(b)** 60 min of addition of PLL, respectively. The lowest trace is the control sample consisting of Au NP alone (volume adjusted). The subsequent traces were obtained with increasing concentration of PLL as indicated by arrow. The corresponding areas under the curve versus PLL concentrations are indicated in **(c)** and **(d)**, respectively.

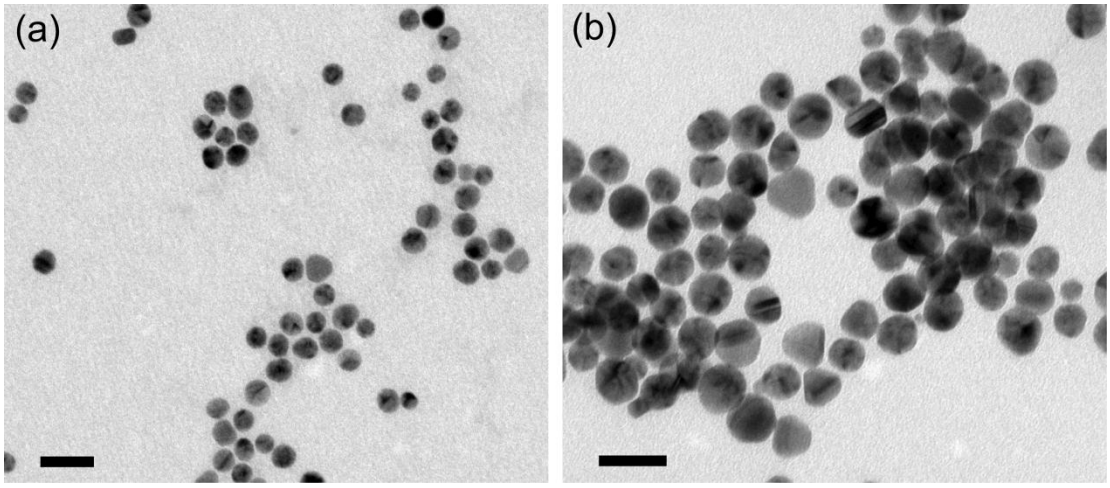

Fig. S3 TEM images of Au NPs in the presence of **(a)** 21 x 10^{-7} % w/v PLL and **(b)** 42 x 10^{-7} % w/v of PLL. Scale bar is 20 nm for the samples.

