Kinetic, metabolic and macromolecular response of bacteria to chronic nanoparticle exposure in continuous culture

F. Faghihzadeh [a], N. M. Anaya [a], C. Astudillo-Castro [b], and V. Oyanedel-Craver [a]*

[a] Department of Civil and Environmental Engineering, University of Rhode Island, Kingston, RI O2881, USA

[b] School of Food Engineering, Pontifical Catholic University of Valparaiso, 716 Waddington, Playa Ancha, Valparaiso 2360100, Chile

* Corresponding author Email: craver@uri.edu , Phone: (401) 874-2784

CONTENTS OF SUPLEMENTARY INFORMATION

Additional details for some aspects of the methodology and results, 11 Figures and 2 Tables. A list of references for *Supporting Information* is included at the end of this document

Additional details for methods

1.1. Experimental conditions and toxicity tests

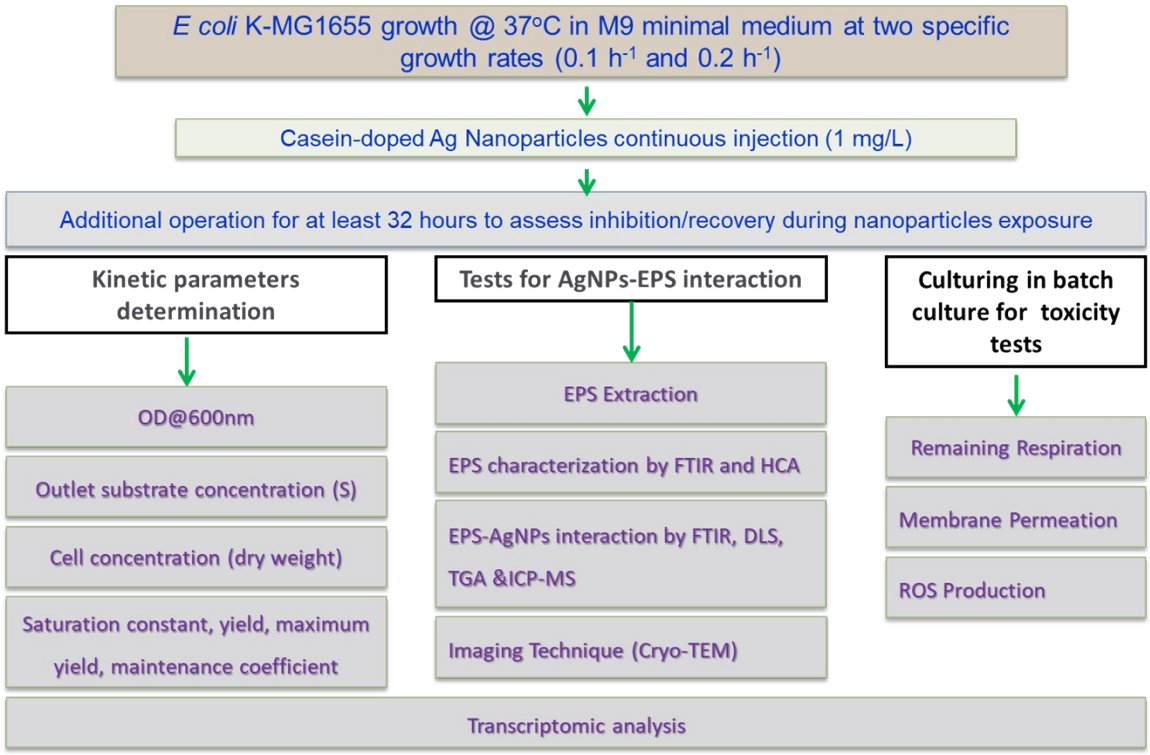

Figure S1. Condition and tests performed in this study

1.2. Kinetic parameters determination in details

1.2.1. Maximum specific growth rate

First we performed the batch culture to find the optimum temperature and optimum substrate concentration for *E.coli* Mg1655 growth. The μ_{max} depends on the culture temperature,[1] hence, temperatures in the range of 37 °C – 40 °C were examined in a batch bioreactor to find the optimum temperature for *E. coli* growth. Also, at high substrate concentration, growth will occur at μ_{max}. Therefore, to determine the optimum substrate concentration, two concentrations of glucose (8 g/L and 20 g/L) as the only carbon source.[1,2] Then, the μ_{max} of *E. coli* was determined by batch culture in optimum growth condition (temperature = 37 and glucose concentration = 8 g/L). For this purpose, direct measurements of μ_{max} carried out in a batch bioreactor (Figure S2b).

Also, in order to obtain the wash out dilution factor the experiment was performed with six bioreactors: four bioreactors inoculated with 300 µl of *E. coli* (OD at 600nm was 1.8) and 2 bioreactors as controls contained only M9 minimal media. A concentration of 8 g/L of glucose as the only carbon source was added into minimal M9 media container. Initial µ was 0.1 h^{-1}

which increased to 0.2 h^{-1} after 20 hours and finally increased to 0.3 h^{-1} after 32 hours. As shown in Figure S2a, after increasing specific growth rate to 0.3 h^{-1}, the cells start to wash out..

As the optimum specific growth rates for the investigating the response of cells at different specific growth rates to AgNPs, 0.1 h^{-1} and 0.2 h^{-1} were selected.

