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Modelling of Bacterial Growth with Shifts in Temperature Using Automated Methods with *Listeria monocytogenes* and *Pseudomonas aeruginosa* as examples

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Abstract

Time to detection (TTD) measurements using turbidometry allow a facile method for the measurement of bacterial growth rates under isothermal conditions. Growth rate measurements were carried out for *Listeria monocytogenes* at 25, 30 and 37^{o}C and for *Pseudomonas aeruginosa* over the temperature range 25 to 45^{o}C. No lags were observed with any of the isothermal studies. The classical 3 parameter logistic model was rearranged to provide the theoretical foundation for the observed TTD. A model was subsequently developed for the analysis of TTD data from non-isothermal studies based on the Malthusian approximation of the logistic model. The model was able to predict the TTD for cultures of *L. monocytogenes* or *P. aeruginosa* undergoing simple temperature shunts (e.g. 25 to 37^{o}C and vice versa), and for a multiple temperature shunt for L. monocytogenes (25-37-25-37^{o}C and 37-25-37-25^{o}C) over a period of 24 hours. In no case did a temperature shunt induce a lag.

1 Introduction

The measurement of microbial growth rates, especially its temperature dependency, is of fundamental importance in food microbiology. For many food pathogens growth above 25 and below 45^{o}C is usually rapid with an optimum around 37^{o}C. Below 5 degrees only a few (often spoilage, e.g. *Pseudomonas aeruginosa*, but pathogens are also represented e.g. *Listeria monocytogenes*) have growth rates that would give rise to concern. This has been shown, for example, by Thomas and O'Bierne (2000) on the temperature abuse of vegetables with respect to spoilage (lactic acid bacteria) and risk (*L. monocytogenes*).

Within the literature several studies have looked at the effect of non-isothermal conditions on microbial growth using established modeling methods (e.g. Baranyi *et al*., 1995; Bovill *et al*., 2000; Dalgaard *et al*., 2002; Giannakourou *et al*., 2005; Koutsoumanis, 2001; Koutsoumanis *et al*., 2006; Li and Torres, 1993; Taoukis *et al*., 1999; Zwietering *et al*., 1994). The aim of many of these studies was to test the ability of using models based on growth data obtained isothermally to predict growth under non-isothermal conditions. Zwietering *et al*., (1994) concluded that, within the exponential phase, the hypothesis of no lag occurrence was accepted statistically in more than 70% of their experiments for *Lactobacillus plantarum*, however within the lag phase, the hypothesis of additional lag occurrence was accepted statistically in more than 90% of their experiments.

Corradini and Peleg (2005) have eloquently questioned the reasoning and conclusions being drawn from the use of the empirical standard primary and secondary models used to interpret and predict data from isothermal and fluctuating temperature studies. They suggest abandoning specific formats and using, instead, a generalized scheme for both primary and secondary modeling, "in the absence of a decisively superior theoretical model… [ad hoc empirical models] have the advantage of being simpler mathematically and free of assumptions that require independent verification".

Automated techniques such as turbidometry tend to come under fire from traditional microbiologists since they cannot directly reproduce the standard microbial growth curve, which the multitude of primary models are fitted to (Augustin *et al.* 1999; Dalgaard *et al.* 1994; McClure *et al.* 1993), yet their very persistence reflects their ease of use, the high quantity and quality of the data obtained and the large savings in consumable costs over that of the traditional (plate-count) methods. We have recently shown that the modified Gompertz and modified logistic models are at odds with the observed time to detection data obtained using turbidometry (Mytilinaios *et al.* 2011). The classic logistic model (and by default the Baranyi equation) were the only models used able to reconstruct the observed TTD data. The three parameter model has a firm (if simple) theoretical foundation. Its application to standard microbiological data results in mismatch due to methodological inadequacies (plate counting) and the presence of lag and so is rarely used in its original form. Herein we further examine the application of the basic logistic model to microbial growth data (obtained as TTD) and use small temperature shifts (or shunts) to examine their effect on the growth rates of *Listeria monocytogenes* and *Pseudomonas aeruginosa*.

2 Methods

2.1 CULTURE PREPARATION:

Pseudomonas aeruginosa (ATCC 15442) or *Listeria monocytogenes* (L-252, an industrial isolate provided by Nestlé Research Centre, Lausanne– obtained from contaminated terrine) was grown overnight in conical flasks containing 80 ml Tryptone Soya Broth, TSB (Oxoid CM 129) shaking continuously at 30°C. The cells were harvested, centrifuged (510g, 10 minutes) and the resulting cell pellets resuspended in 2 ml TSB. The inoculum was standardised by diluting to an approximate OD=0.5 at 600 nm giving approximately $2x10^9$ cfu/ml. This standardised culture was subject to either ten decimal or ten half-fold dilutions in TSB.

2.2 PREPARATION OF MICRO-ARRAY PLATES

Each well in the Bioscreen micro-array plates was filled as follow: all wells except column 10 received 200µl of growth broth (TSB). The wells of column 10 were given 400µl of the appropriate serial dilutions (decimal or half fold), with the highest inoculum (the zero dilution) in well 100. Using a multi-pipette, 200µl were removed from each well of column 10 and transferred into the wells of column 9, mixed by repeated syringing, and then 200µl were removed (using new tips) from the wells of column 9 and transferred to column 8 etc. This was repeated across the plate discarding 200µl after the final mixing in column 1.

From the -5 and -6 decimal dilutions, 0.1 ml of each was transferred and spread onto previously prepared tryptone soya agar (TSA) plates in triplicate and incubated at 30˚C for 2 days. Plates with <300 cfu were counted and the approximate log number of the initial (zero dilution) culture calculated.

For the study of the growth rates, identical plates were placed in different Bioscreens set at particular temperatures. After a given time of incubation the plates were swapped between the machines, without changing the running of the machines. Typical experiments lasted 1 to 2 days. The optical density of the wells was read at 600nm every ten minutes.

One particular effect was noted with Bioscreen data when the plates were removed from a higher incubation temperature to a lower (but not vice-versa); a kink in the OD/time plot due to the temporary presence of condensation on the underneath of the lid of the Bioscreen plates. In general the condensation took between 30 to 50 minutes to evaporate. Thus, TTD which met the OD criterion were censored during the 30 to 50 minutes after the transfer.

