The impact of fold-dilution and diluent volume on the estimation of colony forming units in a bacterial culture

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Abstract

Dilution plating is a fundamental procedure used by researchers for enumerating viable microbial cells. Serial dilution serves an integral part of it that involves stepwise dilutions using different sample volumes. For example, a 10^6 fold dilution can be attained by successively relaying either 100 μL sample six times or 10 μL sample three times or 1 μL sample two times to a final 1 mL volume. Considering the random nature of bacterial distribution in a culture, relaying a higher sample volume seems more accurate; however when the complexity (defined by number of steps involved) is considered, relaying of a lower sample volume seems more accurate. In view of the opposite relationship between sample volume and step complexity, it is pertinent to find out the better approach for enumerating viable microbial cells. Apart from this, a serial dilution can be performed using different diluent volumes to reach a certain final volume (such as 1.0 or 10.0 mL), which might influence the enumeration process. Therefore, the current study was undertaken to find out the impact of fold dilution and diluent volume on bacterial enumeration by using the cultures of two bacteria: *Escherichia coli* and *Ralstonia pseudosolanacearum* that exhibit different colony morphologies. The results revealed that bacterial enumeration is indifferent to fold dilution as well as diluent volume. However, it is recommended to perform a higher fold dilution such as 1000-fold using higher diluent volume to eliminate the procedure of relaying a small volume like 1 μL which requires sophisticated handling. Overall, the study addresses different fundamental yet unexplored aspects of serial dilution process.

Keywords

Dilution plating, CFU (Colony Forming Units), serial dilution, microliter spotting, diluent volume

1. Introduction

Estimation of viable single units in any microbial sample serves the fundamental pillar in microbiology and has been practiced in laboratory for more than one-hundred years after Robert Koch first attempted to enumerate bacterial count from water in 1883 (Ben-David and Davidson, 2014). With bacteriological samples, estimation requires sampling of bacterial population over nutrient agar but their abundance in any sample makes it necessary to begin with diluting it many folds before performing the sampling (Reynold, 2005). The sample is diluted sufficiently so that when plated over nutrient agar, each viable unit forms a distinct colony (cellular aggregate) which can then be counted and estimated in the form of colony forming units (CFU) (Thomas *et al*., 2015). In this method of dilution plating, serial dilution remains the classical technique being employed (Reynold, 2005), where the desirable fold diluted suspension is attained by a series of successive dilutions with the relaying of a specific sample volume in order to produce the respective fold dilution series. Likewise, different sample volumes can be relayed. For example, a 10^6 fold dilution can be attained by stepwise relaying 100 μL or 10 μL or 1 μL sample volume to a final 1 mL volume leading to the respective 10-, 100- and 1000-fold dilution series and requiring six, three and two stepwise dilutions respectively in the process. Considering the random nature of bacterial distribution in a suspension, anomalies are expected in cell counts relayed in the successive dilution steps, with lesser anomaly associated with relaying a higher sample volume. This is attributed to the better tendency of a larger sample size to contain the randomness prevalent in suspension. However apart from sample volume, another variable factor that comes into picture here is the complexity of the process (defined by number of steps involved). In biological experiments, there is a trade-off between complexity of the process involved and accuracy of the result, due to the chances of error associated with each independent step. Evident from our previous study (Bhuyan *et al*. 2023), CFU estimation by a 10 μL spotting of bacterial suspension was found to be more accurate than 100 μL spreading of bacterial suspension on plate. The inaccuracy in the 100 μL spreading was attributed to the complex procedures involving the use of a spreader, ethanol and heat in the spreading process. So although in serial dilution, theoretically, using a higher sample volume (as 100 μL in 10-fold series) aligns better with regards to the random distribution of bacteria; yet using a lower sample volume (as 1 μL in 1000-fold series) appears more accurate considering the

complexity of the process. In view of the opposite relationship between sample volume and step complexity, it is pertinent to observe the impact of fold dilutions in bacterial CFU estimation. Besides this, a given fold dilution series can be performed using different diluent volumes in order to adjust to a certain final volume such as 1 mL or 10 mL. Although the sample to diluent ratio remains constant while scaling up the final volume of dilution, the overall force exerted by the diluent while invert mixing might be different in the two cases. This might pose an impact on the degree of random distribution of bacteria in the diluent, overall affecting the CFU estimation upon sampling. Considering this, it was also pertinent to observe the impact of diluent volume on CFU estimation.

Therefore, the current study was carried out in order to understand the role of two variable factors of serial dilution: (i) fold dilution and (ii) diluent volume being used on CFU estimation of bacteria by carrying out a critical examination of 10-, 100- and 1000-fold dilution series. The studies were conducted using two different bacterial cell suspensions: *E. coli* DH5α and *R. pseudosolanacearum* F1C1 exhibiting two different colony morphologies, with colonies of the former being non-mucoid and latter being mucoid due to the secretion of exopolysaccharides. Microliter spotting approach was employed for the purpose of plating as our previous study (Bhuyan *et al*., 2023) demonstrates a clear advantage of using it over the traditional spread plate method. The present study serves a continuation of this study.

2. Material & Methods

2.1 Bacterial strains and growth conditions

E. coli DH5α (Lab collection) was cultured in Luria Bertani (LB) (Himedia) medium (that contains 1% casein enzyme hydrolysate, 1% NaCl, 0.5% yeast extract) and incubated at 37 °C with 200 rpm shaking condition. *R. pseudosolanacearum* F1C1 (Lab collection) (Kumar *et al*., 2013) was cultured in BG medium (Boucher *et al*., 1985) that contains 1% peptone (Himedia), 0.1% yeast extract (Himedia) and 0.1% casamino acid (SRL), supplemented with 0.5% glucose (Himedia) and incubated at 28 °C with 150 rpm shaking condition. For preparing solid medium, 1.5% agar (Himedia) was added.

2.2 Culturing of strains and serial dilutions

Saturated cultures of the mentioned strains were obtained after incubating for ~16 h in their respective growth conditions as mentioned above; and then serial dilutions were performed in saline (0.9% NaCl) as diluent. Three different approaches were employed for

serial dilution *viz*. (i) a 10-fold, (ii) a 100-fold and (iii) a 1000-fold dilution series. The 10-, 100- and 1000-fold dilution series involves the respective series of diluting 1-part sample in 9-parts diluent, 1-part sample in 99-parts diluent and 1-part sample in 999-parts diluent. These dilutions were performed for two different final volumes: 1 mL and 10 mL. The dilutions for 1 mL were performed in 1.5 mL micro-centrifuge tubes (MCT) (from Abdos) as illustrated in Fig. 1 and for 10 mL, they were performed in test tubes (from Borosil) as illustrated in S.Fig. 1. Tips were changed between different dilutions of the series. For a more reliable dilution, precaution was taken during taking in the sample, dispensing it on the walls of next tube in series and mixing the new suspension obtained as follows: Sample was immediately taken from a well-mixed suspension (to reduce the chances of cellular sedimentation on standby). While taking in the sample, the micro-tip was dipped just below the meniscus, not too deep to reduce bacterial adhesion on the outer surface of tip. The sample was then dispensed carefully on the walls of the next tube, without touching the diluent so that the population adhered on the outer surface of the tip would not have a chance to contact the diluent. Lastly the tip was discarded after dispensing the sample, and the sample on wall was invert-mixed after closing the MCT or covering the mouth with sterile parafilm in case of test-tube. From this suspension, next dilution of the series was similarly performed from a fresh tip.

2.2.1 Dilutions to a final volume of 1 mL

As illustrated in Fig. 1, for a 1 mL final volume, a 10-fold dilution series was performed by adding 100 µL of culture to 900 µL of saline in the first step to obtain a 10^1-fold dilution, subsequently 100 µL of this suspension was added to the next tube in the series containing 900 µL of saline to obtain a 10^2-fold dilution cell suspension. This was repeated until a cell suspension of 10^7-fold dilution was obtained. Similarly, a 100-fold dilution series was performed by adding 10 µL of culture to 990 µL of saline in the first step to obtain a 10^2-fold dilution. In subsequent steps, 10^4- and 10^6-fold dilutions were similarly obtained. Lastly, to proceed with a 1000-fold dilution series, 1 µL of culture was added to 999 µL of saline to obtain 10^3-fold dilution in first step and 10^6-fold dilution in second step. In both the latter dilution series (100-fold and 1000-fold series), the final 10^7-fold dilution was obtained by

performing a 10-fold dilution from their respective 10^6-fold diluted suspensions.