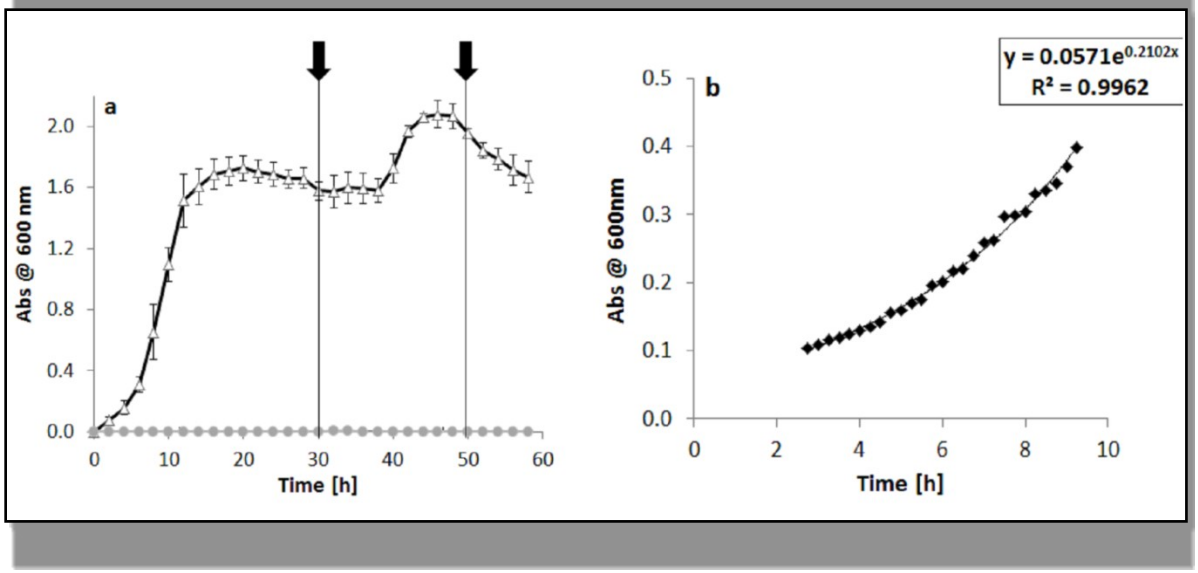

Figure S2. μ_{max} of *E. coli* determined by two methods including batch and continuous cultures. (a) μ_{max} of *E. coli* in continuous culture obtained by increasing specific growth rate (μ). Growth started with μ at 0.1 h^{-1}, increased to 0.1 h^{-1} at 30 hours, and finally increased to 0.3 h^{-1}. Bold black arrows represent the time of increasing μ. Δ represents bacteria control exposed to AgNPs. ● represents a M9 minimal media control to detect contamination. Samples were collected every 2 hours. Bars represent two replicates. (b) μ_{max} of *E. coli* in M9 minimal media in 96 well microplates with glucose concentration of 8 g/L and temperature at 37 °C. Samples were run in triplicate.

1.2.2. Cell dry weight

Then, we found the cell dry weight for each condition by multiplying cell dry weight with the absorbance at OD600. In detail, 1 ml of cell suspension was centrifuged in pre-dried and pre-weighed 1 ml test tubes at 13000 × g for 5 min. After removal of the supernatant, the samples were measured for cell wet weight and then dried at vacuum for at least 24 hours.[47] The dry biomass weight obtained for all four bioreactors. Then, they normalized based on the inside volume of continuous bioreactor's vessels.

1.2.3. Substrate concentration

We determined the outlet substrate concentration (S_{out}) by using Glucose (HK) Assay kit. The glucose consumption rate as an only carbon source were determined by the glucose (HK) assay reagent (Product Code G 3293) which purchased from Sigma Aldrich. The protocols for glucose

concentration determined as given in the manufacturers' instructions. The concentration of glucose in the samples was calculated using the millimolar extinction coefficient or NADH at 340 nm using the equation shown below as given in the manufacturers' instructions.

mg glucose/ml = (ΔA)(TV)(Glucose MW)(F)/ (ε)(d)(SV)(Conversion Factor for µg to mg)

A is the concentration in term of absorbance at 340 nm.

Where; A Total Blank = A Sample Blank + A Reagent Blank , $\Delta A = A_{Test} - A_{Total\ Blank}$, TV = Total Assay Volume (0.02 ml), SV = Sample Volume (1.02 ml), Glucose MW = 180.15g/mole, F = DF = 4 from sample preparation, ε = Millimolar extinction coefficient for NADH at 340nm =6.22, d = Light path (cm), 1000 = Conversion Factor for µg to mg.

1.2.4. Saturation constant

We normalized the outlet substrate concentration (S_{out}) by bacterial absorbance (OD 600). Then we converted the outlet substrate concentration (S_{out}) unit from mg/mL to mmol/L. We prepared a chart calculating K_s , with 1/µ as y axis and 1/ S_{out} as x axis. K_s was determined by multiplying slope with 1/intersect. Also, we checked K_s values with $K_s=S*((\mu_{max}/\mu)-1)$ equation (Table S 1 and Table S 2).

Table S 1. K_s determination for control bioreactors at two specific growth rates.

µ (1/h)	0.10	0.20
S_{out} (mmol/L)	0.78	21.04
1/µ	10.00	5.00
1/ S_{out}	1.28	0.05
Slope	4.05	
Intersect	4.81	
μ_{max}	0.21	
K_s (mmol/L)	0.84	
	Point 1	Point 2
check for µ max=((S+Ks)/S)µ	0.21	0.21
check for Ks=S*((µ max/µ)-1)	0.84	0.84

Table S 2. K_s determination for AgNPs-exposed bioreactors at two specific growth rates.