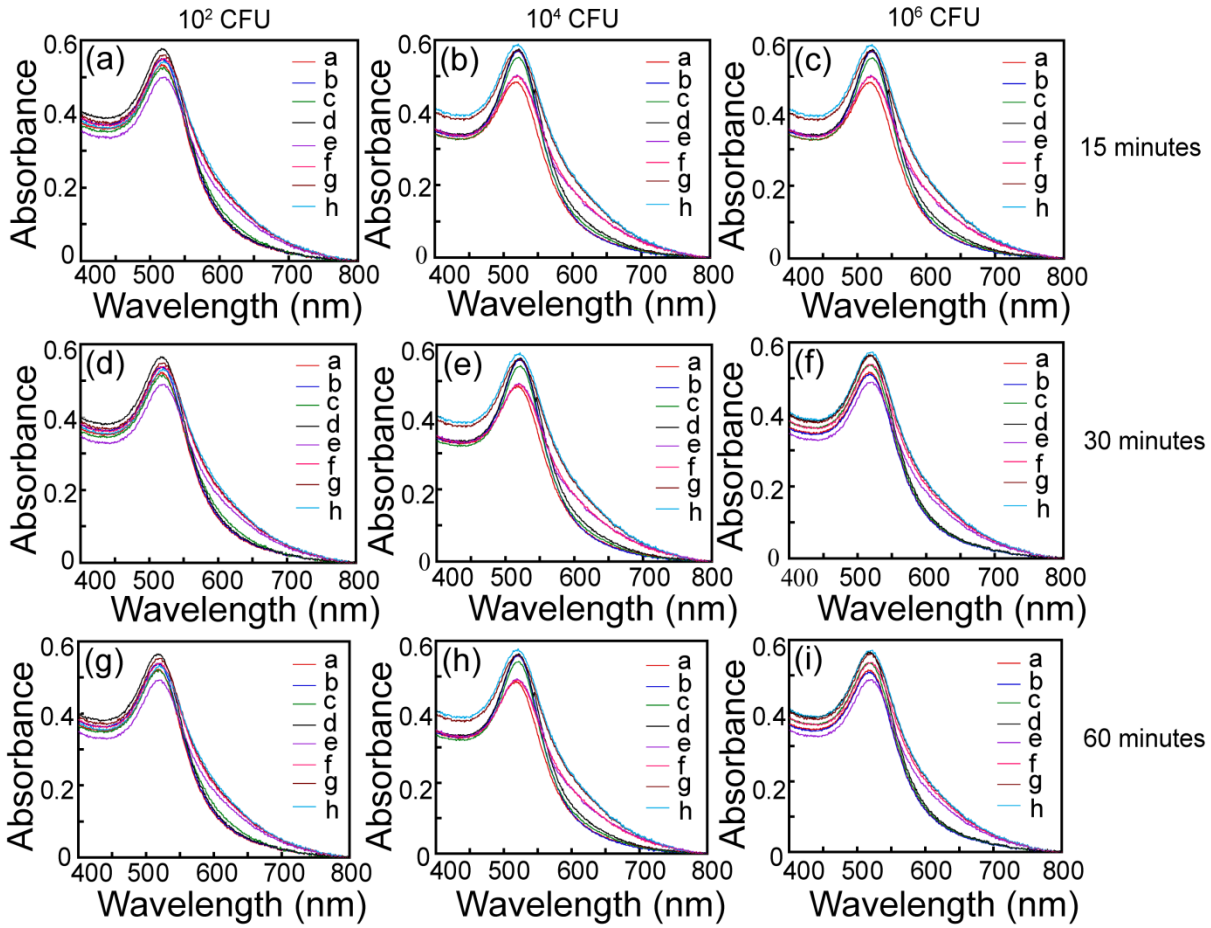

Fig. S4 UV-vis extinction spectrum of Au NP obtained after interaction with unbound PLL. Initially varying cell numbers of *E.coli* MTCC 433 were interacted for 15, 30 and 60 minutes with various concentrations of PLL (traces b-h correspond to 0.25 x 10^{-5}, 0.5 x 10^{-5}, 1.0 x 10^{-5}, 3.0 x 10^{-5}, 3.5 x 10^{-5}, 4.0 x 10^{-5} and 5.0 x 10^{-5} % w/v PLL). The control sample is represented by trace a (Au NP alone).

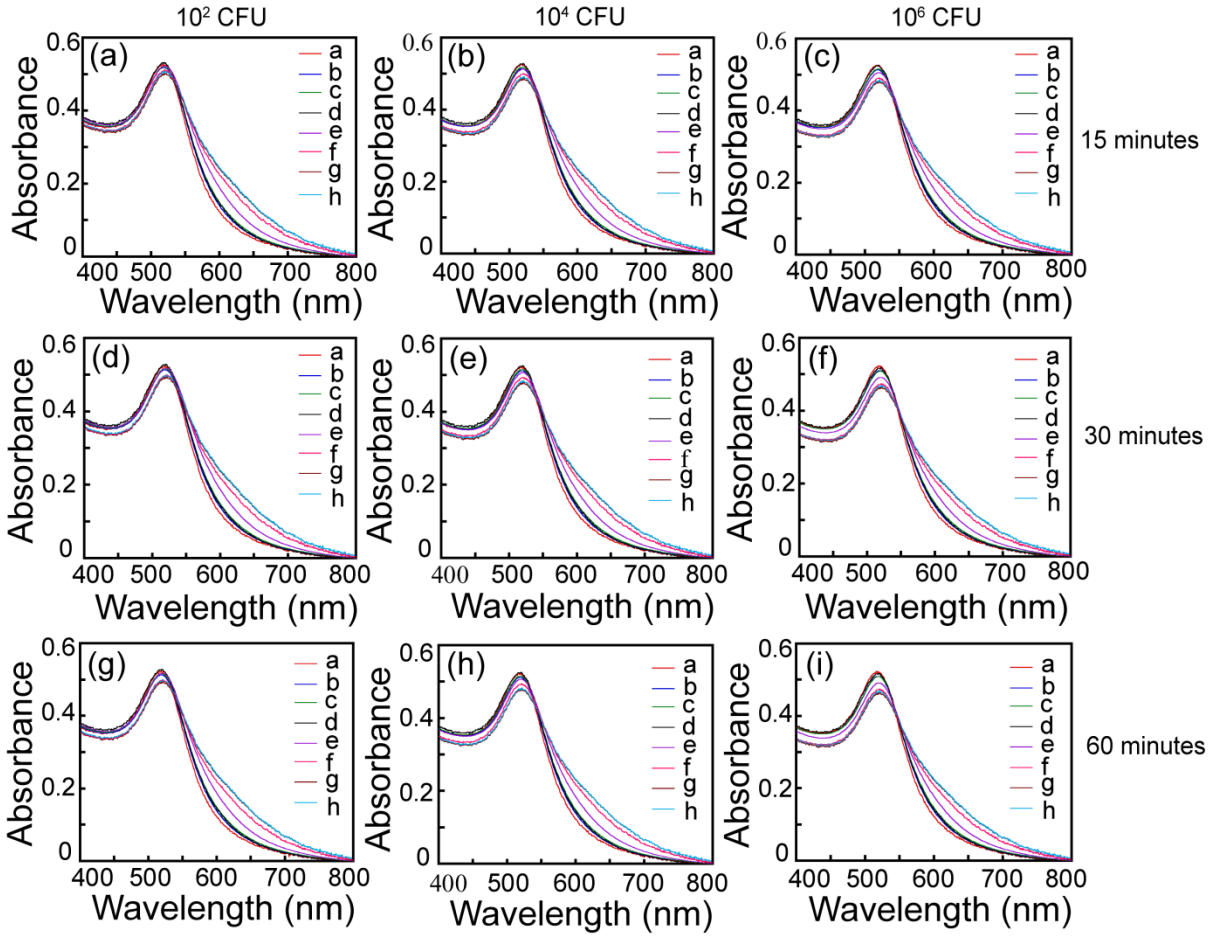

Fig. S5 UV-vis extinction spectrum of Au NP obtained after interaction with unbound PLL. Initially varying cell numbers of *L. monocytogenes* Scott A were interacted for 15, 30 and 60 minutes with various concentrations of PLL (traces b-h correspond to 0.25 x 10^{-5}, 0.5 x 10^{-5}, 1.0 x 10^{-5}, 3.0 x 10^{-5}, 3.5 x 10^{-5}, 4.0 x 10^{-5} and 5.0 x 10^{-5} % w/v PLL). The control sample is represented by trace a (Au NP alone).