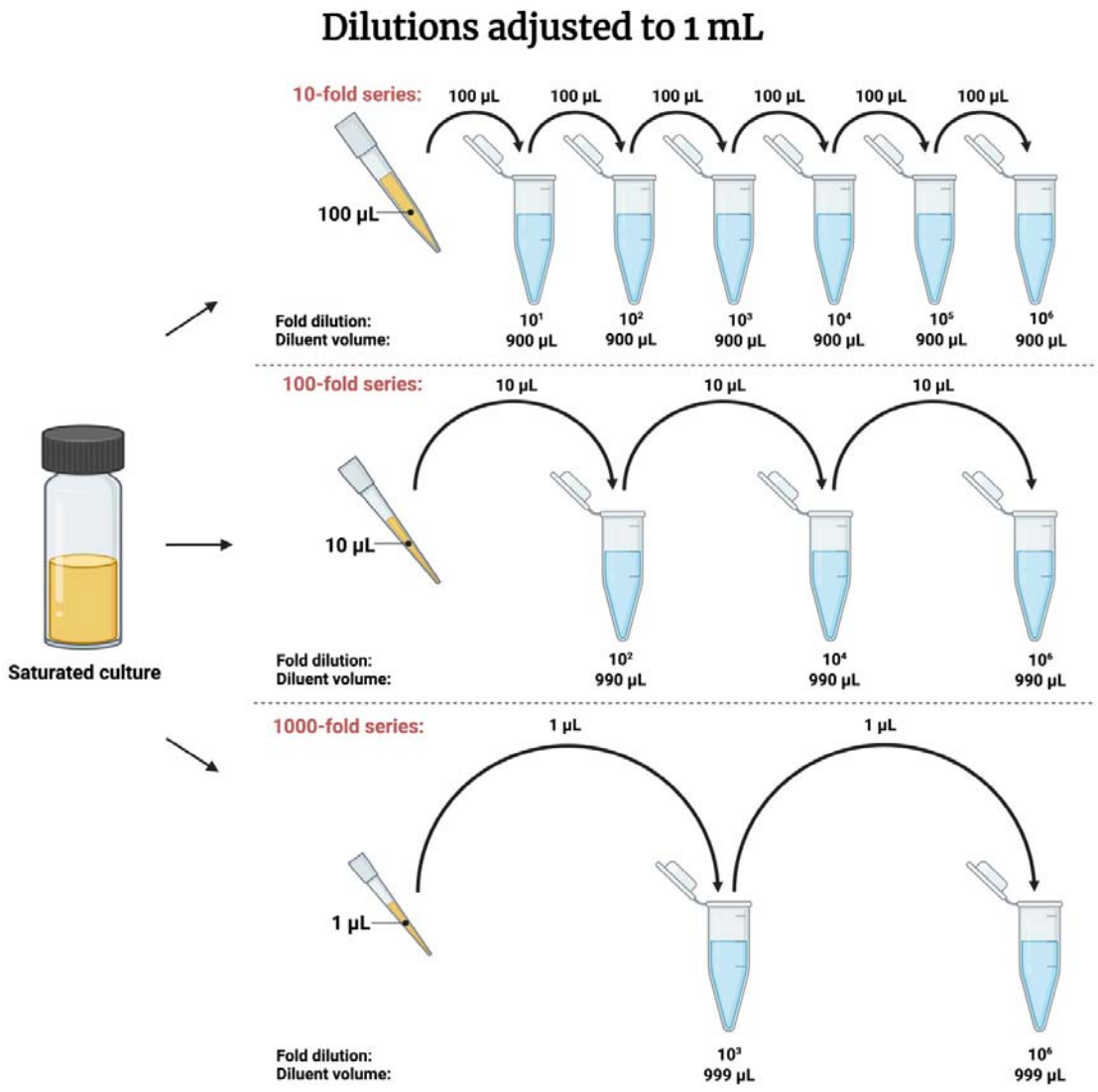

Fig. 1: An illustration of the 10-, 100- and 1000-fold dilution series performed for 1 mL final volume. Bacterial culture is shown in yellow in the screw caped bottle. Diluent (saline) is shown in blue in micro-centrifuge tubes. Three different ways of attaining a 10^6 fold dilution has been shown *viz* a 10-, 100- and 1000-fold dilution series. (i) A 10-fold series involves relaying 100 µL suspension to 900 µL saline in six successive dilutions. (ii) A 100-fold series involves relaying 10 µL suspension to 990 µL saline in three successive dilutions. (iii) A 1000-fold series involves relaying 1 µL suspension to 999 µL saline in two successive dilutions. Created with Biorender.com.

2.2.2 Dilutions to a final volume of 10 mL

Dilutions were performed in a similar way as mentioned above, only the volumes of sample and diluent taken were both up scaled (although same in proportion) for adjusting it to 10 mL volume as illustrated in S.Fig. 1. In a 10-fold dilution series 1 mL of culture was added to 9 mL of saline to obtain a 10^1-fold dilution, which was repeated with subsequent diluents in series to obtain 10^7-fold dilution. In a 100-fold dilution series, 100 µL of culture was added to 9.900 mL of saline to obtain a 10^2-fold dilution. In subsequent steps, 10^4- and 10^6-fold dilutions were similarly obtained. Lastly, in a 1000-fold dilution series, 10 µL of culture was added to 9.990 mL of saline to obtain 10^3-fold dilution and then 10^6-fold dilution in second step. In both the latter dilution series, a 10^7-fold dilution was obtained by performing a 10-fold dilution from their respective 10^6-fold diluted suspensions.

2.3 Plating and colony observation

Plating was done from a 10^6- and 10^7-fold diluted suspensions obtained from each of the series for *E. coli* DH5α and at 10^7- and 10^8-fold diluted suspensions obtained from each of the series for *R. pseudosolanacearum* F1C1. A higher fold dilution was chosen for *R. pseudosolanacearum* F1C1 as (i) with the saturated cultures of the two, the number of colonies observed in this case were always higher than that of *E. coli* DH5α by 2-6 times at same fold dilution (this can also be observed in Table 1) and (ii) *R. pseudosolanacearum* forms typical mucoidal colonies once they appear completely in plate; also they show twitching motility over solid media unlike *E. coli* (Bhuyan *et al*., 2023, 2024b); the latter one forming non-mucoidal colonies. So apparently, colonies of the former one had greater tendency to merge with other such colonies.

For plating, microliter spotting approach was followed (Bhuyan *et al*., 2023). Colonies were observed and counted after 12-14 h of incubation in case of *E. coli* DH5α and after 48 h of incubation in case of *R. pseudosolanacearum* F1C1. Although with the latter one, colonies appear fully after 48 h of incubation (due to a slower generation time), but the simultaneous production of copious amounts of EPS by the bacteria makes it fluidal and difficult to count at this point. So in this case, a prior observation of the colonies was also made at 28 h post incubation, where tiny colonies although not so prominent can be lightly visualized against light and accordingly counted. These were tallied after the colonies completely appear.

2.4 Study on the impact of plating volume on CFU estimation

To study the impact of using different plating/sampling volumes on CFU estimation, ten different spots of each of the different volumes: 5 µL, 10 µL, 15 µL and 20 µL were made in separate nutrient agar plates. These were sampled from a specific suspension which was at 10^6 fold dilution for *E. coli* DH5α and at 10^7 fold dilution for *R. pseudosolanacearum* F1C1, obtained simply from a 10-fold series performed for 1 mL final volume. Plates were then kept under incubation for colony observation. After incubation, average number of colonies per spot was calculated in each case.

2.5 Study on the impact of fold-dilution on CFU estimation

To study the impact of fold dilution series on CFU estimation, all the three dilution series (10-fold, 100-fold and 1000-fold series) were performed for the two final volumes: 1 mL and 10 mL in the independent experiments generated from different cultures. For plating, ten spots of 10 µL each were made from a 10^6- and 10^7- fold diluted suspensions of *E. coli* DH5α and a 10^7- and 10^8- fold diluted suspensions of *R. pseudosolanacearum* F1C1. Plates were then kept under incubation for colony observation. After incubation, average number of colonies per spot was calculated in each case.

2.6 Study on the impact of diluent volume on CFU estimation

For this study, all the dilution series were performed for both 1 mL and 10 mL final volume and were generated from same culture, so that except the factor of final volume for which the dilutions are being performed, all other factors remain constant. This study was performed with *E. coli* DH5α as representative. Plating was done from a 10^6 and 10^7 fold diluted suspension. For each of the three dilution series, ten spots of 10 µL each were made for it being performed at 1 mL and 10 mL final volume in the different halves of same plate to enable clear comparison. The plates were then kept under incubation and later average number of colonies per spot was calculated for all the cases.

2.7 Statistical analyses

2.7.1 Coefficient of Variation (CoV)

For comparing different sample volumes used to estimate bacterial cell density, CoV (which is a measure of variability in a population) was calculated among the colony numbers observed in the ten replicates of each sampling volumes used. First, average number of colonies and standard deviation were calculated for each of the different sample volumes,

then CoV was calculated by dividing standard deviation by average.

$$\mathrm{CoV} = \frac{\text{Standard deviation}}{\text{Average}}$$

2.7.2 Correlation analysis

To study the correlation between different sampling volumes used for bacterial cell density estimation and CoV obtained in the colony count data corresponding to each sampling volume, Pearson's correlation analysis was performed. The Pearson's correlation coefficient (r) was calculated as:

$$r = \frac{\sum(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum(x_i - \bar{x})^2 \sum(y_i - \bar{y})^2}}$$

where x_i = values of x-variable (here, x takes the values of different sample volumes); $\bar{x}$ = mean of the values of x-variable; y_i = values of y-variable (here, y takes the values of CoV corresponding to each values of x); $\bar{y}$ = mean of the values of y-variable

2.7.3 One-way ANOVA

For comparing the mean of sample population of the suspensions obtained from different dilution series, one-way ANOVA test was performed in MS Excel at α=0.05 with H_0 stating that there is no significance difference between means of sample population of suspensions obtained from these different dilution series and H_1 stating that the means of sample population of suspensions obtained from these dilution series are significantly different.

2.7.4 Bonferroni post-hoc test

In cases where one-way ANOVA proved significant difference between the groups, a post-hoc test was performed using Bonferroni corrected p-value to identify which group differ significantly. This was calculated as:

Bonferroni-corrected p-value = α / number of comparative tests performed

When p-value of comparative tests < Bonferroni-corrected p-value, the test is significant.

2.7.4 Mann-Whitney U test

A two-tailed Mann-Whitney U test was performed to compare the mean difference of colony counts obtained with dilutions performed for 1 mL and 10 mL volumes. Test is significant when $U_{Stat} < U_{Crit}$.

U_{Stat} = minimum of the two values, U_1 and U_2.

$$U_1 = n_1 n_2 + \frac{n_1(n_1+1)}{2} - R_1 \quad \text{and} \quad U_2 = n_1 n_2 + \frac{n_2(n_2+1)}{2} - R_2$$

Where, n_1 and n_2 represents sample size of the two groups under comparison; R_1 and R_2 denotes rank sum of the two groups respectively when all of them are arranged in ascending order.

3. Result

3.1 A higher sample volume of a bacterial suspension is a better representation of the population size than a lower sample volume

During CFU counting by dilution method, a specific volume of sample is stepwise relayed to a specific volume of the diluent. As distribution of bacteria is random in a culture, there would be anomalies associated with the colony numbers being relayed. In consideration of the random distribution, the sample volume being relayed during the serial dilution is likely to play an important role in the consistency of bacterial numbers during the stepwise relay process and hence in CFU estimation. To get an insight into the influence of sample volume on consistency of bacterial count, we studied the variation prevalent in colony count in the plating of different sample volumes: 5 μL, 10 μL, 15 μL and 20 μL of a diluted bacterial suspension. The colony numbers obtained in 10 spots of each of these sample volumes is represented in Table 1. To enable clear distinction of colonies in spotted area, the suspension used was at 10^6 fold dilution of the saturated culture in case of *E. coli* and at 10^7 fold dilution in case of *R. pseudosolanacearum*. In case of *E. coli*, the CoV observed in colony numbers obtained with 5 μL, 10 μL, 15 μL and 20 μL spotting were 0.18, 0.12, 0.10 and 0.07 respectively. When the same study was conducted with *R. pseudosolanacearum*, the CoV was found to be 0.42, 0.37, 0.26 and 0.17 respectively for 5 μL, 10 μL, 15 μL and 20 μL spotting. Clearly, for both the independently tested bacterial strains, the coefficient of variation (CoV) of bacterial count in the different spots made of a given sampling volume was observed to be highest with the lowest sampling volume used (5 μL) and lowest with the highest sampling volume used (20 μL). Similar observations were recorded in four repetitive

experiments (S.Table1). This is in concordance with the view of random distribution of bacteria in a culture; as with a randomly distributed population, a larger sampling size is supposed to give more consistency in density estimation. As expected, a strong negative correlation was found between sampling volumes of the suspension used and coefficient of variation (CoV) of bacteria count, with the same trend being observed for both the bacterial species (r = -0.97 for *E. coli* and r = -0.99 for *R. pseudosolanacearum*) (Fig. 2). This suggests that higher the sampling volume of the suspension, greater the consistency in bacterial cell count and lesser the variability. Hence, higher sampling volume is a better representation of the bacterial population.