µ (1/h)	0.10	0.20
S_{out} (mmol/L)	1.38	28.24

1/μ	10.00	5.00
1/ S_{out}	0.73	0.04
slope	7.24	
intersect	4.74	
μ_{max}	0.21	
K_s (mmol/L)	1.53	
	Point 1	Point 2
check for μ max=((S+Ks)/S)μ	0.21	0.21
check for Ks=S*((μ max/μ)-1)	1.53	1.53

1.2.5. Substrate mass balance

Also, mass balance for substrate in bioreactors at steady state checked by the following equation:

$$\frac{ds}{dt} = S_{in} - S_{out} - S_{consumed}$$

By applying steady state assumption and rewriting the consumed substrate as the yield coefficient divided by biomass we arrive at following equation:

$$Y_{\frac{x}{s}} = \frac{X}{S_{in} - S_{out}}$$

For each case, the left and right hand side of above equation are as follows:

For control bioreactor at 0.1 h^{-1} => 0.025 = 1.06/(44-0.78) mmol/L

For control bioreactor at 0.2 h^{-1} => 0.027 = 0.62/(44-21.04) mmol/L

For AgNPs-exposed bioreactor at 0.1 h^{-1} => 0.016= 0.7/(44-1.38) mmol/L

For AgNPs-exposed bioreactor at 0.1 h^{-1} => 0.018= 0.29/(44-28.24) mmol/L

1.2.6. Maximum Yield and maintenance coefficient

The maximum yield and maintenance coefficient calculated by the linear regression of 1/μ as x axis and 1/yield as y axis. Slope is the maintenance coefficient and 1/intersect is the maximum yield.

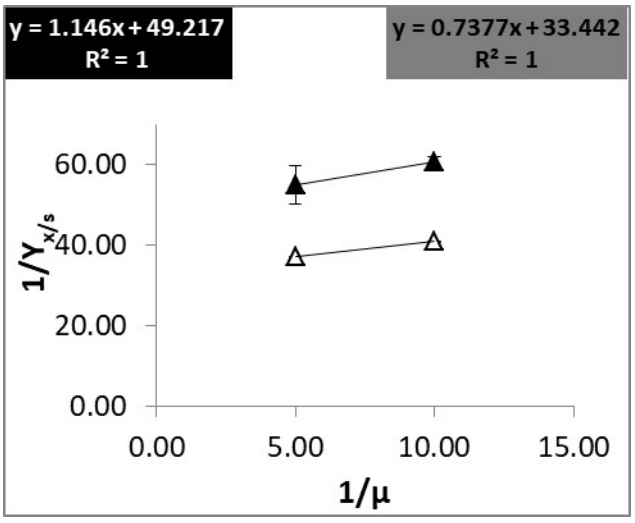

Figure S3. Relation of yield coefficient ($Y_{x/s}$) of *E. coli* to specific growth rate (μ) using double reciprocal linear transformation. Δ, Grey line and grey box represents control bacteria (CB). ▲, Black line and black box represent AgNPs-exposed bacteria (EB).

1.3. *E.coli* number of generation in continuous culture and batch culture

For continuous culture at steady state phase during the nanoparticles exposure time, cell doubling times (T_d) were calculated as the batch culture. The population of bacteria reduced after the treatment with toxicant which caused the reduction in the rate of cell multiplication[4]. Hence, the doubling time calculation of the conventional bioreactor (T_d = 0.693/D) was not applicable. We used the following formula to obtain the doubling time and number of generations:

$$N_t = N_0 * (1 + r)^{Td}$$

Where:

N_t: The amount at time t

N_0: The amount at time 0

r: Growth rate

T_d: Doubling time

We obtained the doubling time of 0.28 h at 0.2 h^{-1}, and 0.15 h at 0.1 h^{-1} the number of generation for 32 hours of contact time was 114 at 0.2 h^{-1} and 206 at 0.1 h^{-1}. In batch test, since the contact time was 5 hours, the number of generation was 17.

1.4. Continuous injection of nanoparticles to bioreactors

AgNPs were continuously injected in to the system to achieve 1 mg/L concentration in bioreactors. Three single syringe infusion pumps were utilized for continuous injection of AgNPs into the bioreactors (Figure S 4). Based on conservation of mass for AgNPs and continuity of the flow we can write:

$$Q_1 + Q_2 = Q_3 \qquad \textbf{Equation 1}$$

$$C_1Q_1 + C_2Q_2 = C_3Q_3 \qquad \textbf{Equation 2}$$

Solving for conservation of mass for AgNPs, based on the Equation1 and Equation 2, as well as $C_1 = 0$, so $C_1Q_1 = 0$, We arrived Equation 3.

$$C_3 = C_2Q_2/\ Q_3 \qquad \textbf{Equation 3}$$

Where Q_1 is M9 minimal culture media inflow rate, C_2 and Q_2 are AgNPs concentration and flow rate, from syringe infusion pump, respectively, as well as Q_3 is the outflow and C_3 is concentration of AgNPs in outflow. Based on this formulation the concentration of AgNPs in outflow is determined and assumed to be equal to AgNPs concentration inside the reactor (Table S 3).

Table S 3. Estimated values for constant concentration of AgNPs inside bioreactors for two specific growth rates

μ (h^{-1})	V mL	C_1 mg/L	Q_1 mL/min	C_2 mg/L	Q_2 mL/min	C_3 mg/L	Q_3 mL/min
0.1	45	0	0.1	11	0.01	1	0.11
0.2	40	0	0.13	14	0.01	1	0.14