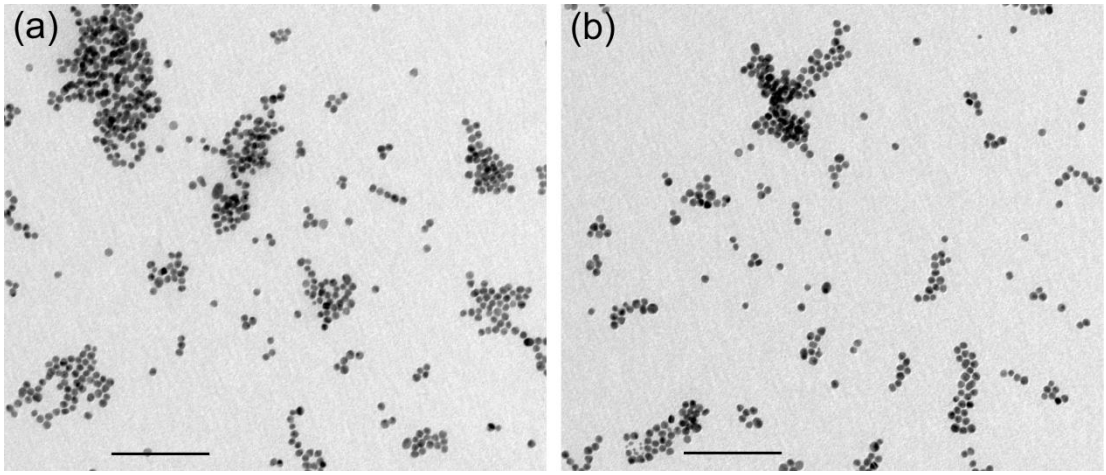

Fig. S6 TEM images of aggregates of Au NP obtained with unbound PLL following interaction of 3.5 x 10^{-5} % w/v PLL with **(a)** 10^2 CFU and **(b)** 10^4 CFU of *E.coli* MTCC 433. Scale bar is 50 nm for the samples.

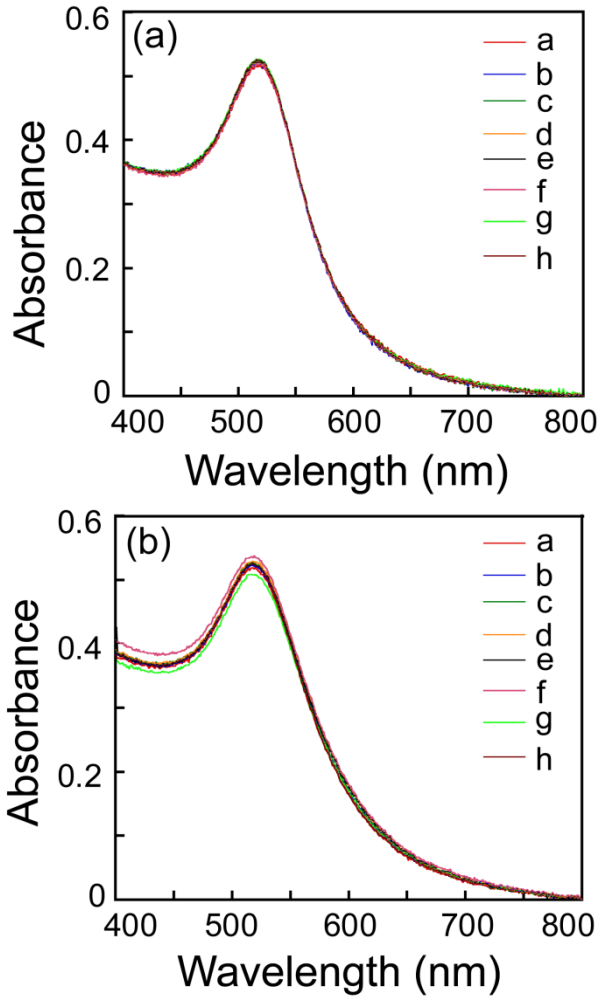

Fig. S7 UV-vis extinction spectrum of Au NP interacted with cell bound PLL. (a) *E.coli* MTCC 433, (b) *L. monocytogenes* Scott A. Traces a-g correspond to 0.25 x 10^{-5}, 0.5 x 10^{-5}, 1.0 x 10^{-5}, 3.0 x 10^{-5}, 3.5 x 10^{-5}, 4.0 x 10^{-5} and 5.0 x 10^{-5} % w/v PLL, Trace h: Au NP alone (volume adjusted).