Table 1: Colony numbers obtained in different plating volumes of the two different bacterial cell suspensions.

Colony count data of 10 spots								
Bacterial samples	***E. coli* DH5α**				***R. pseudosolanacearum* F1C1**			
Dilution factors	**10^6**				**10^7**			
Spotting volume	**5 µL**	**10 µL**	**15 µL**	**20 µL**	**5 µL**	**10 µL**	**15 µL**	**20 µL**
	10	27	47	44	7	6	11	14
	11	24	42	40	4	6	10	16
	12	21	36	49	1	10	17	15
	15	28	39	45	4	8	11	12
	12	26	36	42	5	6	9	17
	13	24	34	44	2	6	11	15
	12	28	42	45	4	8	9	19
	18	19	39	49	5	14	12	11
	15	25	38	41	4	4	6	15
	14	24	36	42	6	8	10	19
Mean	13.2	24.6	38.9	44.1	4.20	7.60	10.60	15.30
SD	2.35	2.91	3.87	3.07	1.75	2.80	2.80	2.63
CoV	**0.18**	**0.12**	**0.10**	**0.07**	**0.42**	**0.37**	**0.26**	**0.17**
CFU estimate	$2.6x10^9$	$2.5x10^9$	$2.6x10^9$	$2.2x10^9$	$8.4x10^9$	$7.6x10^9$	$7.1x10^9$	$7.7x10^9$

Standard deviation (SD), Coefficient of Variation (CoV) and CFU estimate calculated for parent culture in each of these cases are represented.

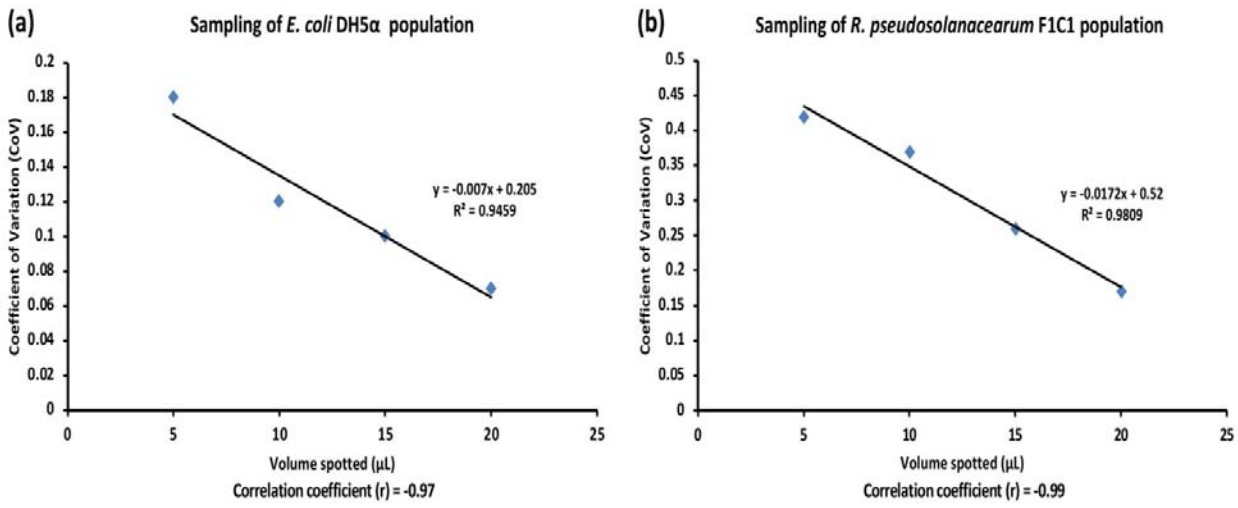

Fig. 2: Correlation between suspension volume spotted (X- axis) and coefficient of variation (CoV) obtained among the colony numbers (Y-axis) of (a) *E. coli* and (b) *R. pseudosolanacearum*. Pearson's correlation coefficient (r) is represented for each case. A strong negative correlation has been observed in each case.

3.2 Stepwise relaying of 1 mL, 100 µL and 10 µL (but not 1 µL) are equally accurate for CFU estimation of E. coli DH5α

In theory, to make a 10^6 fold dilution of an original culture different dilution series such as ten or hundred or thousand fold can be used which has been shown in Fig. 1. As evident from the Fig, in case of a higher fold dilution series such as thousand, only 1 µL of the bacterial suspension is relayed twice to achieve 10^6 fold dilution whereas in case of a lower fold dilution series such as ten, 100 µL of bacterial suspension is relayed six times. In the intermediary hundred fold dilution series, 10 µL of bacterial suspension is relayed thrice. In this way, the three different approaches of serial dilution are different with respect to (i) volume of the sample relayed in the series; and (ii) complexity of the series (number of steps involved). As mentioned earlier, in view of the random distribution of bacteria, relaying of a higher sample volume (as in 10-fold series) seems a better approach for CFU estimation. However, in view of the complexity involved, relaying of a lower sample volume (as in 1000-fold series) seems better. To find out the more accurate dilution series considering this trade-off, we estimated the CFU of *E. coli* culture using these three dilution series *viz* a 10-fold, a 100-fold and a 1000-fold dilution series as discussed below.

The CFU of bacterial suspension obtained from the three different dilution series (a 10-, 100-, and 1000-fold dilution series) is represented in Table 2a and a representative result

is shown in Fig. 3a. The 10 μL spotting of 10^6 fold dilution obtained from a 10-fold and a 100-fold dilution series produced average 11.9 ± 4.1 and 11.3 ± 4.5 numbers of colonies per spot respectively; however that obtained from a 1000-fold dilution series produced average 28.6 ± 1.8 numbers of colonies per spot. Similarly with 10^7 fold dilution average number of colonies per spot obtained from a 10- and 100-fold dilution series were 1.4 ± 1.0 and 1.1 ± 1.2 respectively; however a 1000-fold dilution series produced average 3.5 ± 1.4 number of colonies per spot. A p-value < 0.05 was indicative of significant differences among the groups, and thus a post-hoc test was followed in each case to identify the group being significantly different. This was observed to be the 1000-fold series in almost all of these cases. The results indicated that a 10-fold and 100-fold dilution series are producing similar CFU count whereas the 1000-fold dilution series is giving a higher CFU count than the other two. This trend was further observed in all the three repetitive experiments (Table 2a). In addition the CFU count by the 1000-fold dilution series was inconsistent. This was perplexing for us to find out if a 1000-fold dilution series is more accurate than the other two dilution series. As the results by 1000-fold dilution series were inconsistent, we thought pipetting a smaller volume like 1 μL might be a more complex procedure while taking in as well as dispensing out the suspension. To avoid any bias related to pipette, we repeated the experiments using pipettes from three neighboring labs for pipetting 1 μL volume. In this case also, an inconsistently higher CFU count was observed with the 1000-fold series. This led us to assume that 1 μL pipetting indeed is more complex and prone to inconsistencies than 10 μL and 100 μL pipetting. This might be because a minor deviation in either taking in and/or dispensing out the 1 μL suspension would contribute a larger percentage of change in the total volume.

To compare the accuracies of these dilution series after eliminating the complex factor of 1 μL pipetting, all the three series were re-performed this time to a final 10 mL volume by relaying stepwise 1 mL, 100 μL and 10 μL respectively in the 10-, 100- and 1000-fold dilution series. The CFU now obtained with each of these dilution series are represented in Table 2b and a representative result is shown in Fig. 3b. The 10 μL spotting of 10^6 fold dilution obtained from a 10-, 100- and 1000-fold dilution series produced average 13.7 ± 4.2, 14.7 ± 2.1 and 16.7 ± 3.9 numbers of colonies per spot respectively. Similarly with the 10^7 fold dilution, average number of colonies obtained from the 10-, 100- and 1000-fold dilution series were 1.6 ± 1.4, 2.6 ± 1.8 and 2.0 ± 0.9 respectively. This trend was further observed in all the three repetitive experiments. It was interesting to observe this time a similar number of

colonies with all the three dilution series. A p-value > 0.05 as obtained in one-way ANOVA was indicative of no significant differences between the colony numbers obtained from the different dilution series. This proves a 1000-fold dilution series to be equally accurate as the 10-fold and 100-fold dilution series provided that pipetting of 1 μL is not carried out.

Table 2(a): Average colony numbers (along with standard deviations) of *E. coli* DH5α obtained from different dilution series performed to a final 1 mL volume.

Dilutions adjusted to a total 1 mL volume (*E. coli* DH5α)							
Dilution factors		**10^6**			**10^7**		
Dilution series		**10 fold (A)**	**100 fold (B)**	**1000 fold (C)**	**10 fold (A)**	**100 fold (B)**	**1000 fold (C)**
Experiment 1	**Colony Count**	11.9 ± 4.1	11.3 ± 4.5	28.6 ± 1.8	1.4 ± 1.0	1.1 ± 1.2	3.5 ± 1.4
	One-way ANOVA	F (2, 27) = 71.64, p = 1.6 x 10^{-11}			F (2, 27) = 11.60, p = 2.3 x 10^{-4}		
	Bonferroni post-hoc test	(C) > (A), (B); (A) □ (B)			(C) > (A), (B); (A) □ (B)		
Experiment 2	**Colony Count**	9.5 ± 1.6	8.3 ± 3.4	28.8 ± 5.2	1.8 ± 1.2	1.8 ± 1.3	3.5 ± 2.0
	One-way ANOVA	F (2, 27) = 108.21, p = 1.3 x 10^{-13}			F (2, 27) = 4.08, p = 2.8 x 10^{-2}		
	Bonferroni post-hoc test	(C) > (A), (B); (A) □ (B)			(A) □ (B) □ (C)		
Experiment 3	**Colony Count**	22.5 ± 3.3	19.9 ± 3.5	45.4 ± 5.1	3.4 ± 1.8	2.9 ± 1.6	7.5 ± 2.0
	One-way ANOVA	F (2, 27) = 120.33, p = 3.5 x 10^{-14}			F (2, 27) = 19.15, p = 6.6 x 10^{-6}		
	Bonferroni post-hoc test	(C) > (A), (B); (A) □ (B)			(C) > (A), (B); (A) □ (B)		

$F_{statistic}$ and p-value calculated from One-way ANOVA and interpretation of Bonferroni post-hoc tests for the different comparable groups are represented.