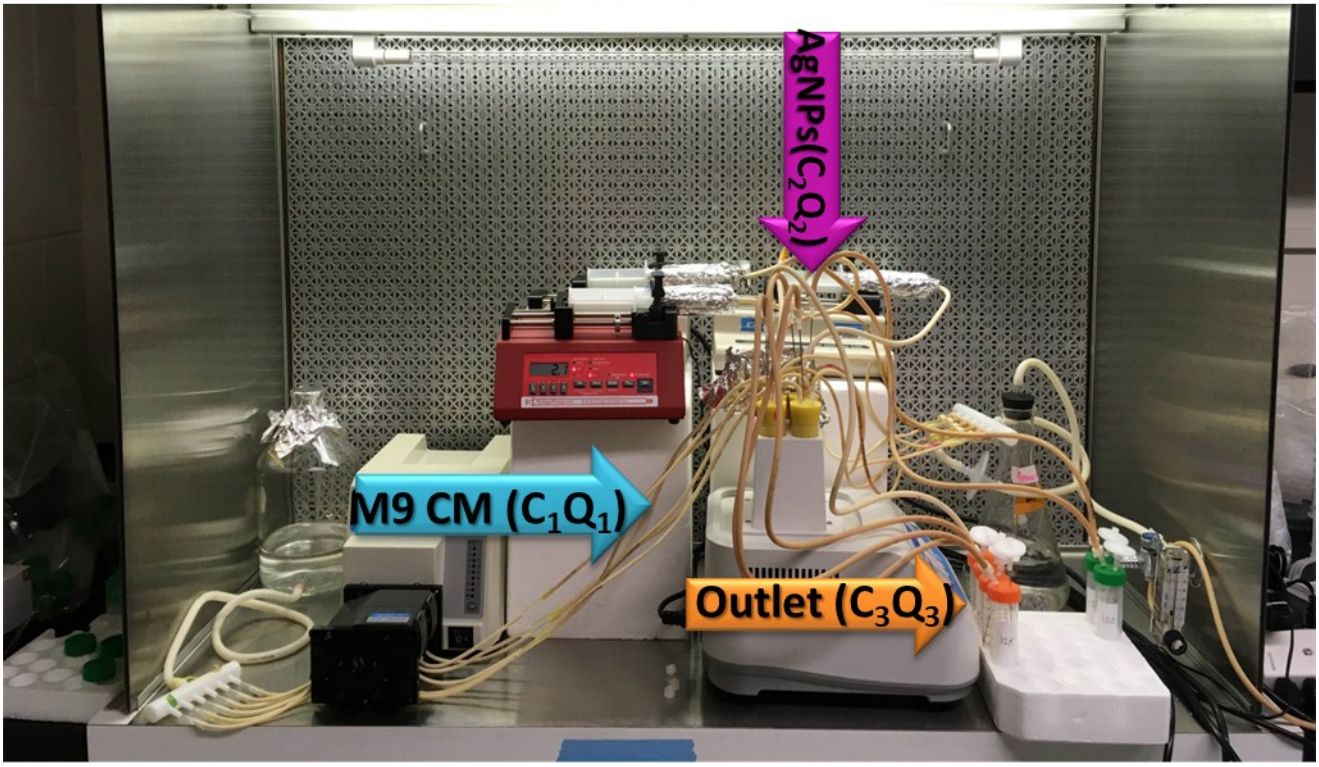

Figure S 4. Continues culture of *E.coli* K-12 with continues injection of AgNPs

Through continuous injection, AgNPs concertation inside the reactor was kept at constant value from time of injection to the end of experiment, allowing us to make consistent comparisons at multiple exposure times. Samples were collected at 4 hours intervals from bioreactors. After centrifugation for 20 minutes at 6000 rpm, the supernatant that includes ES and AgNPs was separated from bacteria. Then, the ES and AgNPs solution was used for measuring the particle hydrodynamic diameter size distribution and zeta potential (ζ) of the suspensions as well as measuring AgNPs and silver ions concentrations.

1.5. Testing designed primers for transcriptomic analysis

To ensure that the primer dimer would not be formed, multiple primer pairs were analyzed for the possibility of primer dimer formation. We ordered the primer pairs which showed the lowest possibility to form self and/or hetero dimers. Next, a series of dilutions for the cDNA template was tested for qPCR efficiency and the melting curves were carefully analyzed. Since, there was only a single melting point for each primer pair it was established that the amplification was specific and only one target amplicon for each primer pair was being generated. The melting temperatures of the qPCR product ranged from 81.81 °C to 86.84 °C. The primer dimer formation was ruled out by the absence of melting point at temperature between 65 °C and 70 °C.

Furthermore, we used $\Delta\Delta C_T$ method for gene regulation calculation. First, for both control bioreactors and AgNPs-exposed bioreactors, we determined the ΔC_T by subtracting the mean C_T value of each gene from mean C_T value of housekeeping gene (*rrsB*). Then, in order to calculate the $\Delta\Delta C_T$ we subtracted the ΔC_T of genes at control bioreactors from the ΔC_T of genes at AgNPs-exposed bioreactors. Finally, the regulation of each gene (R) is obtained by $2^{-\Delta\Delta CT}$

Table S4. Primers of target genes

Target genes	Forward Primer	Reverse Primer
cpsB	GTT GGC TCC TGG TCT TCA TTA	CAG GCC AGA TTC AGC ATA CA
copA	CGA TCC GTT GCG TAG TGA TAG	CCT CAT CAA TCC CTG CTT CTT
CueO	TGC TGC ATC CGT TCC ATA TC	CAC TTC GCT GAC ATT ACC TTC T
CusA	GAC GCC ACG CTG GAT AAT AA	CAG GGT GAA GAT CGG GAT AAA C
fabR	ATG GTT GAT GAG AGC GGT TTA	CCG GAA GGC GTT AGG ATT ATT
ompF	CGC TAC GCC GAT CAC TAA TAA	ACC AGA TCA ACA TCA CCG ATA C
rrsB	GTC AGC TCG TGT TGT GAA ATG	CCC ACC TTC CTC CAG TTT ATC
soxS	ATC AGA CGC TTG GCG ATT AC	GAG ACA TAA CCC AGG TCC ATT G
zwf	CCA AGC TGG ATC TGA GCT ATT C	ACC CAT TTC CAG GCT TCT TC

1.6. ROS generation measurement using 2',7' –dichlorofluorescin diacetate (DCFH-DA)

First, $2x10^5$ cells/well were cultured in a 96-well black plate. After washing the cells with buffer 1X, supernatant in the microplate was replaced with DCFH-A (10 µM) and incubated for 1 hour at 37 °C. Finally, cells were washed with buffer 1X and exposed with given concentration of AgNPs. Procedures were performed in the dark. Each 96-well microplate consists of blanks (AgNPs 1 mg/L and 10 mg/L), AgNPs exposed bacteria, and control bacteria. Blanks were subtracted from AgNPs exposed bacteria, allowing us to make consistent comparison with control bacteria.