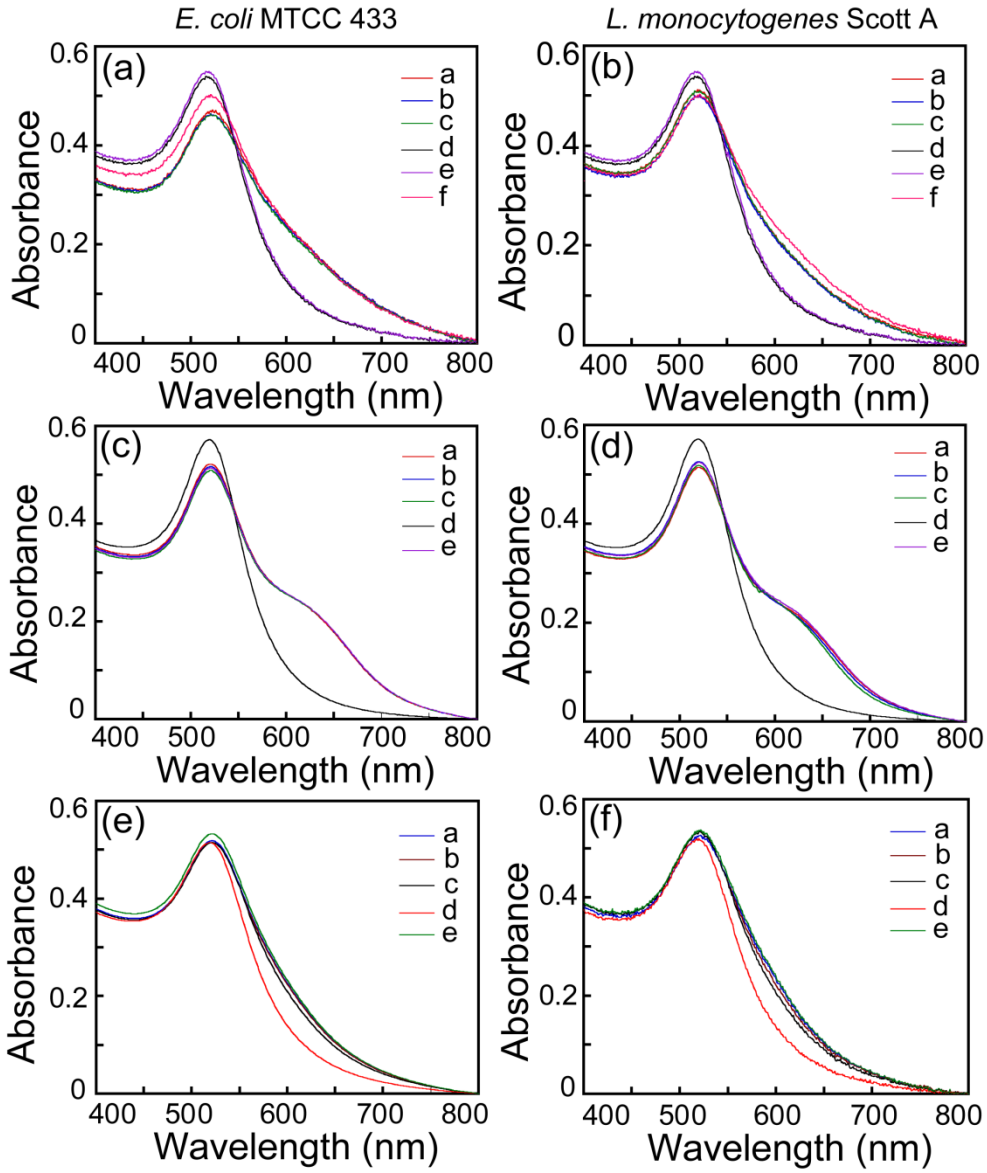

Fig. S8 UV-vis extinction spectrum for control experiments. **(a-b):** Experiment I; **(c-d):** Experiment II; **(e-f):** Experiment III. For experiment I: traces a-c correspond to 10^2, 10^4 and 10^6 CFU, respectively; trace d: Au NP alone (volume adjusted), trace e: 10^6 CFU bacteria interacted with Au NP alone, trace f: Au NP interacted with 3.5 x 10^{-5} % w/v PLL. For experiment II and III: traces a-c correspond to 10^2, 10^4 and 10^6 CFU, respectively; trace d: Au NP alone (volume adjusted) and trace e: Au NP interacted with 3.5 x 10^{-5} % w/v PLL.