Table 2(b): Average colony numbers (along with standard deviations) of *E. coli* DH5α obtained from different dilution series performed to a final 10 mL volume.

Dilutions adjusted to a total 10 mL volume (*E. coli* DH5α)							
Dilution factors		**10^6**			**10^7**		
Dilution series		**10 fold**	**100 fold**	**1000 fold**	**10 fold**	**100 fold**	**1000 fold**
Experiment 1	**Colony Count**	13.7 ± 4.2	14.7 ± 2.1	16.7 ± 3.9	1.6 ± 1.4	2.6 ± 1.8	2.0 ± 0.9
	One-way ANOVA	F (2, 27) = 1.87, p = 0.17			F (2, 27) = 1.20, p = 0.32		
Experiment 2	**Colony Count**	17.5 ± 5.0	18.3 ± 4.5	21.5 ± 2.7	1.7 ± 1.5	2.3 ± 1.2	1.6 ± 1.0
	One-way ANOVA	F (2, 27) = 2.57, p = 0.10			F (2, 27) = 0.95, p = 0.40		
Experiment 3	**Colony Count**	16.0 ± 3.9	17.6 ± 5.2	18.8 ± 2.3	1.8 ± 0.9	1.7 ± 1.2	2.0 ± 1.2
	One-way ANOVA	F (2, 27) = 1.23, p = 0.31			F (2, 27) = 0.20, p = 0.82		

$F_{statistic}$ and p-value calculated from One-way ANOVA for the different comparable groups are represented.

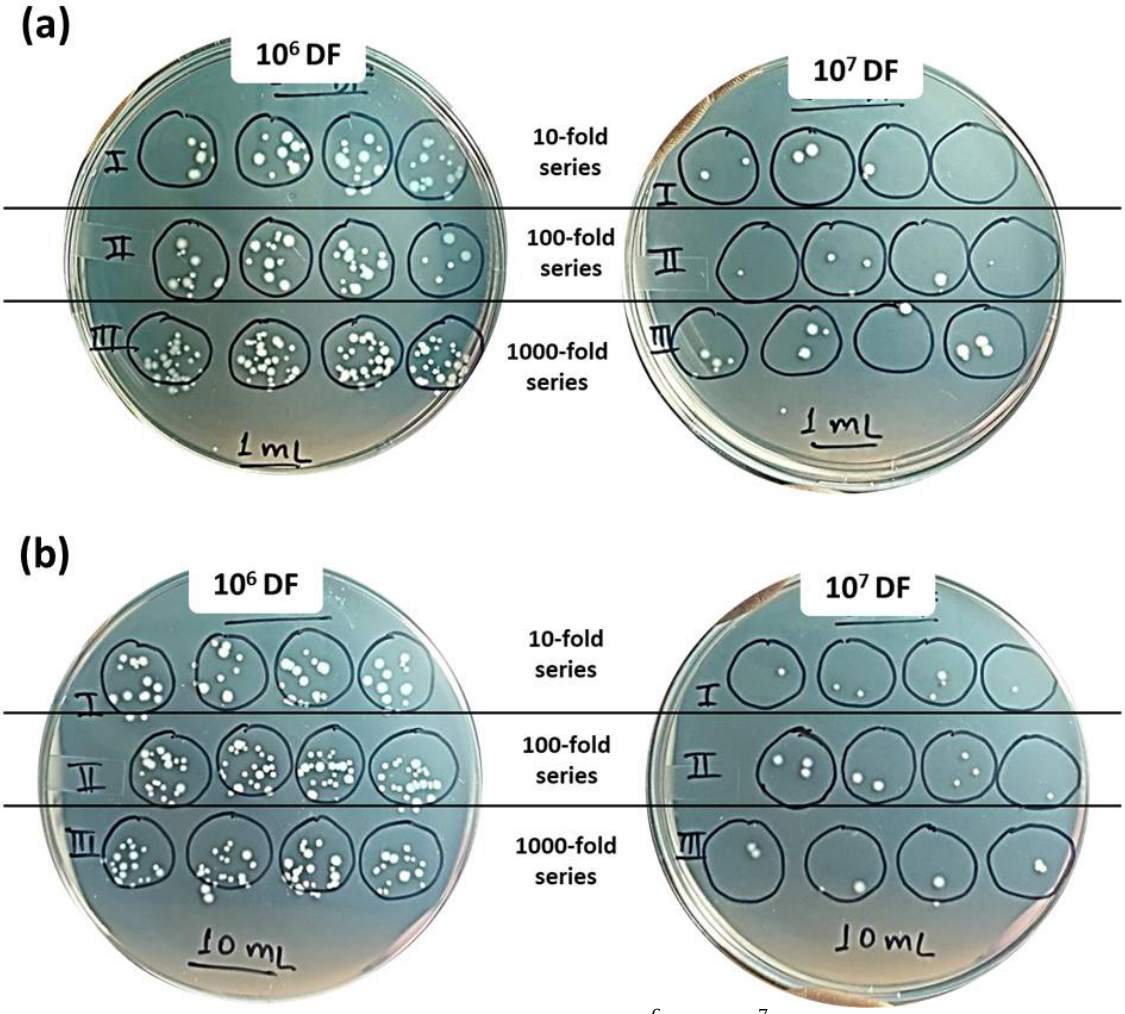

Fig. 3: *E. coli* colonies at selected fold dilutions (10^6 and 10^7) obtained with the different dilution series. These series were performed first to a final volume of (a) 1 mL and later to a final volume of (b) 10 mL from independent cultures. At 1 mL final volume, a clearly greater number of colonies were observed with the 1000-fold series; however, at 10 mL final volume, similar numbers of colonies were observed with all the three dilution series.

3.3 Stepwise relaying of 1 mL, 100 μL and 10 μL (but not 1 μL) are equally accurate for CFU estimation of R. pseudosolanacearum F1C1

We further performed this CFU estimation study for a different bacterium: *R. pseudosolanacearum* which in contrast to *E. coli*, forms mucoidal colonies on plate due to the production of exopolysaccharides. This study was undertaken to know whether the accuracies observed previously with the different dilution series holds true for the other bacterial suspension too. Unlike *E. coli*, a higher fold dilution (10^7 and 10^8) was chosen for plating this time as the mucoidal nature of the colonies and twitching motility exhibited by *R.*

pseudosolanacearum over solid media (Bhuyan *et al*., 2024b) leads towards a greater tendency for the colonies to merge among each other. Also the CFU/mL of the saturated culture of *R. pseudosolanacearum* was always observed to be higher than that of *E. coli* by 2-6 times (this can also be observed in Table1). So plating a higher fold dilution in this case will enable clear distinction of the colonies. When the dilutions were performed to a final volume of 1 mL, average number of colonies observed per 10 μL spot of 10^7 fold dilution were 6.0 ± 2.5, 5.4 ± 1.0 and 14.1 ± 3.5 respectively with a 10-, 100- and 1000-fold dilution series (Table 3a). Similarly with 10^8 fold dilution, average number of colonies per spot obtained from a 10-, 100-, and 1000-fold dilution series were 0.6 ± 0.8, 0.4 ± 0.7 and 2.5 ± 1.3 respectively. A p-value < 0.05 was indicative of significant differences among the groups, and thus a post-hoc test was followed in each case to identify the group being significantly different. This was observed to be the 1000-fold series in almost all of these cases. A representative result is shown in Fig. 4(a). Aligning with our previous observation, CFU recorded with the 1000-fold series were again observed to be significantly higher and were inconsistent than that recorded with the other two dilution series. This further highlight the potential complexities associated with 1 μL pipetting in the 1000-fold dilution series and so to eliminate it, the dilutions were re-performed to a final volume of 10 mL as done previously with *E. coli* suspension. In this case, average number of colonies observed per 10 μL spot of 10^7 fold dilution were 9.8 ± 1.9, 8.4 ± 4.3 and 7.2 ± 2.0 respectively with a 10-, 100- and 1000-fold dilution series (Table 3b). Similarly with 10^8 fold dilution, average number of colonies per spot obtained from a 10-, 100-, and 1000-fold dilution series were 0.3 ± 0.7, 0.5 ± 0.7 and 0.2 ± 0.4 respectively. It was interesting to note that the colony numbers observed were similar in this case and this trend was repetitive in all the three experiments. A representative result is shown in Fig. 4(b). A p-value > 0.05 as obtained in one-way ANOVA was indicative of no significant differences between the colony numbers obtained from the different dilution series. This was again in concordance with our previous observation of *E. coli* CFU estimation. This establishes 1000-fold dilution series to be equally accurate as the 10-fold and 100-fold dilution series when 1 μL pipetting is not carried out and validates our understanding on the inconsistencies and complexities associated with 1 μL pipetting. In summary, when performing the dilutions to a final volume of 1 mL, a 10- and 100-fold series proved to be effective for CFU estimation and when performing them to a final volume of 10 mL, all the 10-, 100- and 1000-fold dilution series proved to be equally effective for CFU estimation. But considering the time, cost and energy efficiency of the approach with no compromise on accuracy of CFU estimation, a 1000-fold dilution series shall be preferred

when performing the dilutions for 10 mL final volume and a 100-fold dilution series shall be preferred when performing the dilutions for 1 mL final volume. This is because a relatively lower number of steps are involved in these processes compared to the 10-fold series.

Table 3(a): Average colony numbers (along with standard deviations) of *R. pseudosolanacearum* F1C1 obtained from different dilution series performed to a final 1 mL volume.

***R. pseudosolanacearum* F1C1**							
Dilution factors		**10^7**			**10^8**		
Dilution series		**10 fold**	**100 fold**	**1000 fold**	**10 fold**	**100 fold**	**1000 fold**
Experiment 1	**Colony Count**	6.0 ± 2.5	5.4 ± 1.0	14.1 ± 3.5	0.6 ± 0.8	0.4 ± 0.7	2.5 ± 1.3
	One-way ANOVA	F (2, 27) = 35.95, p = 2.4 x 10^{-8}			F (2, 27) = 14.34, p = 5.7 x 10^{-5}		
	Bonferroni post-hoc test	(C) > (A), (B); (A) □ (B)			(C) > (A), (B); (A) □ (B)		
Experiment 2	**Colony Count**	7.1 ± 2.5	4.9 ± 1.4	21.9 ± 3.3	0.8 ± 0.6	0.2 ± 0.4	2.0 ± 1.6
	One-way ANOVA	F (2, 27) = 135.21, p = 8.6 x 10^{-15}			F (2, 27) = 8.34, p = 1.5 x 10^{-3}		
	Bonferroni post-hoc test	(C) > (A), (B); (A) □ (B)			(C) □ (A); (A) □ (B); (C) > (B)		
Experiment 3	**Colony Count**	3.7 ± 2.1	1.8 ± 0.9	11.9 ± 3.3	0.6 ± 0.7	0.3 ± 0.5	1.7 ± 0.9
	One-way ANOVA	F (2, 27) = 53.79, p = 3.8 x 10^{-10}			F (2, 27) = 10.05, p = 5.5 x 10^{-4}		
	Bonferroni post-hoc test	(C) > (A), (B); (A) □ (B)			(C) > (A), (B); (A) □ (B)		

$F_{statistic}$ and p-value calculated from One-way ANOVA and interpretation of Bonferroni post-hoc tests for the different comparable groups are represented.