2. Additional Results

2.1. AgNPs concentration inside of the continuous culture

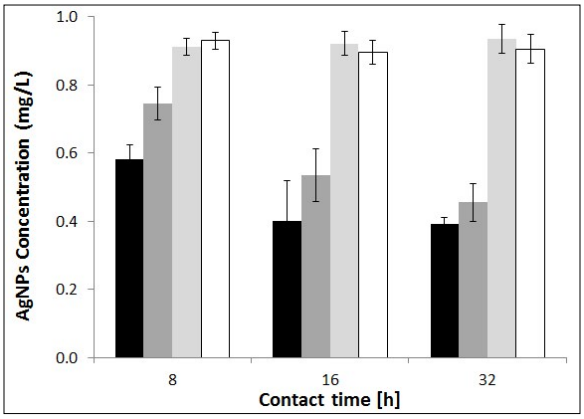

Figure S5. Changes of the AgNPs concentration inside of the continuous culture for both specific growth rates are displayed after 8, 16, and 32 hours after AgNPs injection. Black bars represent 0.1 h^{-1} and dark grey bars represent 0.2 h^{-1} for exposed bacteria. Light grey bars represent control reactors at 0.1 h^{-1}. White bars represent control reactors at 0.2 h^{-1}. For control reactors M9 minimal medium plus AgNPs were used. Error bars represent the error between three technical replicates from duplicate bioreactors.

2.2. Dissolution rate of nanoparticles in continuous bioreactors

Dissolution experiments consisted of measuring the concentration of Ag ion released in ES from both growth rates. Ag ions release was quantified for ES with AgNPs (from continuous bioreactors), M9 minimal medium, and distilled water (control) (Figure S6). The initial concentration of Ag ions released in DI water was 0.031 ± 0.005 mg/L but over time after 12 hours, the released ion concentration increased to 0.08 ± 0.006 of the total silver concentration (1 mg/L). In contrast, initial ion released in M9 minimal culture media slightly increased over 12 hours of contact. The effect of ES from both specific growth rates on the initial Ag ion release was not detectable for both 0.1 h^{-1} and 0.2 h^{-1}, although, after 12 hours of exposure the concentration of ions released in ES of 0.1 h^{-1} and 0.2 h^{-1} were almost the same (Figure S6). The low rate of AgNPs dissolution in M9 minimal culture media and ES clarified that bacteria that responded to the inhibitory effect of AgNPs were not correlated with ions released by the nanoparticles and their interference in the cellular metabolic pathways.

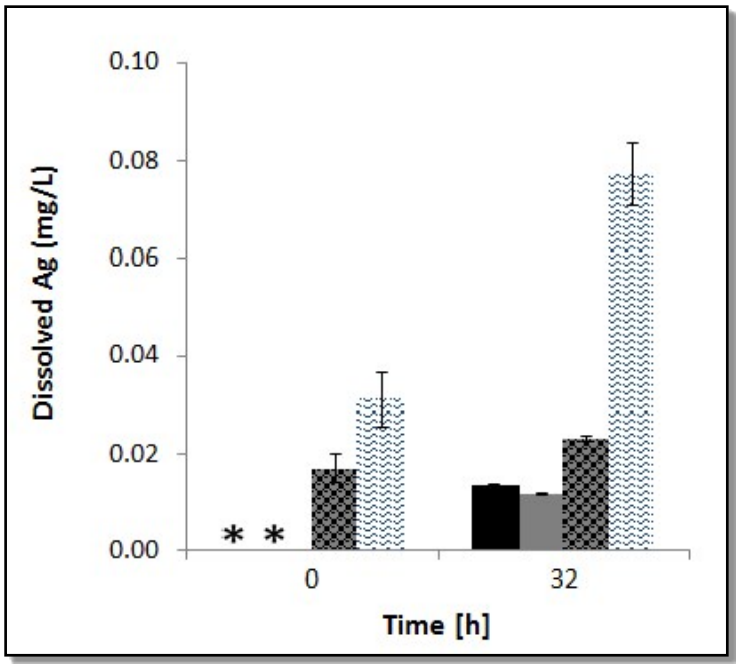

Figure S6. Ag ions released from AgNPs at concentrations of 1 mg/L DI water, M9 minimal media, ES from EB-0.1, and ES from EB-0.2 at time zero and after 32 hour contact time at 37 °C. Wave patterns represent AgNPs suspended in DI water, sphere patterns show AgNPs suspended in fresh M9 minimal media, and grey filled represent AgNPs exposed to ES from EB-0.2 and black filled correspond to AgNPs suspended in from EB-0.1. Star sign means that Ag ion concentrations were not detectable. Samples run in triplicate.