2.3 DATA ANALYSIS

From the resulting Bioscreen OD/time data, the background OD due to the media was removed from each. A time to detection (TTD) criterion of OD = 0.2 was then used on the background corrected data: TTD were found using linear interpolation between OD/time values which straddled the OD = 0.2 value.

2.4 MODEL DEVELOPMENT

Theoretical Background: From the classical logistic equation

$$N = \frac{M}{1 + \left(\frac{M}{N_0} - 1\right) e^{-\mu t}} \qquad (1)$$

where μ is the specific growth rate and M is the maximum population density (also known as the carrying capacity, cfu/ml), the time taken (TTD_N) to reach a specific population level (N) from a given initial value (N_0, cfu/ml) is given by

$$TTD_N = -\frac{1}{\mu}\ln N_0 + \frac{1}{\mu}\ln\left(\frac{N(M-N_0)}{M-N}\right) \quad (2)$$

The TTD_N is defined as the time to reach a given detection threshold (e.g. an optical density of 0.2) for which N_d is the equivalent microbial numbers per ml. If the assumption that $M>>N_0$ is made then this can be approximated by

$$TTD = -\frac{1}{\mu}\ln N_0 + \frac{1}{\mu}\left\{\frac{N_d M}{M-N_d}\right\} \quad (3)$$

When N_0 =1, the TTD is given by the right hand expression of eq.3, if $M>>N_d$ then this can be approximated by N_d/μ. Hence, a plot of the initial inoculum against the TTD will give a gradient equal to the negative reciprocal of the growth rate, the TTD intercept at N_0 = 1, is the time taken for one organism to reach the TTD criterion. This expression can be considered as the basis of the methodology of Cuppers and Smelt (1993) described below. In the presence of a lag equation 2 can be supplemented with a lag term, else the Baranyi equation can be used, but the required parameters have to be found using a more involved numerical technique.

Geometric or Malthusian model

For a given set of environmental conditions a plot of the log of the initial inoculum size against the time to detection (TTD) gives a straight line relationship with gradient equal to the reciprocal of the specific growth rate (Cuppers and Smelt 1993). In the absence of a lag the line will intersect the log initial inoculum axis at the detection value for the given OD criterion used.

If two microtitre plates each containing identically prepared multiple inocula are incubated at different temperatures then each inoculum will grow at a rate dictated by the temperature and media conditions. The difference between the two plates will be governed only by the differential effect of the temperature. For each initial inoculum the time to detection can be given by

$$TTD = m(\log I - \log I_D) \quad (4)$$

Where log I is the log of the initial inoculum used, log I_D is the log of the detection number and m is the gradient of the TTD, log *I* plot.

We hypothesise that if at time t_i the incubation temperature is changed then, whether in the absence or not of an induced lag, the growth rate will increase or decrease to meet the demands of the new incubation temperature. In the absence of any induced lag Figure 1 shows the expected result on the time to detection/log initial inoculum plot. Incubation at temperature T_0 gives an observed gradient of M_0; at time t_i, the temperature is changed to T_1 (where, in this case, T_0 is more optimal for growth than T_1), the gradient changes to M_1, commensurate with the new incubation temperature.

From simple geometrical arguments it can be shown that the TTD for multiple changes of temperature is given by equation 5.

For time: $t_i < t < t_{i+1}$, for i= 0,1,2,.., where $t_0 = 0$,

——— (5)

For example,

For i = 0

For i = 1, —

For i = 2, —— ——

If lags are induced at t_i, then the expression for t_i can be replaced by $t_i = t_i + \lambda_i$. For a given experiment the values of t_i are fixed.

Since the models are simple linear models, the majority of the modelling was carried out using Excel (Microsoft) and the data analysis add-in package.

3 Results

3.1 ISO-THERMAL STUDIES

A plot of the optical density/time curves for multiple inocula of *Listeria monocytogenes 252* (from 1.2 x10^9 cfu ml^{-1} to 1x10^4 cfu ml^{-1}) incubated at 25°C is shown in Figure 2 (without background correction carried out). From analyses of the curves for inocula which had initial OD greater than the background it was evident that there was no lag present. A plot of the time to detection defined as the time to reach an OD = 0.2 against the log of the initial inoculum gave a straight line plot (Figure 3a). The gradient of the fitted line is the reciprocal of the growth rate, and the intercept on the TTD axis is the time taken for one organism to reach the detection criterion. For zero lag the line cuts the log initial inoculum axis at the log inoculum size equivalent to the optical density of the TTD criterion. The microbial density obtained from direct plating of an OD = 0.2 at 600nm in the Bioscreen was equivalent to 9.09 $\log_{10}$ cfu ml^{-1} (95% CI: 8.97 – 9.21). Similar findings with respect to lag and the fit of the TTD data were found with data obtained at 30 and 37°C (Table 1).

Pseudomonas aeruginosa was analysed in a similar manner but over a wider range of temperatures (Table 1). An optimum growth temperature of approximately 39°C was apparent; with a specific growth rate of 1.51 ln cfu/ml/hr. None of the studies conducted showed the presence of a lag. Figure 3b shows data obtained at 37 and 30°C, the fitted lines cut the log inoculum axis at the detection number threshold value. The data shown for growth at 37°C has approximately constant variance until the initial inoculum level is less than 10^2 cfu/ml. Below this level the variance increases. To preclude the need for weighted regression data below this threshold were censored in the regression fits.

3.2 NON-ISOTHERMAL STUDIES

Two temperature shunt studies

Listeria monocytogenes: Figure 4 shows the observed TTD data from two plates incubated initially for 400 mins at 25 and 37°C and then transferred to the Bioscreens incubating at 37 and 25°C respectively; Figure 5 shows a similar experiment done with incubation at 30 and 37°C. The gradients of the lines are given in Table 2 and in each case the gradients obtained follow approximately those found from the isothermal data (Table1). Further, there were no indications of induced lags after the plates were exchanged. A lag would result in a vertical gap between the two rates.

Pseudomonas aeruginosa: Table 4 gives the observed gradients and intercepts for a temperature shunt of 25 to 39°C and vice versa and also for 34 and 39°C. Figure 6 shows the data obtained from the Bioscreen incubating at 37°C for the 37 to 30°C shunt. The initial incubation at 37°C gave a gradient of -95.2 (-99.90 to -90.50 : 95% CI) mins/$\log_{10}$ cfu/ml; the gradient of the plate initially incubated at 30°C but placed into the 37°C incubator after 300mins was -92.96 (-96.9 to -89.02 : 95% CI) mins/$\log_{10}$ cfu/ml. The horizontal separation of the two was calculated as 0.887 $\log_{10}$cfu/ml., i.e. the plate incubated at 30°C was growth retarded relative to growth at 37°C by just less than 1 log cfu/ml after 300 minutes of incubation.