Table 3(b): Average colony numbers (along with standard deviations) of *R. pseudosolanacearum* F1C1 obtained from different dilution series performed to a final 10 mL volume.

***R. pseudosolanacearum* F1C1**							
Dilution factors		**10^7**			**10^8**		
Dilution series		**10 fold**	**100 fold**	**1000 fold**	**10 fold**	**100 fold**	**1000 fold**
Experiment 1	**Colony Count**	9.8 ± 1.9	8.4 ± 4.3	7.2 ± 2.0	0.3 ± 0.7	0.5 ± 0.7	0.2 ± 0.4
	One-way ANOVA	F (2, 27) = 1.97, p = 0.16			F (2, 27) = 0.62, p = 0.55		
Experiment 2	**Colony Count**	4.3 ± 1.7	4.8 ± 1.8	4.1 ± 1.3	0.5 ± 0.7	0.5 ± 0.7	0.3 ± 0.5
	One-way ANOVA	F (2, 27) = 0.51, p = 0.61			F (2, 27) = 0.32, p = 0.73		
Experiment 3	**Colony Count**	3.3 ± 2.5	3.2 ± 1.7	3.9 ± 1.5	0.5 ± 0.8	0.3 ± 0.5	0.5 ± 0.5
	One-way ANOVA	F (2, 27) = 0.37, p = 0.70			F (2, 27) = 0.32, p = 0.73		

$F_{statistic}$ and p-value calculated from One-way ANOVA for the different comparable groups are represented.

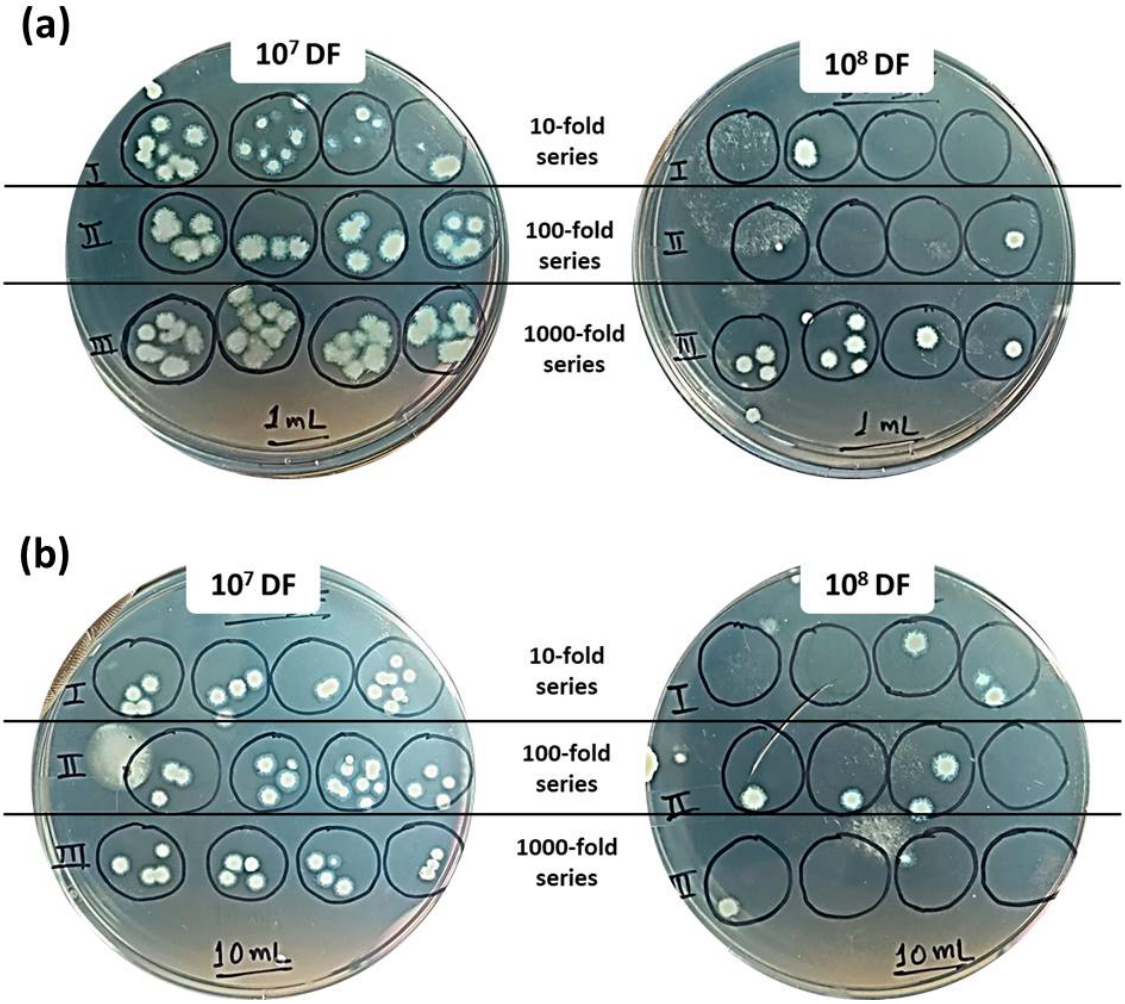

Fig. 4: *R. pseudosolanacearum* colonies at selected fold dilutions (10^7 and 10^8) obtained with the different dilution series. These series were performed first to a final volume of (a) 1 mL and later to a final volume of (b) 10 mL from independent cultures. At 1 mL final volume, a clearly greater number of colonies were observed with the 1000-fold series; however, at 10 mL final volume, similar numbers of colonies were observed with all the three dilution series.

3.4 CFU estimation is indifferent to the diluent volume used in serial dilution

To observe if diluent volume used in serial dilution has an impact on the CFU estimation of bacteria, each of the 10-, 100- and 1000-fold dilution series were performed from a saturated *E. coli* culture using different diluent volumes to reach the final volume of 1 mL and 10 mL respectively. CFU observed with the plating of 10^6 and 10^7 fold dilutions obtained from each of the three dilution series adjusted to the different final volumes are represented in Table 4 and a representative result is shown in Fig. 5. With 10^6 fold dilution, a

10-fold series yielded 21.9 ± 3.0 and 18.1 ± 3.4, a 100-fold series yielded 19.7 ± 3.3 and 19.1 ± 3.4, and a 1000-fold series yielded 46.9 ± 5.3 and 19.9 ± 2.6 average numbers of colonies for the final 1 mL and 10 mL dilution volumes respectively. Similarly, with 10^7 fold dilution, a 10-fold series yielded 2.6 ± 1.1 and 2.4 ± 1.3, a 100-fold series yielded 2.5 ± 1.3 and 1.9 ± 1.0, and a 1000-fold series yielded 5.6 ± 2.1 and 1.9 ± 1.3 average numbers of colonies for the final 1 mL and 10 mL dilution volumes respectively. Clearly, the colony numbers observed with the 1 mL and 10 mL dilution volumes were similar for most of the times for a 10-fold and 100-fold series; however they were greatly different in all the cases for the 1000-fold series. This suggests that the two diluent volumes used for dilution brought no contrasting difference in the bacterial density being relayed in the 10-fold and 100-fold dilution series. However, the relaying in 1000-fold series was contrastingly different in the two diluent volumes used. Here, the relaying of 1 μL (in final 1 mL dilution volume) produced significantly higher number of colonies than the relaying of 10 μL (in final 10 mL dilution volume); the latter yielding comparable colonies to that of the 10-fold and 100-fold series performed in either diluent volumes. It correlates to our previous understanding of the complexities associated with 1 μL pipetting as in case of the 1000-fold series performed to a final 1 mL volume. Overall, this suggests that the diluent volume used for dilution doesn't affect the accuracy of CFU estimation, given that pipetting of 1 μL is not being practiced.

Table 4: Comparison of the colony numbers (along with standard deviations) of *E. coli* obtained from each of the dilution series adjusted to the final volume of 1 mL and 10 mL, generated from same culture.

Average colonies per spot												
Dilution factors	**10^6**						**10^7**					
Dilution series	**10 fold**		**100 fold**		**1000 fold**		**10 fold**		**100 fold**		**1000 fold**	
Final volume of dilutions	**1 mL**	**10 mL**	**1 mL**	**10 mL**	**1 mL**	**10 mL**	**1 mL**	**10 mL**	**1 mL**	**10 mL**	**1 mL**	**10 mL**
Experiment 1	21.9* ± 3.0	18.1* ± 3.4	19.7 ± 3.3	19.1 ± 3.4	46.9* ± 5.3	19.9* ± 2.6	2.6 ± 1.1	2.4 ± 1.3	2.5 ± 1.3	1.9 ± 1.0	5.6* ± 2.1	1.9* ± 1.3

Experiment 2	21.8 ± 5.1	20.6 ± 4.1	28.3* ± 5.4	20.5* ± 4.6	62.7* ± 8.9	27.7* ± 2.8	2.4 ± 1.9	2.6 ± 1.7	2.7 ± 2.2	2.0 ± 1.3	6.6* ± 2.4	2.8* ± 1.7
Experiment 3	27.6 ± 3.0	26.3 ± 3.9	36.5 ± 5.1	33.5 ± 4.4	86.9* ± 7.4	36.8* ± 7.9	3.0 ± 1.2	2.9 ± 1.3	5.7* ± 3.0	3.2* ± 1.1	15.7* ± 3.4	4.3* ± 1.3

Asterisk (*) denotes a significant difference ($U_{statistic} < U_{critical}$) between the colony numbers obtained with dilutions for 1 mL and 10 mL volumes, as calculated from a two-tailed Mann-Whitney U-Test at 95% confidence interval.