2.3. AgNPs stability and hydrodynamic diameter size distribution

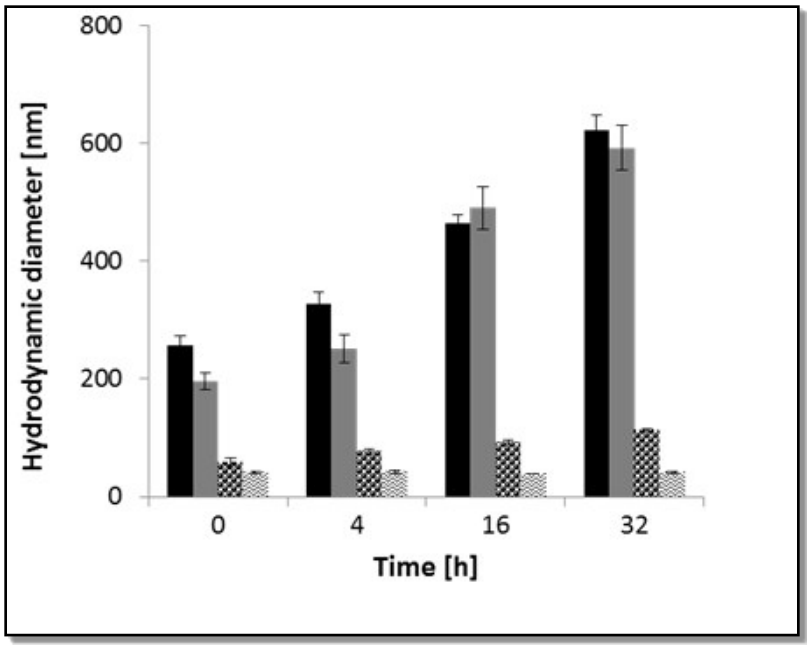

Figure S7. AgNPs stability in DI water, M9 minimal media, ES from EB-0.1, and ES from EB-0.2 after 32 hours of exposure. Wave patterns represent AgNPs suspended in DI water, sphere patterns show AgNPs suspended in fresh M9 minimal media, and grey filled represent AgNPs in ES from EB-0.2 and black filled correspond to AgNPs suspended in ES from EB-0.1. Samples run in triplicate.

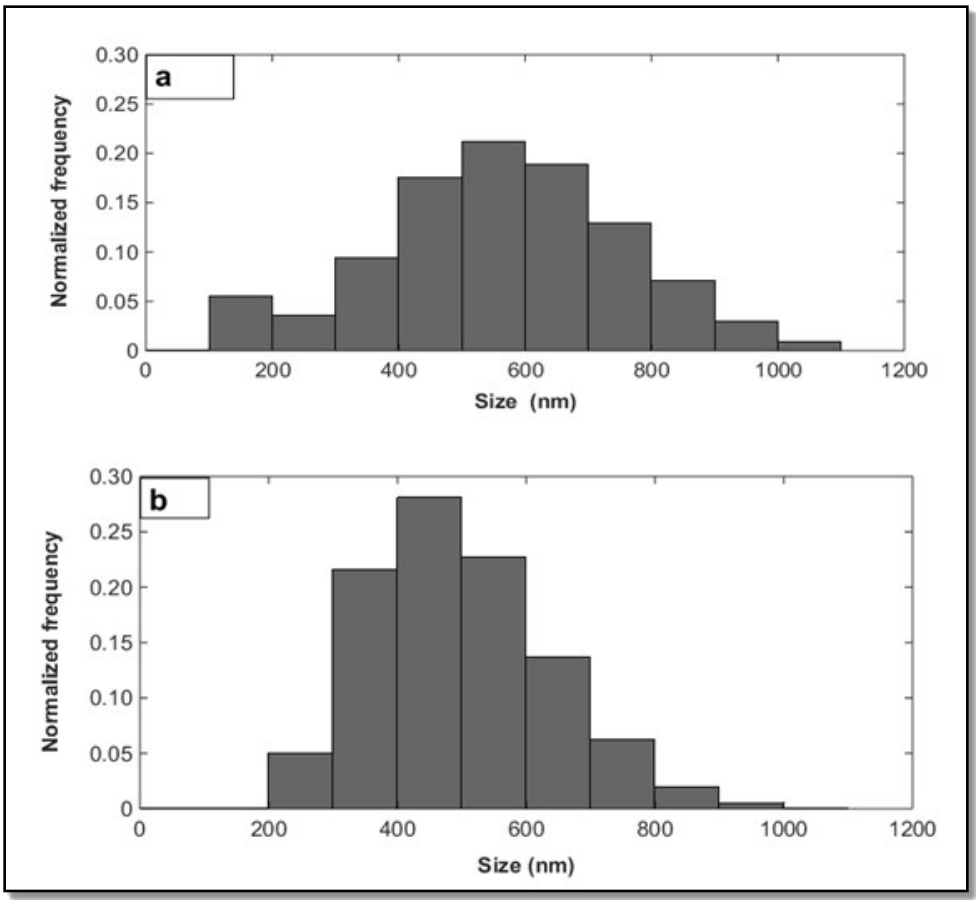

Figure S8. AgNPs size distribution in ES of continuous bioreactor products. (a) AgNPs in ES at EB-0.1 and (b) AgNPs in ES at EB-0.2 h both after 32 hours of contact time.

2.4. AgNPs zeta potential

Table S5. Zeta potential characterization of AgNPs suspension in different solutions.

Suspensions	AgNPs Zeta Potential (mV)
DI water + AgNPs	-26 ± 1.53
M9 minimal media + AgNPs	-22 ± 1.12
AgNPs at ES from EB-0.1	-11 ± 1.23
AgNPs at ES from EB-0.2	-14 ± 1.34