Multiple Temperature Shunts: Figure 7 shows the observed times to detection for multiple initial inocula of *L.monocytogenes* undergoing either a 37-25-37-25°C or a 25-37-25-37°C temperature incubation sequence, changing temperatures after 360, 500 and 900 minutes. The observed gradients were -107.9, -179.3,-105.2,-NA for the 37-25-37-25°C sequence and -193.5,-104.7,-172.6,-110.0 for the 25-37-25-37°C sequences respectively. Superimposed on Figure 7 are the predicted values from the Geometric model, the TTD predictions of which are based on the growth rate data given in Table 1.

Modelling

The Geometric model (Eq. 5)can be either used to predict the outcome of hypothetical experiments –as was done for the multiple temperature shunt with *Listeria* shown in Figure 7, or can be used to

fit the observed data by minimising the sum of squares of the errors. Another method of using the predictive capacity of the model is to predict the TTD observed from a single Bioscreen incubating at a given temperature, when identical plates are moved in or out of the machine. Figure 8 shows a prediction of the pattern of TTD/log initial inocula from the single Bioscreen incubating at 37^{o}C. Using the growth rates described in Table 1, in 360 minutes the model predicts that 3.27 logs of growth will occur in this plate, whereas the other plate incubating in the other machine at 25^{o}C will increase by only 1.97 logs. When the latter plate is placed in the machine at 37^{o}C, if there are no lags then over the next 240 minutes there will be further increase of 2.18 logs in this plate. By calculating the log increase in the numbers of *L. monocytogenes* at 37^{o}C and that incubated at 25^{o}C and then subsequently placed at 37^{o}C, the pattern shown in Figure 8 was obtained. The observed data are overlain on the predicted lines. No lags were observed.

4 Discussion

The simple, classical 3-parameter logistic model can model the time to detection data obtained from turbidometric experiments using multiple initial inocula incubated iso-thermally. In all cases studied no lags were observed either from the OD/incubation time plots or from the plotted or modelled data. Plots of the log initial inoculum against the TTD cut the log I_0 axis at 9.22 log_{10} cfu/ml (95% CI 9.05 – 9.4 log_{10} cfu/ml) and at 9.15 log_{10} cfu/ml (95% CI 8.9 – 9.4) for *L. monocytogenes* and *P. aeruginosa* respectively. The detection number (N_d) for each species was confirmed by plate counting and from calibration curves of OD against microbial numbers. In the presence of a lag the plot would fail to cross the axis at the N_d, and a vertical separation equal to the lag between the x-axis and the TTD of the N_d would be present. This was not observed in any of the isothermal studies performed.

The linear approximation (eq.4) to the logistic expression (eq.2) assumes that $M>>N_0$ and also that $M>>N_d$; when $N_0 = N_d$, TTD = 0. If $M < 10N_d$ then curvature of the observed TTD occurs, if $M < 3 N_d$ then this curvature is substantial and the mismatch between eq.4 and eq.2 becomes significant. In the cases studied here, $M > 10N_d$ in all cases and this curvature was not observed. If the detection threshold is increased, e.g. use of a higher OD threshold, then curvature is observed. Conversely, lowering the threshold would reduce any observed curvature. Equation 4 does not require the estimation of the maximum population density (MPD) and in the absence of a lag is a two parameter model. Rearranging eq. 4 results in the Malthusian approximation of biological growth – i.e. growth without limit. The value of M is, however, used in the full form of the logistic model. M can be obtained through plate count, from dilutions of the MPD culture to produce a calibration curve using OD or from using the phenomenon of curvature discussed above.

When a temperature shunt was applied to growing bacteria, the cultures reduced or increased their growth rate commensurate with the incubation temperature. When cultures were shunted

281 from a lower temperature to a higher temperature there was no evidence of an induced lag and
282 growth continued at the rate dictated by the new temperature. When cultures were shunted from a
283 higher to a lower temperature condensation on the inside of the plate lid occurred and this led to
284 unusable data for a period after the shunt (the period depended on the temperature difference).
285 From the observed, fitted and predicted data it can be concluded that no induction of lag occurred
286 when moving from the higher to the lower temperatures used: the intercept of the regression lines
287 for each temperature coincide at the time of the temperature shunt, if lags were present this would
288 not occur (e.g. Figure 7).
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Table 1. Isothermal Rate data

Organism	Temp/^{o}C	gradient	LCI	UCI	Intercept	LCI	UCI	r^2	Obs
Listeria monocytogenes	25	-183.40	-185.94	-180.86	1687.6	1671.7	1703.6	0.993	153
	30	-130.30	-131.90	-128.70	1221.4	1211.0	1231.7	0.994	153
	37	-109.98	-110.85	-109.10	999.22	993.90	1004.6	0.997	160
Pseudomonas aeruginosa	25	-202.24	-205.33	-199.15	1908.7	1889.2	1928.1	0.991	161
	27	-140.73	-143.54	-137.92	1295.1	1279.1	1311.1	0.984	157
	30	-123.84	-125.24	-122.43	1122.4	1114.3	1130.5	0.994	154
	34	-99.17	-100.80	-97.54	883.05	873.74	892.34	0.989	157
	35	-96.04	-97.52	-94.57	889.61	880.60	898.62	0.991	149
	37	-91.80	-92.86	-90.75	846.13	840.00	852.27	0.995	157
	39	-91.38	-93.26	-89.49	826.70	818.78	834.78	0.987	122
	42	-100.84	-102.67	-99.01	923.14	911.90	934.37	0.988	147
	45	-125.81	-128.41	-123.21	1137.8	1121.7	1153.9	0.983	160

Table 2. Temperature shifts: *Listeria monocytogenes*

		25 to 37°C					37 to 25°C			
		Coefficients	LCL	UCL	r^2 (obs)		Coefficients	LCL	UCL	r^2 (obs)
t < 400 mins	Intercept	1758.9	1645.7	1872.0	0.943	Intercept	972.97	954.82	991.12	0.984
	m0	-194.75	-209.15	-180.35	(47)	m0	-107.88	-110.46	-105.30	(112)
t > 400 mins	Intercept	1121.9	1110.1	1133.7	0.988	Intercept	1441.0	1418.5	1463.4	0.992
	m1	-104.25	-106.47	-102.02	(103)	m1	-198.8	-204.40	-193.21	(45)