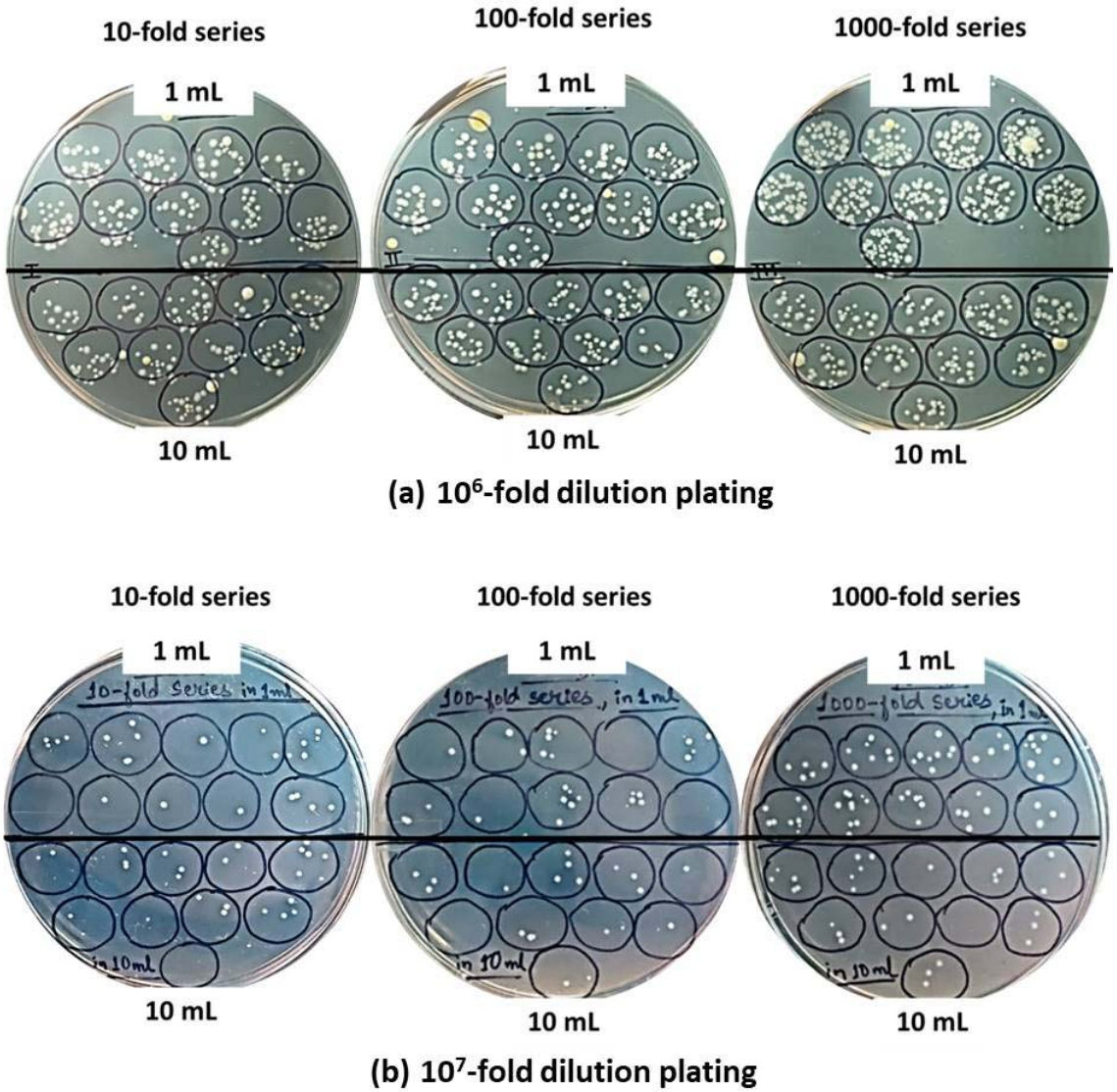

Fig. 5: Comparison of colony numbers of *E. coli* generated from same culture but attained using two different diluent volumes of serial dilution in order to adjust to 1 mL and 10 mL volumes. Plating at (a) 10^6- and (b) 10^7-fold dilution was done. Each petri-plate corresponds

to a specific dilution series where comparison is made between upper and lower halves containing colonies from 1 mL and 10 mL final dilution volumes respectively. In each of the case, colony numbers observed with 1 mL and 10 mL final volume of dilutions were similar for 10-fold and 100-fold series. However a contrasting difference was observed with 1000-fold series, where a clearly greater number of colonies were observed in the case of 1 mL final volume of dilution.

4. Discussion

In serial dilution process to reach a certain dilution fold, different specific suspension volumes can be relayed stepwise. Considering the random distribution of bacteria, a higher volume of cell suspension is expected to better contain the randomness of population and give more consistency in cell density being relayed in each step. But relaying a higher suspension volume leads to a lower fold dilution in a single step, thereby requiring more steps to reach the desired dilution fold. This increases the complexity of the process. Alternately, relaying of a lower suspension volume leads to a higher fold dilution in a single step and requires fewer steps to reach the desired dilution fold. This reduces the complexity of the process. So, although relaying of a higher suspension volume seems advantageous considering the random distribution of bacteria but is disadvantageous considering the complexity of the process and alternately, relaying of a lower suspension volume seems disadvantageous considering the random distribution of bacteria but is advantageous considering the complexity of the process. In order to know whether relaying a higher or lower volume of cell suspension provides accuracy in CFU estimation of bacteria, a critical examination of 10-, 100- and 1000-fold dilution series was carried out in the current study. When performed to a final volume of 1 mL, a 10-, 100- and 1000-fold dilution series requires stepwise relaying of 100 μL, 10 μL and 1 μL suspension volumes respectively. When performed to a final volume of 10 mL, a 10-, 100- and 1000-fold dilution series requires stepwise relaying of 1 mL, 100 μL and 10 μL suspension volumes respectively. The studies were performed with two different bacterial species: *E. coli* DH5α and *R. pseudosolanacearum* F1C1, exhibiting two different colony morphologies, with colonies of the former one being non-mucoidal, having distinct boundaries and that of the latter being mucoidal, having fluidal morphology and a greater tendency to merge with other such colonies. The study revealed that all the three dilution series are equally accurate in terms of CFU estimation and in practice all of them are reliable. However, it is recommended to use higher diluent volume to perform a higher fold dilution. Example, in our study, a 1000-fold

dilution series proved good when performed to a final 10 mL volume, but inappropriate when performed to the final 1 mL volume. The inaccuracies observed in this case were perhaps attributed to relaying a small sample volume, *i.e.*, 1 μL, which is replaced by 10 μL upon scaling up the final volume 10-times. 1 μL relaying was found to be associated with significantly higher and inconsistent colony count compared to relaying 100 μL and 10 μL. It might be because 1 μL requires a more sophisticated handling. Furthermore, equal accuracies observed with the three dilution series after eliminating the factor of 1 μL pipetting might be because the advantage of a greater pipetted volume in 10-fold series was balanced by the disadvantage of a more complexity; similarly the advantage of a lesser complexity in 1000-fold series was balanced by the disadvantage of a smaller pipetted volume; and the 100-fold series being moderate in both respect. But considering the time, cost and energy efficiency of the approach with no compromise on accuracy of CFU estimation, a 1000-fold dilution series shall be preferred when performing the dilutions for 10 mL final volume and a 100-fold dilution series shall be preferred when performing the dilutions for 1 mL final volume. This is because a relatively lower number of steps are involved in these processes compared to the 10-fold series. The study was carried forward to understand if diluent volumes used for serial dilution affects bacterial CFU estimate. For this, each of the 10-, 100- and 1000-fold dilution series were performed using two different diluent volumes to maintain the final volume of 1 mL and 10 mL respectively. Results revealed that the diluent volume used for dilutions doesn't affect CFU estimation. It is only the relaying of a small suspension volume (such as 1 μL) that bring in-congruencies and yield a higher than expected CFU count.

Besides highlighting the accuracies and complexities of various dilution series to make a conclusive comment on the practice that shall be followed and the ones that shall be avoided and understanding the impact of diluent volume on CFU estimation, our study offers a clear demonstration on the random distribution pattern of bacteria. Had it been uniform, all the sampling volumes would have been equally consistent in terms of colony count. Whereas, the findings of our study that indicates that a higher sample volume gives more consistent bacterial count, is a clear indicative of randomness prevalent in the population that can't be contained in a smaller sample volume. On this note, more the sampling size required for bringing consistency in colony count, more the degree of randomness prevalent in the bacterial distribution. Our study serves a good and easy model (in terms of reproducibility and cost efficiency) for students who want to observe the random nature of bacterial distribution. The plus point here is that a greater number of replicates (10 or even more) can

be obtained in just a single solid agar plate, and a greater number of sampling is always better for addressing the randomness prevalent in the population. Many-a-times, researchers performing microbiological experiments find out the average number of colonies in a sampling population from triplicates, generally obtained from spread-plate method, which requires three separate solid agar plates. Even sometimes, due to less understanding of the mathematics of bacterial distribution, students performing the experiment consider singular plating for estimating cell density. The randomness in bacterial distribution can't be contained in just a single replicate. Hence, taking one replicate or three proves insufficient for getting precise cell density estimate of bacteria. In this context, our study by virtue of a greater number of sampling in a single plate becomes advantageous for depicting the randomness if any is prevalent in a population. Further, the precision of pipettes or the sample-adherence ability of tips are some of the additional aspects that could be examined by this reproducible study.

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Conflict of interest

The authors declare no conflict of interest in the subject matter discussed in this manuscript.

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Figure Legends

Fig. 1: An illustration of the 10-, 100- and 1000-fold dilution series performed for 1 mL final volume. Bacterial culture is shown in yellow in the screw caped bottle. Diluent (saline) is shown in blue in micro-centrifuge tubes. Three different ways of attaining a 10^6 fold dilution has been shown *viz* a 10-, 100- and 1000-fold dilution series. (i) A 10-fold series involves relaying 100 μL suspension to 900 μL saline in six successive dilutions. (ii) A 100-fold series involves relaying 10 μL suspension to 990 μL saline in three successive dilutions. (iii) A 1000-fold series involves relaying 1 μL suspension to 999 μL saline in two successive dilutions.

Fig. 2: Correlation between suspension volume spotted (X- axis) and coefficient of variation (CoV) obtained among the colony numbers (Y-axis) of (a) *E. coli* and (b) *R. pseudosolanacearum*. Pearson's correlation coefficient (r) is represented for each case. A strong negative correlation has been observed in each case.