2.5. ES concentration and characteristic at different specific growth rates

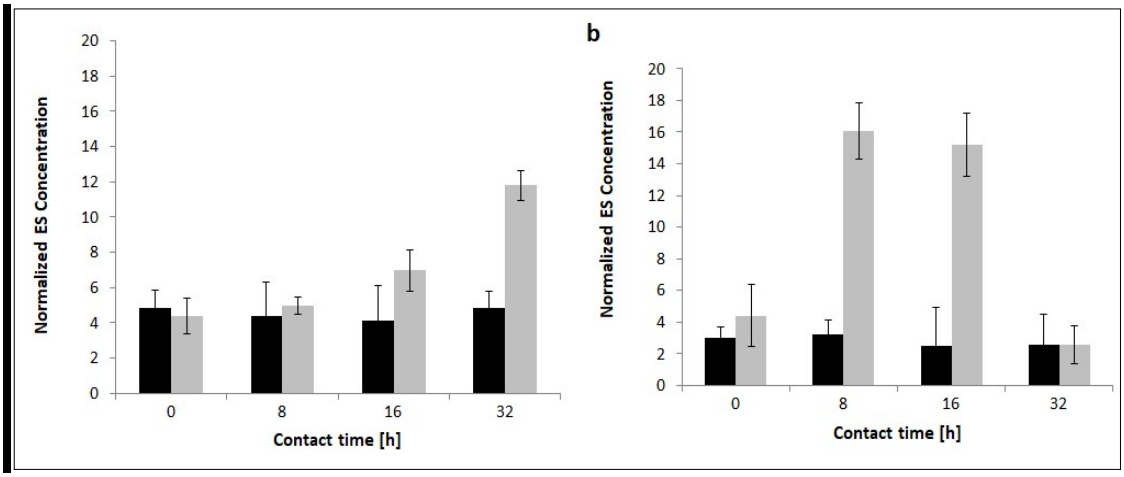

Figure S9. ES concentration normalized to OD600 at different contact times, zero contact time means before the starting injection, 8, 16, and 32 represents the time in hours of contact between nanoparticles and bacteria inside continuous culture at two specific growth rates. (a) Normalized ES concentration at 0.1 h^{-1} and (b) Normalized ES concentration at 0.2 h^{-1}. Black and grey marks show the ES from CB and ES from EB, respectively. Bars represent the error between three technical replicate from two replicates.

ES can affect the inhibitory effectiveness of AgNPs through two mechanisms; physiochemical alteration of nanoparticles' surface modifications and nanoparticles' immobilization in ES matrix.[7] In here, it was considered the heterogeneity between the compositions of ES at different specific growth rates (Figure S10). Therefore, after characterization of ES compositions by ATR-FTIR,[8] variation of ES composition among the tested conditions was assessed using HCA.[9]

ATR-FTIR spectra which looked at a comparison of the ES before and after reaction with AgNPs demonstrated changes in the organic moieties and functional groups of the ES. For the ES at 0.1 h^{-1}, the band around 1640 cm^{-1} is ascribed to C=O stretching (amide I), which completely disappeared in ES of 0.2 h^{-1}. Also, the band at 1550 cm^{-1} is ascribed to the N-H bending and C-N stretching (amide II) in peptides vanished in ES of 0.2 h^{-1}. The bands near 1046 cm^{-1} are assigned to the stretching vibration of C-O-C in sugar derivatives after AgNPs exposure shifted to 1060 cm^{-1} at 0.1 h^{-1} and 1070 cm^{-1} at 0.2 h^{-1} due to carbohydrate backbones. These results assessed that the ES contains mainly protein, saccharides, and carboxylates. These findings are in agreement with Wang *et al*.[7] They reported the weakness of bands related to amide II and carboxylic acids after reaction with zinc oxide nanoparticles in ES. Furthermore, the band corresponding to the C-O-C group of sugar derivatives (1046 cm^{-1}) becomes shifted in ES after AgNPs exposure, implying the possibility of trivial reducing sugar adsorbing these nanoparticles.

In this study, HCA was based on the baseline-normalized spectra of all ES obtained from continuous culture. HCA results for the pairwise analysis of the fingerprint (1800-900 cm^{-1}) region of ES released from bacteria without and with nanoparticles are shown in Figure S10. ES extracted from 0.1 h^{-1} segregated distinctly from the ES from control groups of 0.2 h^{-1}. Higher heterogeneity values in the dendogram was obtained in ES from 0.2 h^{-1} compared to 0.1 h^{-1}, which indicates that the composition of ES released by CB-0.1 during their time spent in the continuous culture/during nanoparticle exposure were more stable than those obtained from CB-0.2. Moreover, the heterogeneity between ES from EB-0.2 showed the higher similarity to ES composition of other samples (ES from CB-0.1 and ES from EB-0.1). Generally, in the control group ES concentration and composition was function of growth rate. In addition, ES from AgNPs exposed bacteria changed in composition at the higher growth rate condition and obtained similar compositions to ES from exposed bacteria at low growth rate condition.

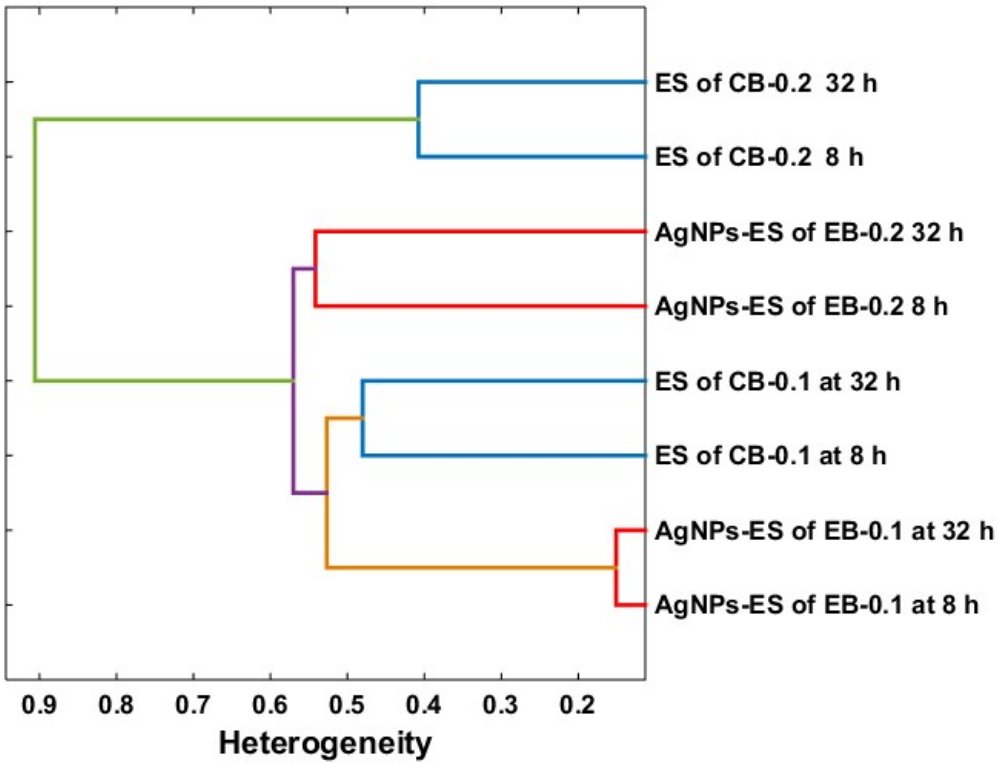

Figure S10. Hierarchical cluster analysis of the ES composition from control bacteria (CB) and ES from AgNPs-exposed bacteria (EB) at two different specific growth rates during 8 hours and 32 hours of contact time. Blue line represents only time impacts on compositional differences between ES of control bacteria (CB). Red line shows heterogeneity between the AgNPs-ES samples from AgNPs-exposed bacteria (EB) during different time of treatment with AgNPs.