		30 to 37°C					37 to 30°C			
		Coefficients	LCL	UCL	r^2 (obs)		Coefficients	LCL	UCL	r^2 (obs)
t < 410 mins	Intercept	1333.6	1302.5	1364.6	0.985	Intercept	983.14	975.06	991.23	0.997
	m0	-147.31	-151.55	-143.07	(72)	m0	-109.30	-110.48	-108.12	(108)
t > 410 mins	Intercept	1068.5	1057.7	1079.4	0.991	Intercept	1166.1	1144.5	1187.7	0.986
	m1	-106.60	-108.82	-104.38	(84)	m1	-145.56	-151.02	-140.10	(44)

Table 3. Temperature shifts: *Pseudomonas aeruginosa*

		25 to 39°C					39 to 25°C			
		Coefficients	LCL	UCL	r^2 (obs)		Coefficients	LCL	UCL	r^2 (obs)
t < 300 mins	Intercept	2081.9	1783.8	2380.0	0.91	Intercept	854.35	820.60	888.1	0.951
	m0	-228.26	-264.20	-192.33	(19)	m0	-91.89	-96.58	-87.21	(80)
t > 300 mins	Intercept	1035.8	1025.4	1046.1	0.987	Intercept	1639.5	1600.7	1678.2	0.972
	m1	-91.79	-93.57	-90.00	(140)	m1	-208.55	-216.8	-200.31	(75)

		34 to 39°C					39 to 34°C			
		Coefficients	LCL	UCL	r^2 (obs)		Coefficients	LCL	UCL	r^2 (obs)
t < 300 mins	Intercept	907.07	866.26	947.89	0.954	Intercept	811.66	832.49	854.58	0.980
	m0	-99.12	-104.81	-93.43	(61)	m0	-89.00	-91.96	-86.03	(75)
t > 300 mins	Intercept	868.04	855.48	880.59	0.982	Intercept	872.48	854.58	890.38	0.973
	m1	-92.64	-95.24	-90.02	(93)	m1	-100.47	-104.55	-93.39	(69)

Table 4. Observed parameters for the 37/25/37/25 and 25/37/25/37 temperature shunts of *Listeria monocytogenes*

time	Temp	Parameter	Estimate	LCI	UCI	r2 (obs)
		log Id	9.48	9.26	9.71	
<360	37	m0	-107.92	-108.36	-106.47	0.998(38)
<600	25	m1	-179.29	-188.04	-170.54	0.992 (17)
<900	37	m2	-104.62	-106	-103.25	0.998(52)
>900	25	m3	-	-	-	no obs

time	Temp	Parameter	Estimate	LCI	UCI	r2 (obs)
		log Id	9.58	7.98	11.52	
<360	25	m0	-193.5	-212.59	-174.4	0.981 (12)
<600	37	m1	-104.73	-106.54	-102.93	0.997(42)
<900	25	m2	-174.69	-189.12	-160.26	0.971 (21)
>900	37	m3	-109.52	-114.69	-104.35	0.984 (33)

Caption to Figures

Figure 1.The geometrical basis for equation 5: for a given rate (M0), over the time period T_x initial inocula between $\log I_D - \log I_x$ will reach the detection threshold of log I_d. At T_x the rate is changed (M1) and the TTD now follow the new rate. If a lag is induced a vertical separation at T_x equal to the time of lag will be present before growth recommences.

Figure 2. Optical density/incubation time curves for *Listeria monocytogenes* incubated at 25°C.

Figure 3a. Observed TTD data for multiple initial inocula of *L.monocytogenes* (L252) at 25 (triangles), 30 (squares) and 37°C(circles).The solid lines are the fitted regression lines (Table 1)

Figure 3b. Observed TTD data for multiple initial inocula of *P.aeruginosa* ATCC 15442) at 30 (squares) and 37°C(circles). The solid lines are the fitted regression lines (Table 1)

Figure 4. Observed TTD data for *L.monocytogenes* incubating at 37 or 25°C with a temperature shunt to 25°C or 37°C (open circles and open squares respectively) after 400 minutes incubation at the initial temperature. Solid lines are the fitted regression lines (Table 2).

Figure 5. Observed TTD data for *L.monocytogenes* incubating at 37 or 30°C with a temperature shunt to 30°C or 37°C (open circles and open squares respectively) after 410 minutes incubation at the initial temperature. Solid lines are the fitted regression lines (Table 2).

Figure 6. Observed TTD data (symbols)for an initial plate of multiple inocula of *P.aeruginosa* incubating at 37°C for 300minutes, replaced by an identical filled plate after incubation at 30°C for 300 minutes. The solid lines are the predicted TTD based on the data of Table 1 and the use of the logistic model to calculate the expected increase in numbers in both plates.

Figure 7. Observed TTD data for *L.monocytogenes* incubating at 37-25-37-25°C (open circles) or 25-37-25-37°C (open squares) with temperature shunts occurring at 360, 600 and 900 minutes. The solid lines are the predicted values based on the data of Table 1, with Nd = 9.4.

Figure 8. Observed TTD data (symbols) and predicted data (solid line) from a single Bioscreen incubating at 37°C for multiple inocula of *L. monocytogenes* undergoing plate changes to and from another machine incubating at 25°C (see figure 7). The solid lines are the predicted TTD based on the data of Table 1 and the use of the logistic model to calculate the expected increase in numbers in both plates during the periods of incubations at 37 and 30°C.