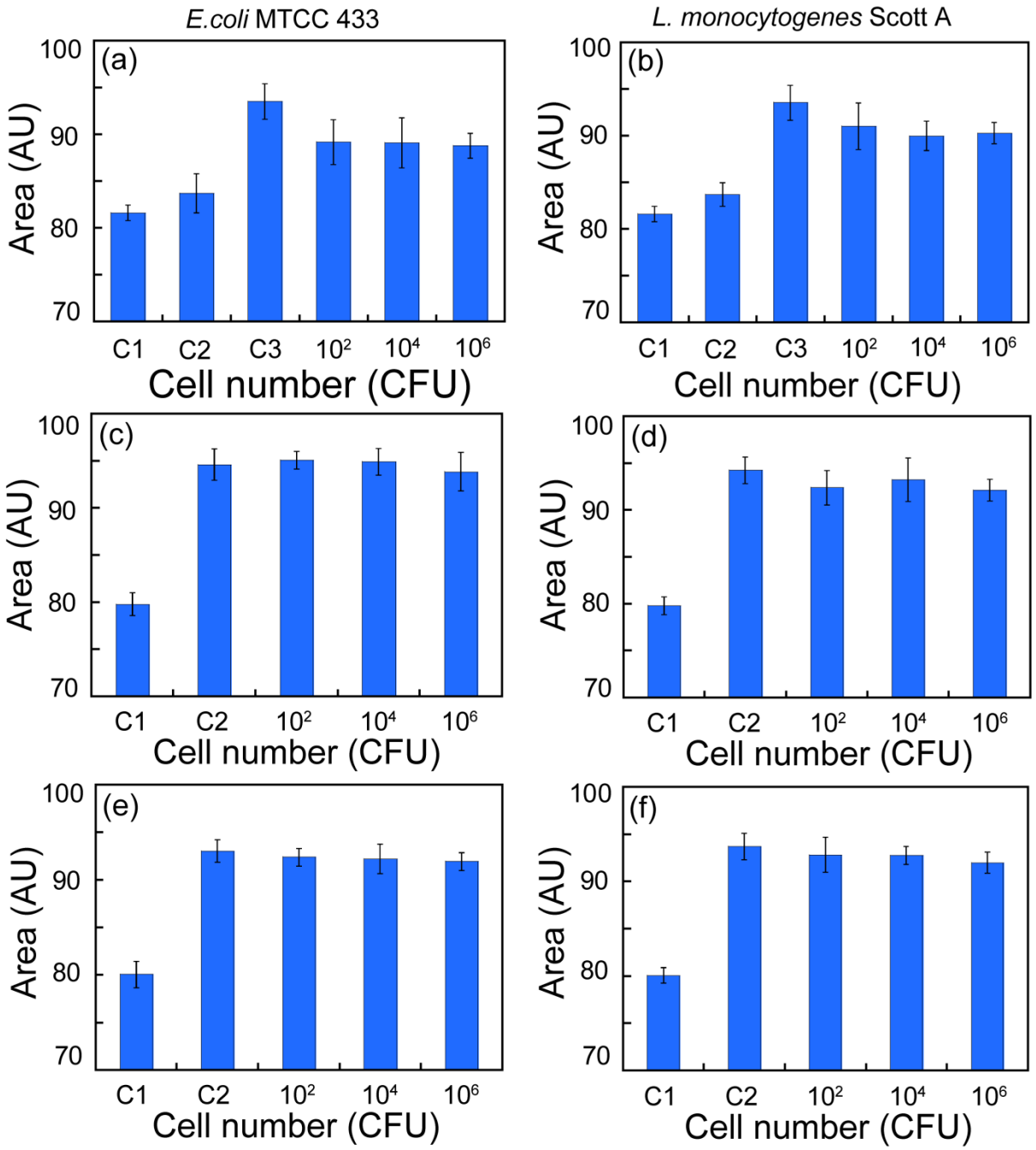

Fig. S9 Area under UV-vis extinction spectrum obtained for various control experiments. **a-b:** Experiment I; **c-d:** Experiment II; **e-f:** Experiment III. For a-b, C1: Au NP alone (volume adjusted) C2: 10^6 CFU bacteria (in 0.4 mL) interacted with 1.5 mL Au NP and C3: Au NP interacted with 3.5 x 10^{-5} % w/v PLL. For c-f: C1: Au NP alone (volume adjusted) and C2: Au NP interacted with 3.5 x 10^{-5} % w/v PLL.