Green line shows dissimilarity between the ES of CB-0.2 and other samples (ES of CB-0.1, AgNPs-ES of EB-0.1, and AgNPs-ES EB-0.2). Purple line represents dissimilarity between ES of CB-0.1 and AgNPs-ES of EB-0.1, and AgNPs-ES of EB-0.2. Orange line shows the heterogeneity between ES of CB-0.1, and AgNPs-ES of EB-0.1.

2.6. **Thermogravimetric analysis (TGA)**

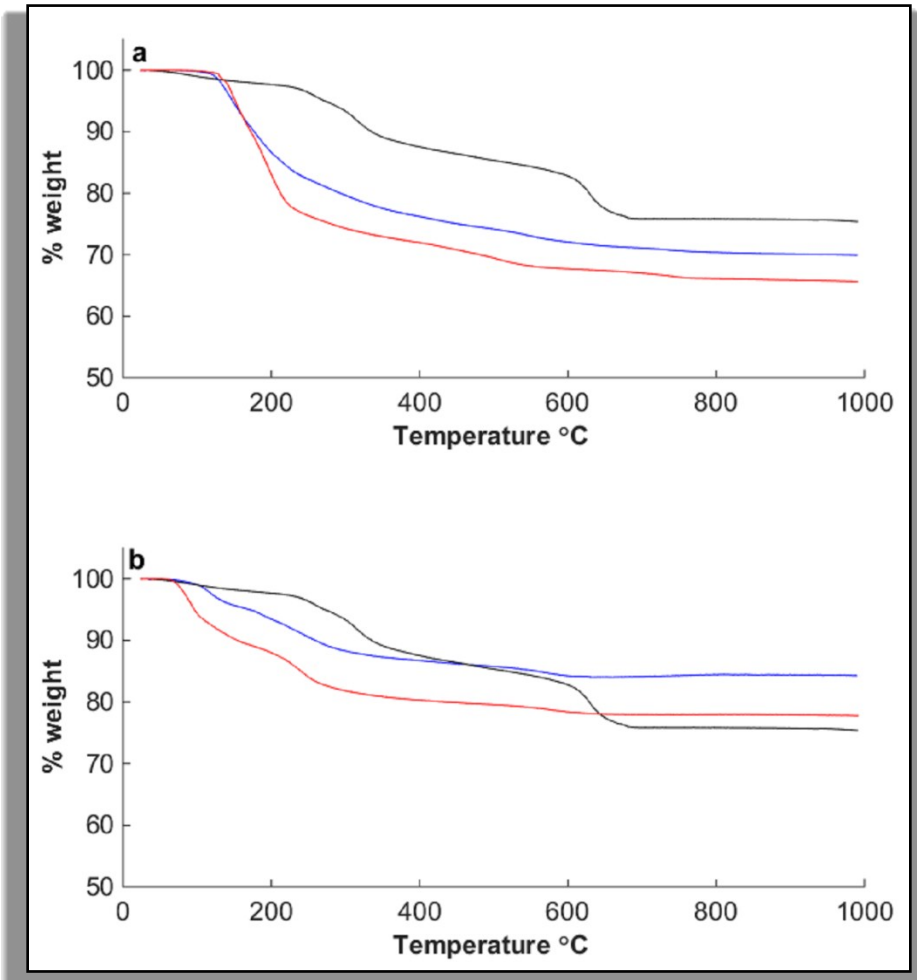

Figure S11. Thermogravimetric analysis (TGA) of ES-AgNPs of the AgNPs-exposed bacteria (EB) and ES of the control bacteria (CB) from different specific growth rates. (a) Thermal stability of AgNPs-ES from EB-0.1 and ES from CB-0.1, (b) thermal stability of AgNPs-ES from EB-0.2 and ES from CB-0.2. Thermal stability of AgNPs-casein was obtained as a reference to compare with interacted AgNPs-ES. Black, blue, and red lines represent TGA of AgNPs, AgNPs-ES, and ES, respectively.

REFERENCES FOR SUPLEMENTARY INFORMATION err

1 R. M. Maier, I. Pepper and C. Gerba, *Environ. Microbiol.*, 2000, 43–59.
2 G. Molin, *Eur. J. Appl. Microbiol. Biotechnol.*, 1983, **18**, 303–307.
3 J. Glazyrina, E.-M. Materne, T. Dreher, D. Storm, S. Junne, T. Adams, G. Greller and P. Neubauer, *Microb. Cell Factories*, 2010, **9**, 42.
4 M. O. Tovey and D. Brouty-Boyé, *Exp. Cell Res.*, 1979, **118**, 383–388.
5 Q. Wang, F. Kang, Y. Gao, X. Mao and X. Hu, *Sci. Rep.*, 2016, **6**, 21379.
6 J. Zeng, J.-M. Gao, Y.-P. Chen, P. Yan, Y. Dong, Y. Shen, J.-S. Guo, N. Zeng and P. Zhang, *Sci. Rep.*, 2016, **6**, 26721.
7 R. Gurbanov, N. Simsek Ozek, A. G. Gozen and F. Severcan, *Anal. Chem.*, 2015, **87**, 9653–9661.