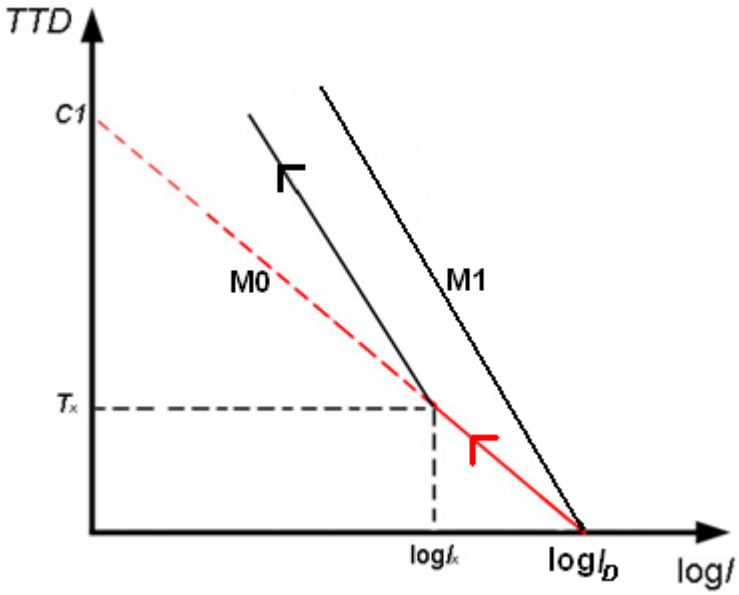


Figure 1.

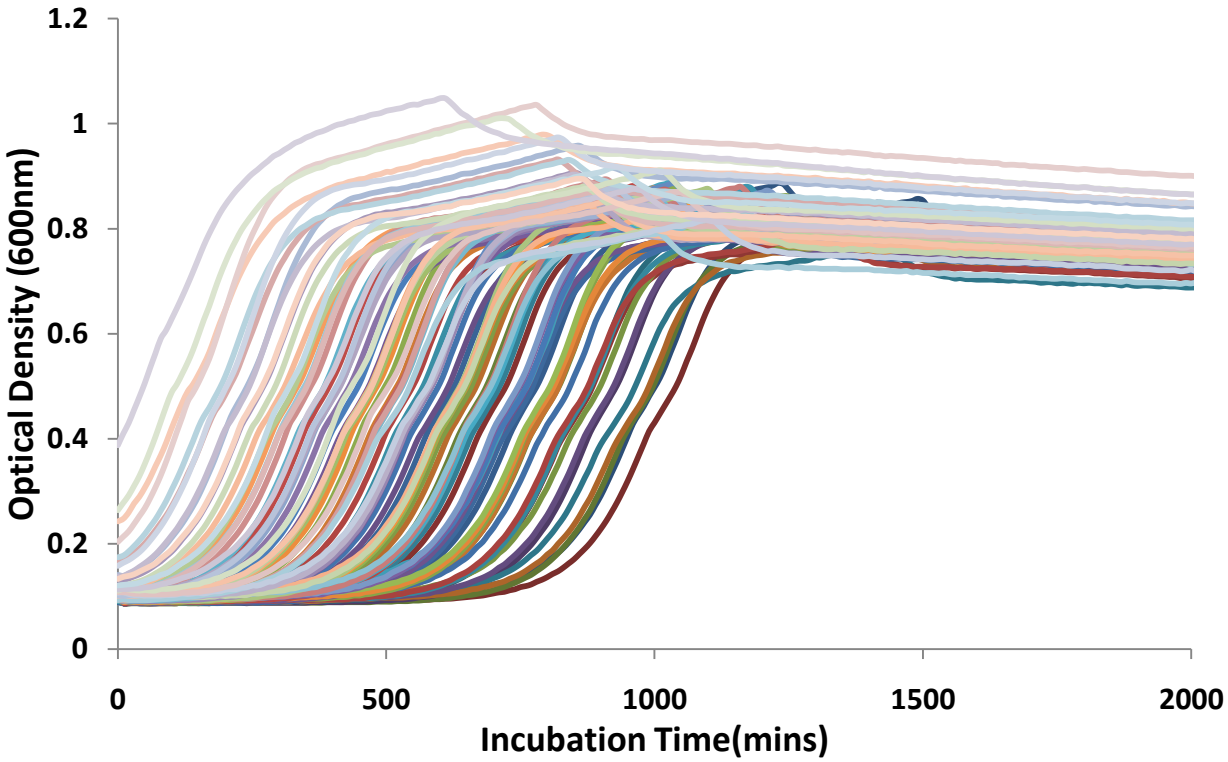
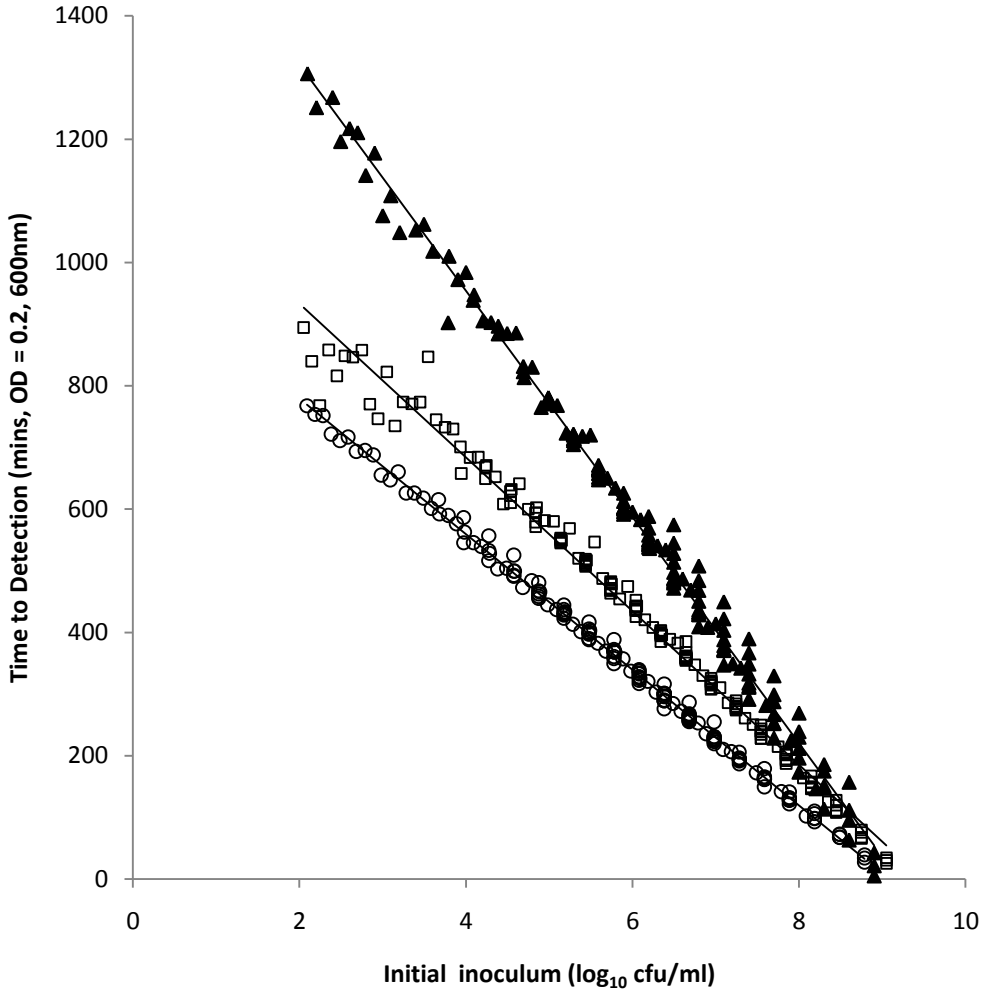


Figure 2.