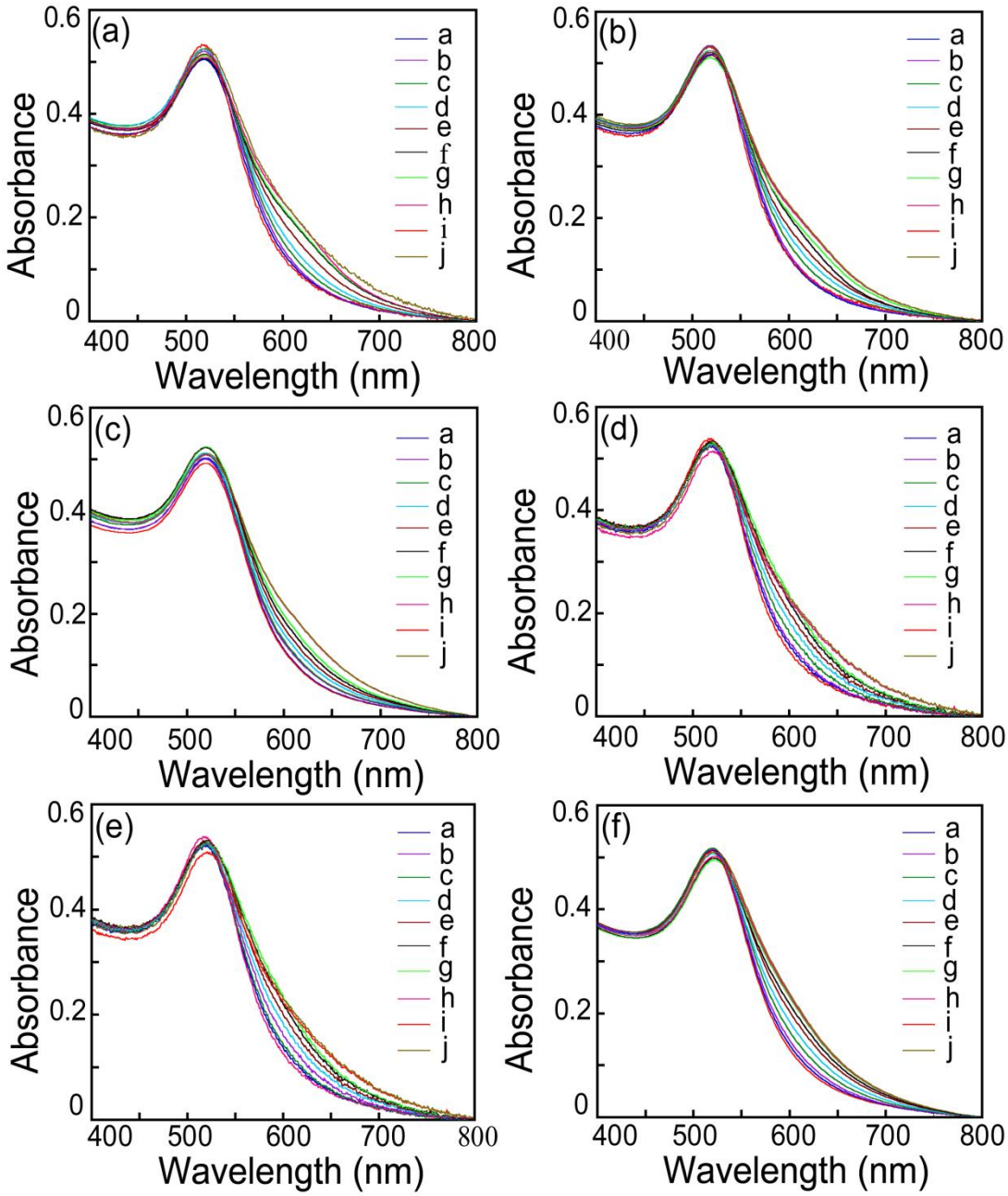

Fig. S10 UV-vis extinction spectrum for estimation of bacterial cell numbers by Au NP aggregation. **(a)** *Escherichia coli* MTCC 433, **(b)** *Listeria monocytogenes* Scott A, **(c)** *Bacillus cereus* MTCC 1305, **(d)** *Pseudomonas aeruginosa* MTCC 2488, **(e)** *Staphylococcus aureus* MTCC 740 and **(f)** *Enterobacter aerogenes* MTCC 2822. Traces a-h correspond to decreasing cell numbers (10^7 – 1.0 CFU), trace i: Au NP alone (volume adjusted) and trace j: Au NP interacted with 3.5 x 10^{-5} % w/v PLL.

Table S1 Measurement of area under UV-vis extinction spectrum as a function of bacterial cell numbers

Bacterial strains	**[$] Equation for area under UV-vis extinction spectrum (AU) versus cell number (CFU)**
Escherichia coli MTCC 433	$y = -1.762x + 95.50$ ($R^2 = 0.976$)
Pseudomonas aeruginosa MTCC 2488	$y = -1.873x + 96.24$ ($R^2 = 0.970$)
Enterobacter aerogenes MTCC 2822	$y = -1.746x + 94.46$ ($R^2 = 0.987$)
Listeria monocytogenes Scott A	$y = -1.598x + 94.38$ ($R^2 = 0.976$)
Bacillus cereus MTCC 1305	$y = -1.665x + 93.98$ ($R^2 = 0.990$)
Staphylococcus aureus MTCC 740	$y = -1.936x + 97.14$ ($R^2 = 0.972$)

[$] Equations for the plots indicated in **Fig. 4**.

Table S2 Measurement of subtractive-aggregation as a function of bacterial cell numbers

Bacterial strains	**[$] Equation for subtractive-aggregation area (ΔAagg) versus cell number (CFU)**
Escherichia coli MTCC 433	$y = 1.762x – 2.475$ ($R^2 = 0.976$)
Pseudomonas aeruginosa MTCC 2488	$y = 1.873x – 1.928$ ($R^2 = 0.970$)
Enterobacter aerogenes MTCC 2822	$y = 1.746x – 0.932$ ($R^2 = 0.987$)
Listeria monocytogenes Scott A	$y = 1.598x – 1.357$ ($R^2 = 0.976$)
Bacillus cereus MTCC 1305	$y = 1.665x – 0.952$ ($R^2 = 0.990$)
Staphylococcus aureus MTCC 740	$y = 1.936x – 2.824$ ($R^2 = 0.972$)

[$] Equations for the plots indicated in **Fig. 6**. $\Delta Aagg = Aagg_{Max} – Aagg_{Unbound\text{-}PLL}$. $Aagg_{Max}$ is obtained from the area under the curve for Au NP aggregation by 3.5×10^{-5} % w/v PLL (corresponding to trace j in Fig. S10a-f). $Aagg_{Unbound\text{-}PLL}$ is obtained from the area under curve for Au NP aggregation in presence of varying bacterial cell numbers (corresponding to trace a-h in Fig. S10a-f).