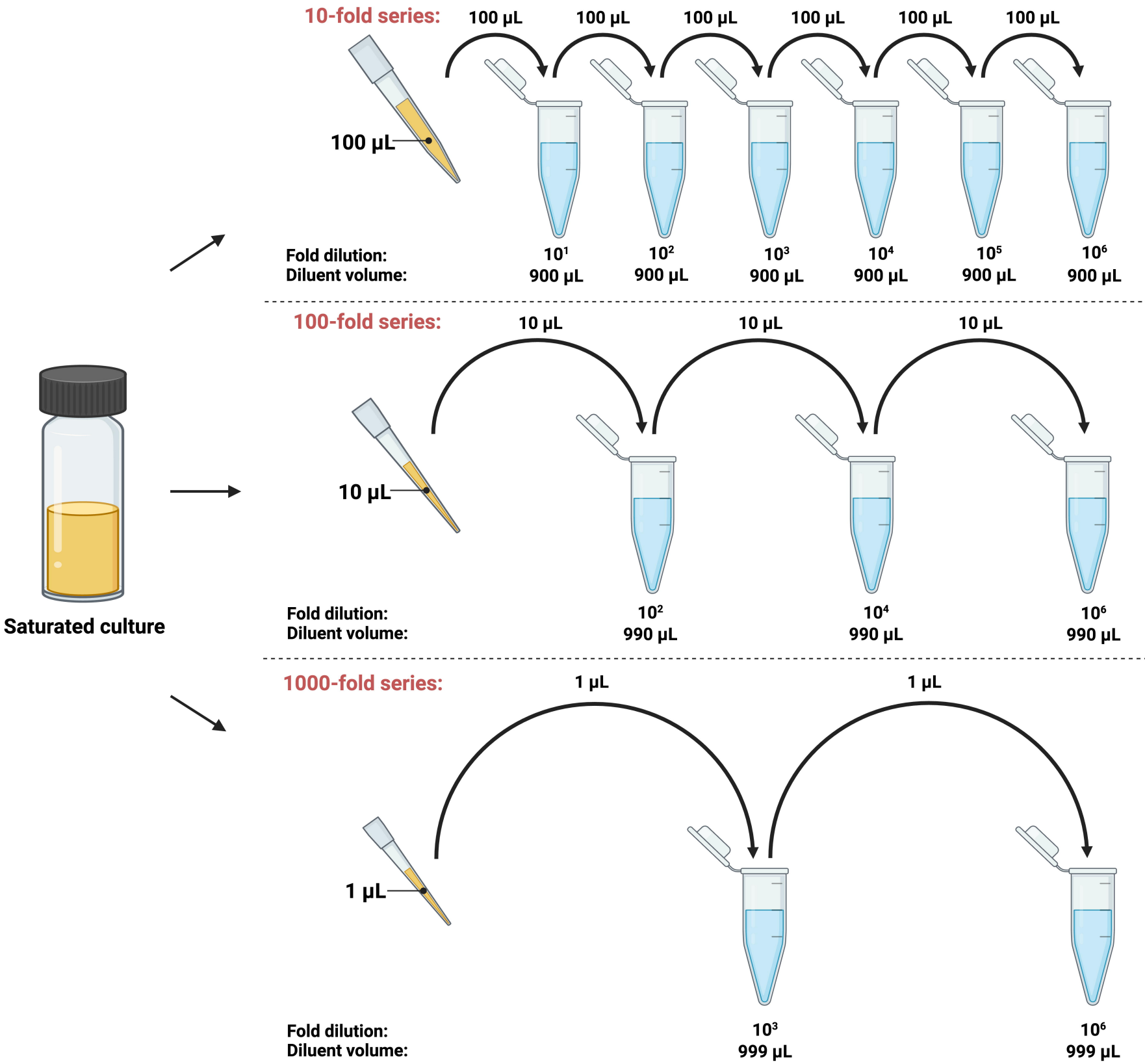
Fig. 3: *E. coli* colonies at selected fold dilutions (10^6 and 10^7) obtained with the different dilution series. These series were performed first to a final volume of (a) 1 mL and later to a final volume of (b) 10 mL from independent cultures. At 1 mL final volume, a clearly greater number of colonies were observed with the 1000-fold series; however, at 10 mL final volume, similar numbers of colonies were observed with all the three dilution series.

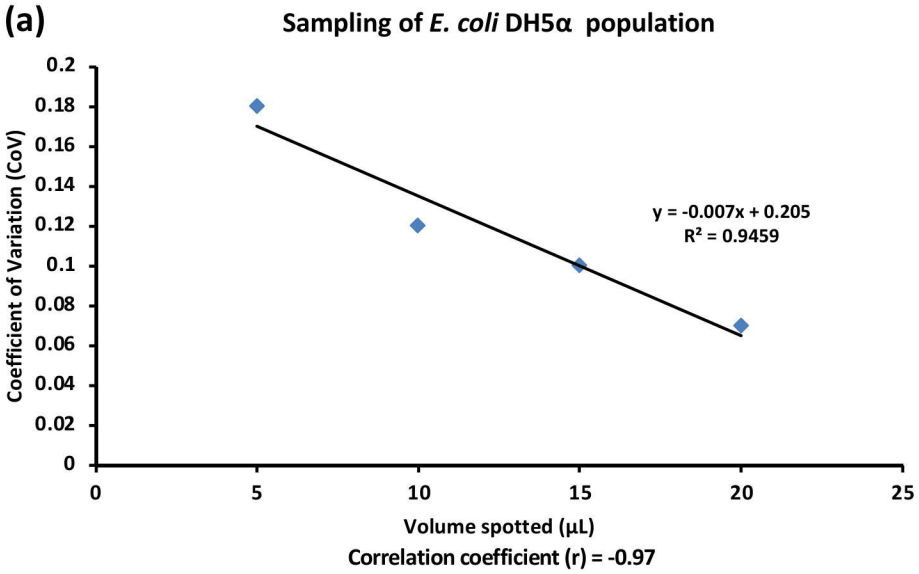
Fig. 4: *R. pseudosolanacearum* colonies at selected fold dilutions (10^7 and 10^8) obtained with the different dilution series. These series were performed first to a final volume of (a) 1 mL and later to a final volume of (b) 10 mL from independent cultures. At 1 mL final volume, a

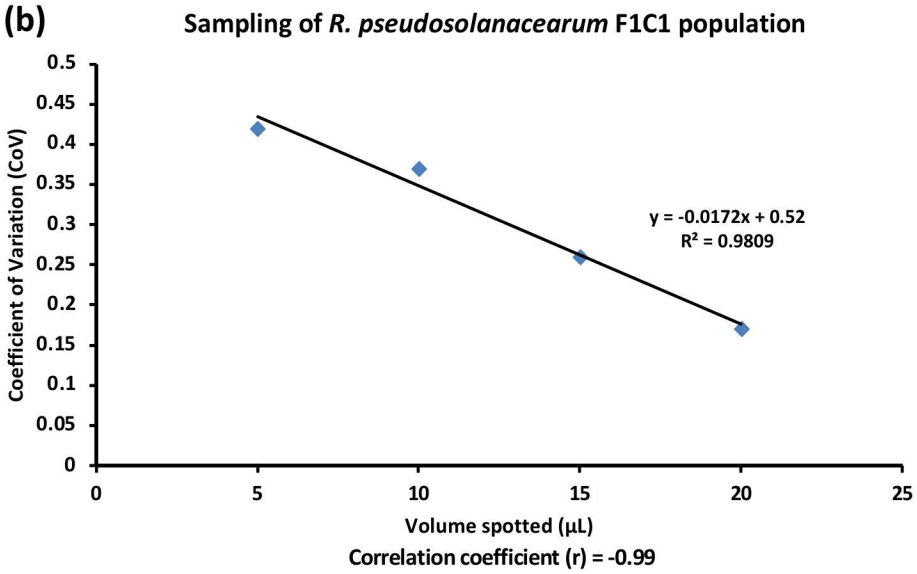
clearly greater number of colonies were observed with the 1000-fold series; however, at 10 mL final volume, similar numbers of colonies were observed with all the three dilution series.

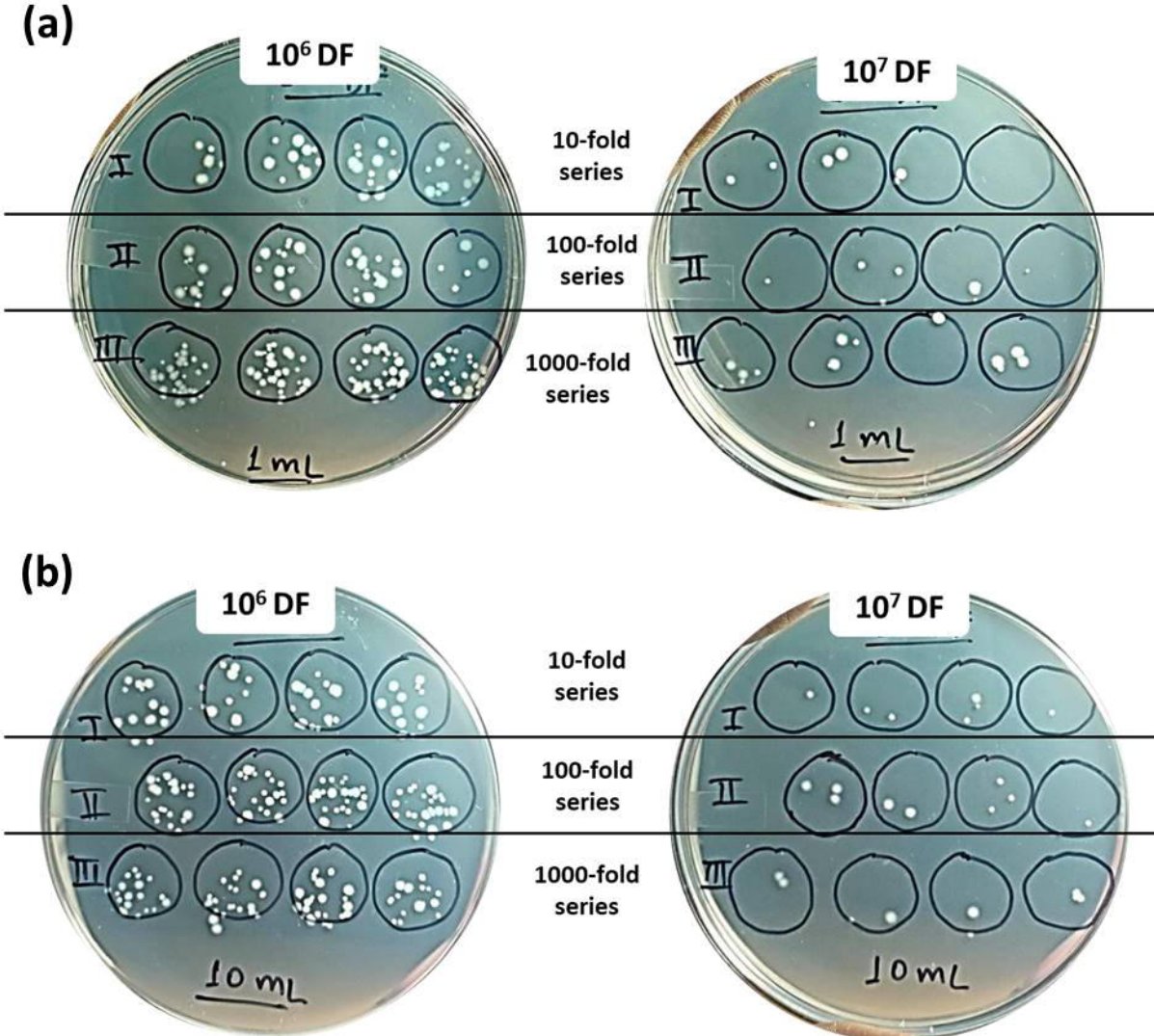
Fig. 5: Comparison of colony numbers of *E. coli* generated from same culture but attained using two different diluent volumes of serial dilution in order to adjust to 1 mL and 10 mL volumes. Plating at (a) 10^6- and (b) 10^7-fold dilution was done. Each petri-plate corresponds to a specific dilution series where comparison is made between upper and lower halves containing colonies from 1 mL and 10 mL final dilution volumes respectively. In each of the case, colony numbers observed with 1 mL and 10 mL final volume of dilutions were similar for 10-fold and 100-fold series. However a contrasting difference was observed with 1000-fold series, where a clearly greater number of colonies were observed in the case of 1 mL final volume of dilution.