Figure 3a



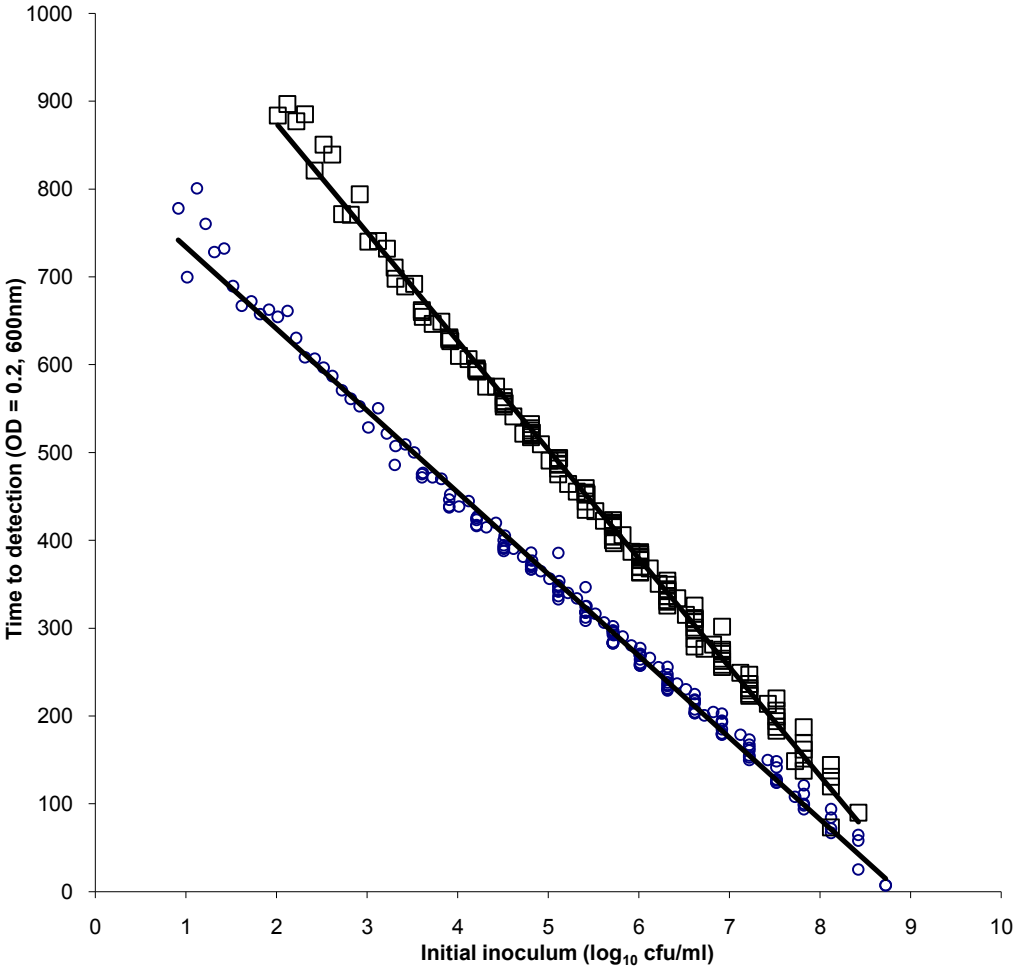


Figure 3b.

Figure 4.