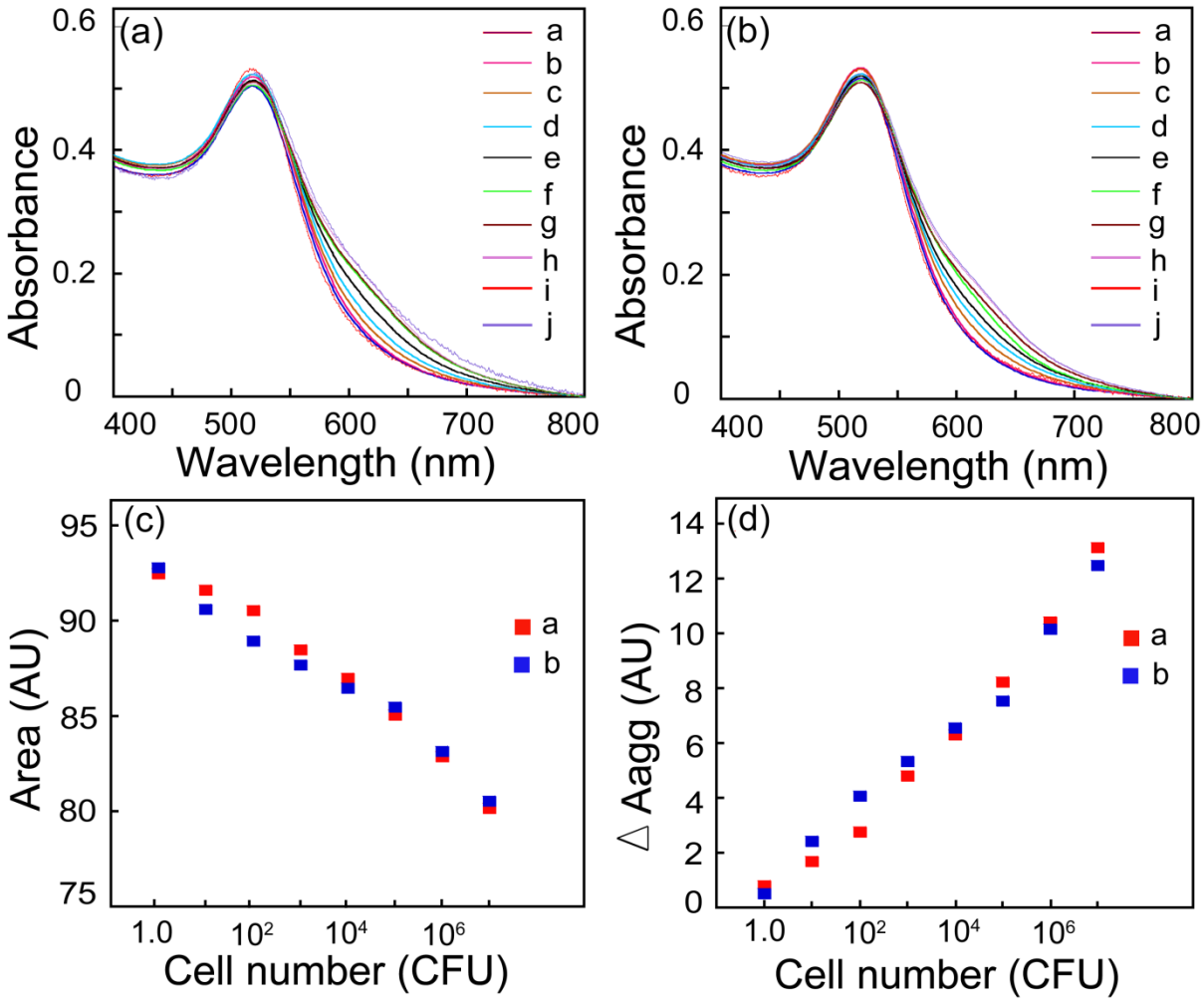

Fig. S11 UV-vis extinction spectrum for Au NP incubated with unbound PLL following separation of bacterial cells by centrifugal filter device (Costar Spin-X centrifuge tube filter). Varying cell numbers of **(a)** *Escherichia coli* MTCC 433 and **(b)** *Listeria monocytogenes* Scott A were taken for the experiments. Traces a-h correspond to decreasing cell numbers (10^7–1.0 CFU), trace i: Au NP alone (volume adjusted) and trace j: Au NP interacted with 3.5 x 10^{-5} % w/v PLL. **(c)** Area under UV-vis extinction spectrum as a function of bacterial cell numbers for a: *Escherichia coli* MTCC 433, b: *Listeria monocytogenes* Scott A. The area represents mean value of three independent measurements. **(d)** Plot for subtractive-aggregation (ΔAagg) versus bacterial cell numbers for a: *Escherichia coli* MTCC 433, b: *Listeria monocytogenes* Scott A.