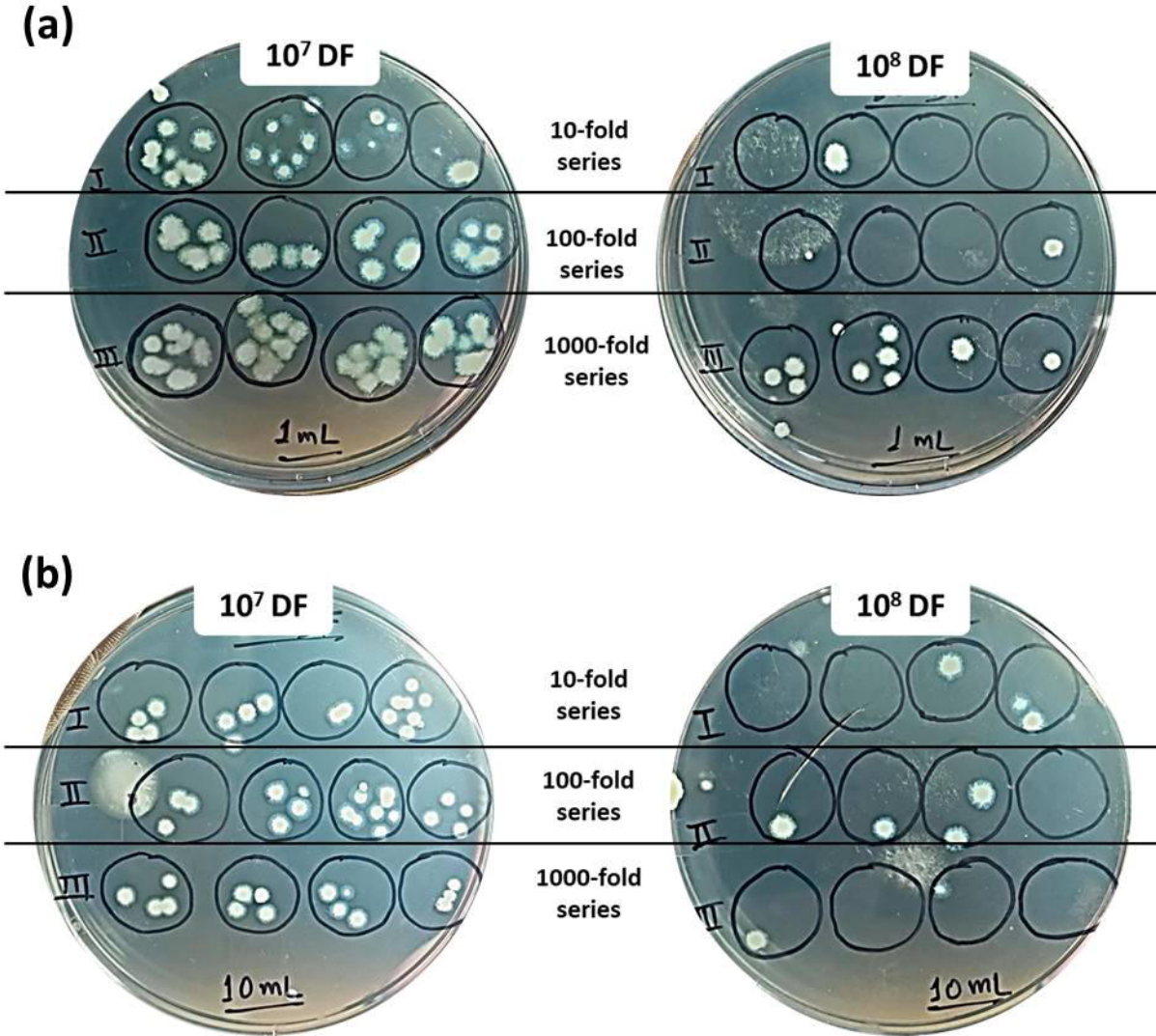
Dilutions adjusted to 1 mL

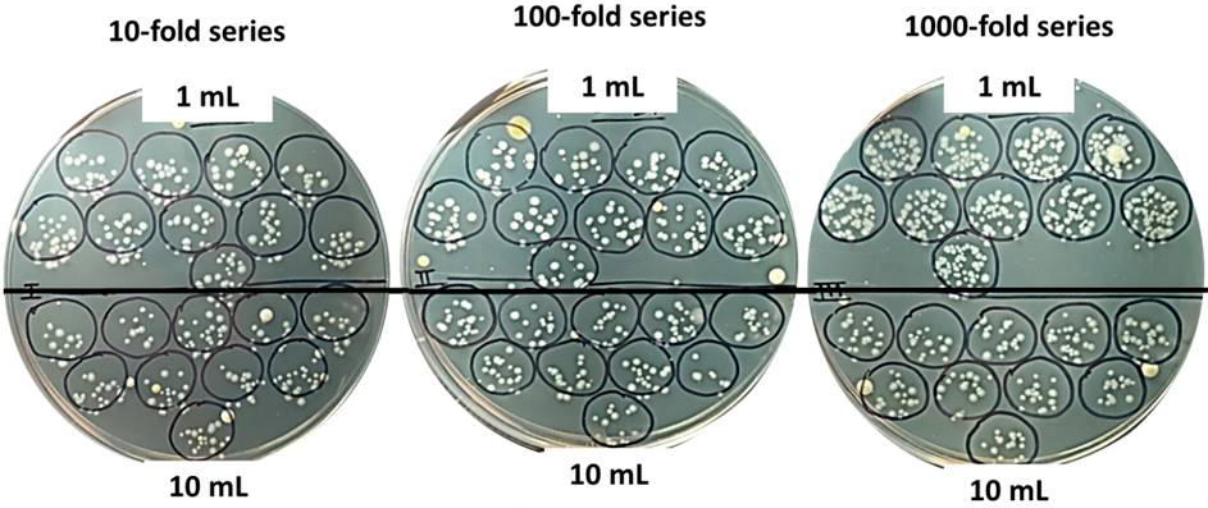



(a)
Sampling of E. coli DH5α population
Coefficient of Variation (CoV)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
20
25
y = -0.007x + 0.205
R² = 0.9459
Volume spotted (µL)
Correlation coefficient (r) = -0.97

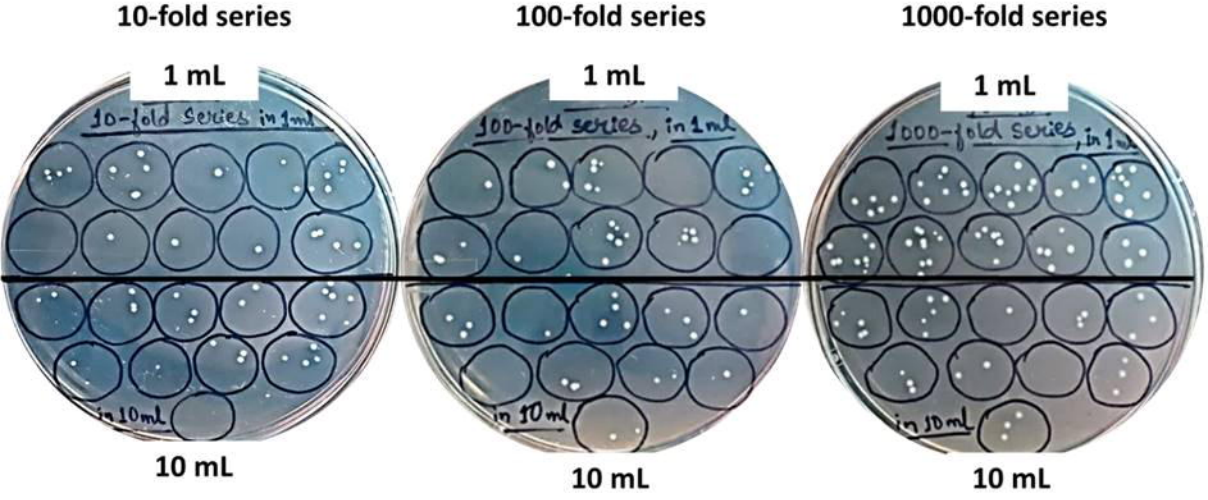

(b)
Sampling of R. pseudosolanacearum F1C1 population
Coefficient of Variation (CoV)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
y = -0.0172x + 0.52
R² = 0.9809
Volume spotted (µL)
Correlation coefficient (r) = -0.99


(a)
10^6 DF
10^7 DF
10-fold series
100-fold series
1000-fold series
I
II
III
1 mL
1 mL
(b)
10^6 DF
10^7 DF
10-fold series
100-fold series
1000-fold series
I
II
III
10 mL
10 mL


(a)
10^7 DF
10^8 DF
10-fold series
100-fold series
1000-fold series
I
II
III
1 mL
1 mL
(b)
10^7 DF
10^8 DF
10-fold series
100-fold series
1000-fold series
I
II
III
10 mL
10 mL



(a) 10^6-fold dilution plating



(b) 10^7-fold dilution plating