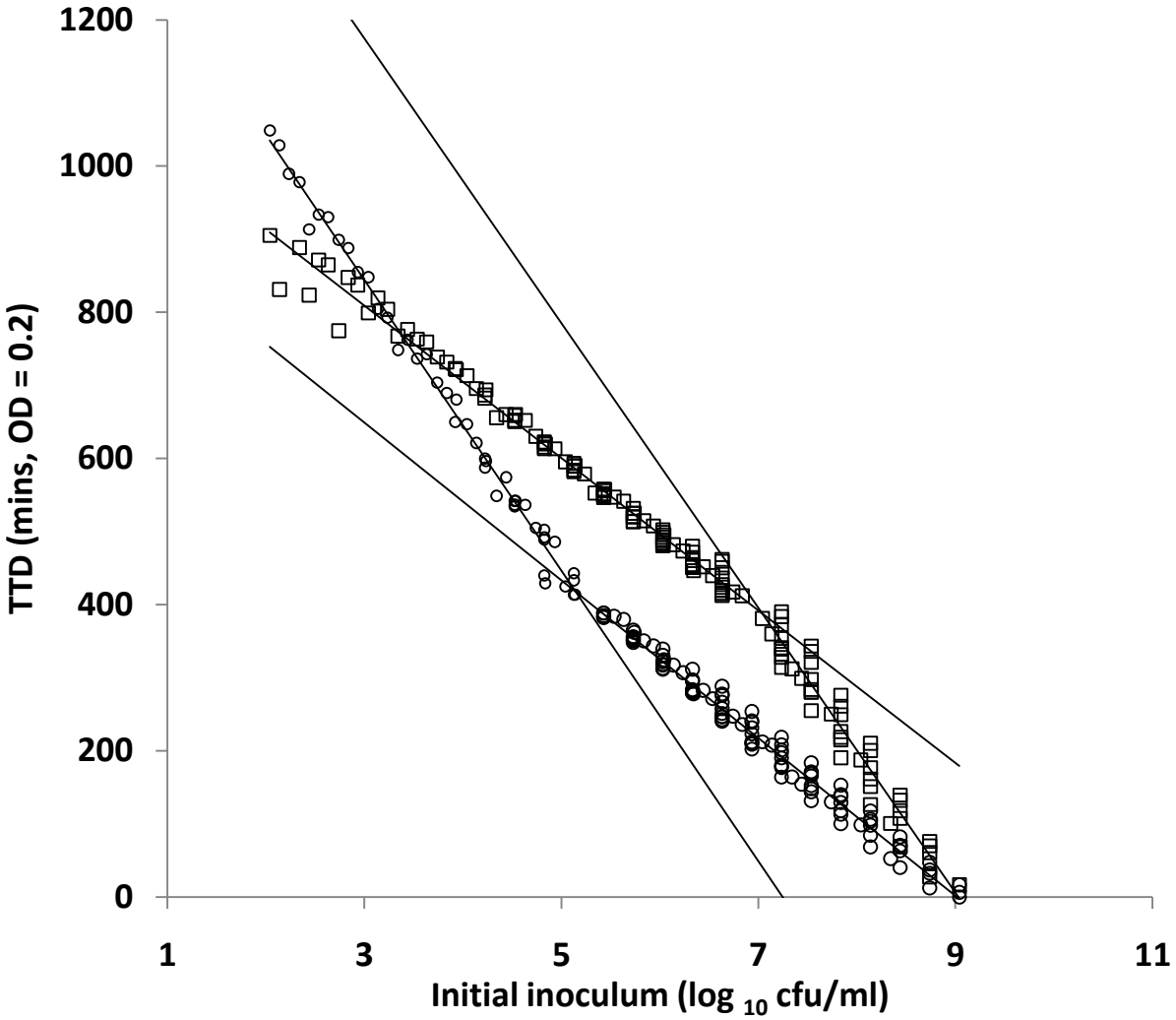


Figure 5.