Table S3 Estimation of viable cell numbers of *E. coli* MTCC 433 following heat treatment.

Heat treatment	**Initial cell number (log_{10} CFU)**	**Cell number after heat treatment estimated by plating (log_{10} CFU)**	**[$] Cell number after heat treatment estimated by subtractive-aggregation (log_{10} CFU)**	**Growth after re-inoculation in fresh media**
55°C/30 min	6.0 4.0 2.0	4.45 2.54 nd	4.8 2.42 nd	+ + -
65°C/30 min	6.0 4.0 2.0	nd nd nd	nd nd nd	- - -
80°C/30 min	6.0 4.0 2.0	nd nd nd	nd nd nd	- - -
121°C/20 min/15 lb/in^2 (autoclaving)	6.0 4.0 2.0	nd nd nd	nd nd nd	- - -

[$] Cell number for *E.coli* MTCC 433 was estimated using the equation y = 1.762x – 2.475 (Table S2) where y is subtractive aggregation area (ΔAagg) and x is cell number (CFU).

nd: Cell viability was affected by heat treatment and hence could not be estimated by either plating or subtractive aggregation.

+: indicates turbidity in growth media after re-inoculation; - : indicates lack of turbidity in growth media after re-inoculation

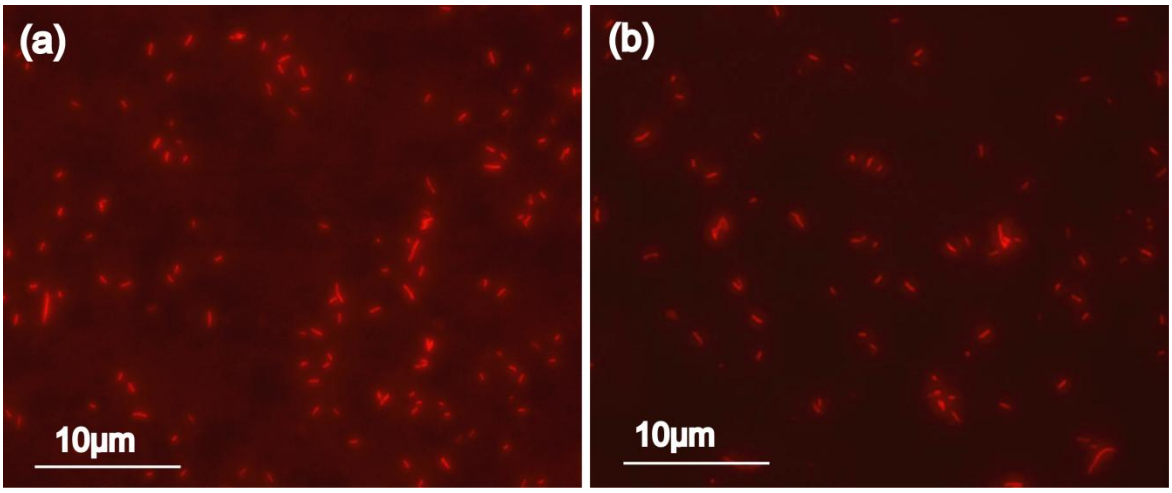

Fig. S12 Propidium iodide stained heat-killed (autoclaving) cells of *E.coli* MTCC 433. **(a)** 10^4 CFU and **(b)** 10^2 CFU.

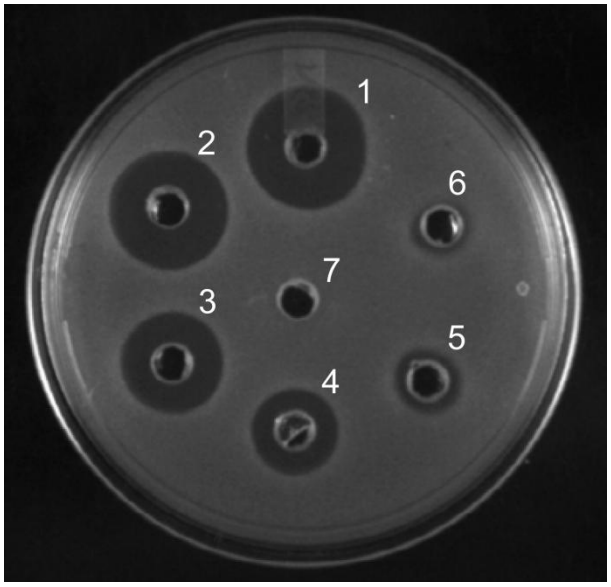

Fig. S13 Agar well diffusion assay to test antimicrobial activity of nisin against *Leuconostoc mesenteroides* NRRL B640. Well nos. 1-6: 1000 IU/mL, 800 IU/mL, 400 IU/mL, 200 IU/mL, 100 IU/mL, and 50 IU/mL nisin; 7: 0.75% NaCl (pH 5.3). Nisin stock solution (10000 IU/mL) was made in 0.75% NaCl (pH 5.3).