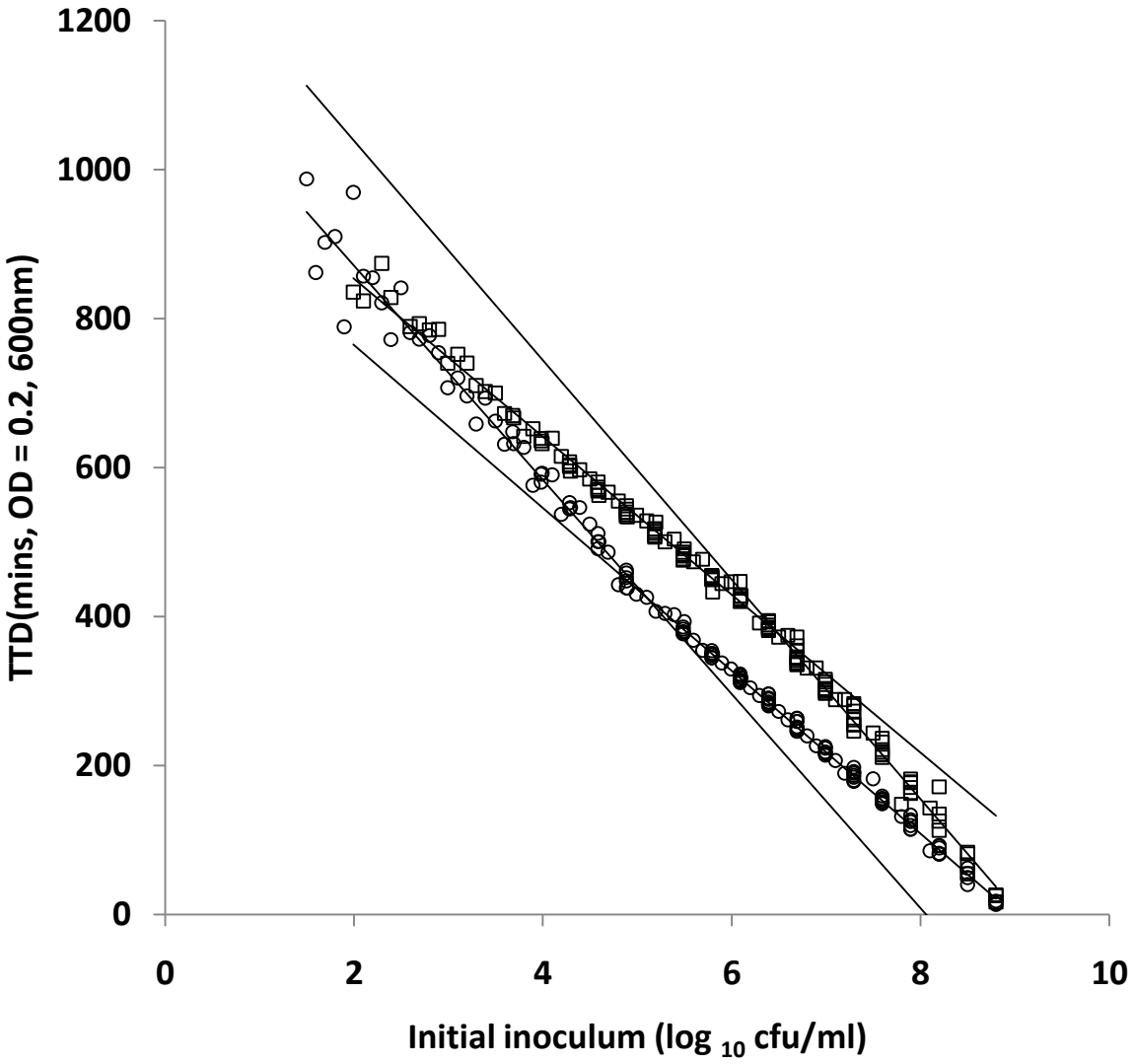


Figure 6

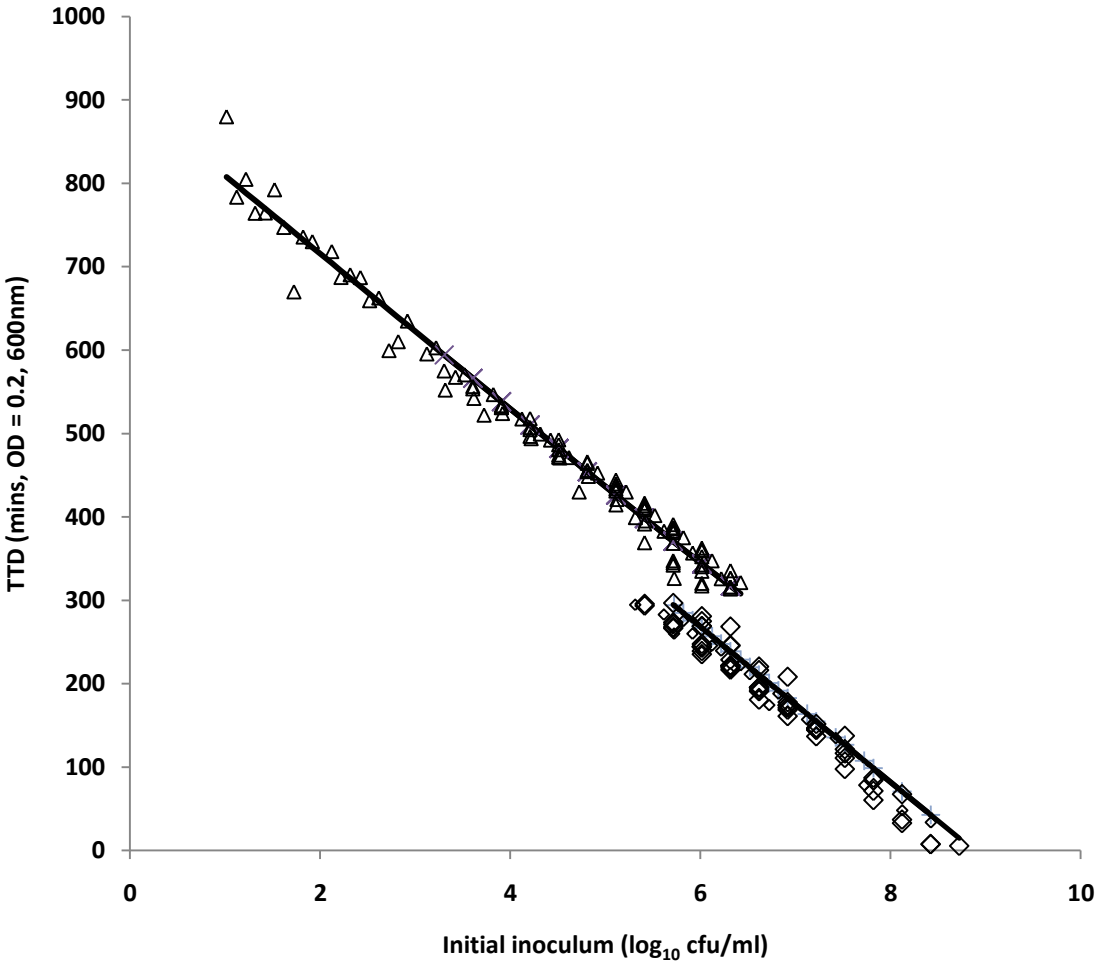


Figure 7

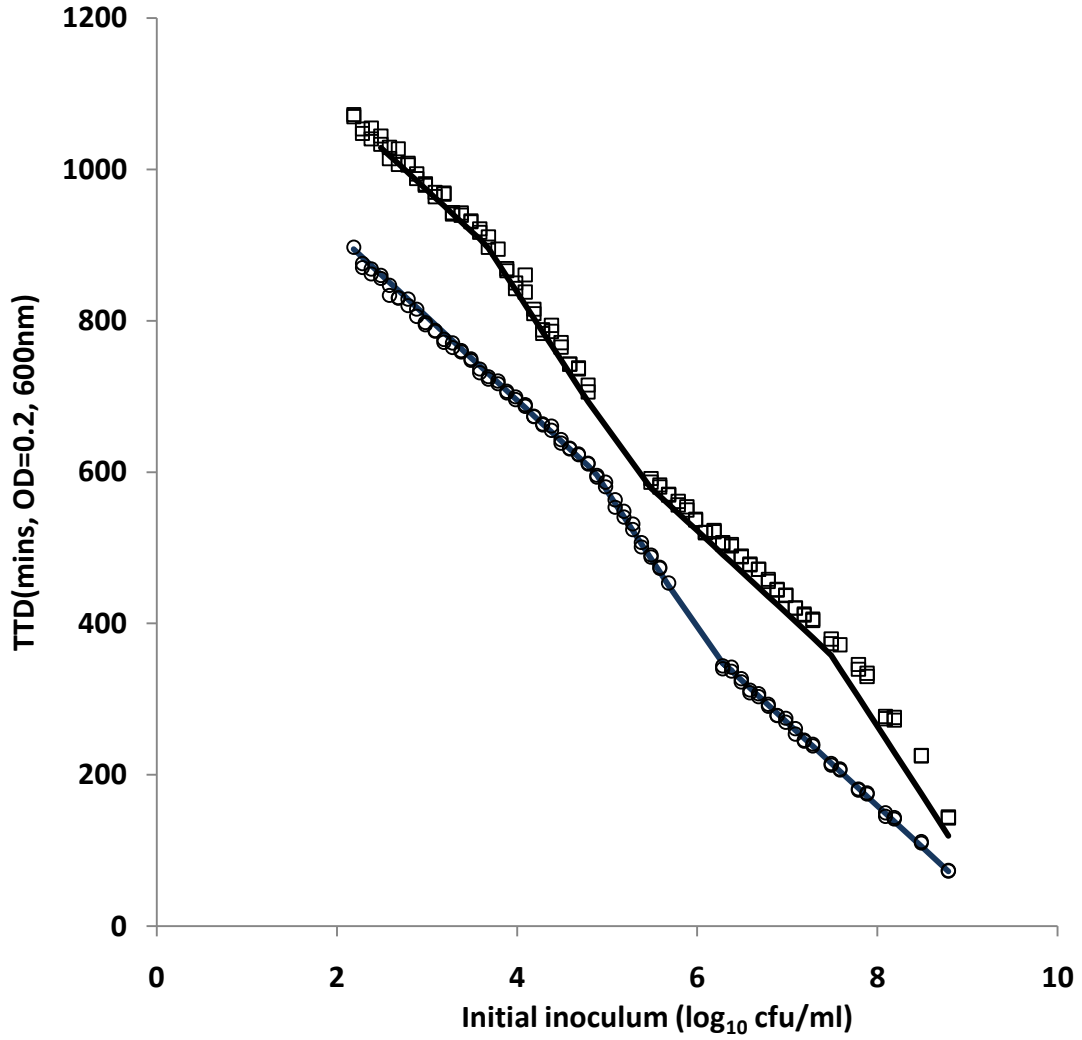


Figure 8