African Journal of Microbiology Research Vol. 4(1) pp. 061-070 January, 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2010 Academic Journals

Full Length Research Paper

Application of methylene blue dye reduction test (MBRT) to determine growth and death rates of microorganisms

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Accepted 29 October, 2009

An efficacy of a compound for antimicrobial characteristics is typically evaluated through experiments to obtain the minimum inhibitory concentration (MIC). Similarly, a minimum activating concentration (MAC) can be obtained for a compound necessary for growth. We report a protocol for the determination of MIC and MAC for antibiotic and exhibition assays, respectively using methylene blue reduction test. The assay has been demonstrated on the growth of *Escherichia coli* on the antibiotic kanamycin, as an inhibitor, and on vitamin B12, as an activator. The slope of discoloration of methylene blue was used to determine the growth and death rates of the organism. The use of the rate constants to determine MIC/MAC values established a rational basis for determination of these relevant constants. The methodology presented here is general and can be easily adapted to other systems. The dose-response curves for extent of death / growth can be used to establish the MIC90 or MAC90 for an inhibitor or an activator, respectively. The methodology presented here rationalizes and standardizes the antibiotic and exhibition assays. The main advantage of the reported assay is the rapid and easy determination of the MIC and MAC values.

Keywords: *Escherichia coli*, vitamin B12, kanamycin, minimum inhibitory concentration (MIC), minimum activating concentration (MAC).

INTRODUCTION

Antibiotic and exhibition assays are common techniques used to establish the efficacy of a compound for antimicrobial properties and growth characteristics, respectively. While inhibition assays establishes the amount of antibiotics necessary to kill a specific organism, exhibition assay determines the amount of nutritional compound necessary for enhancing the growth of an organism. Inhibition assays enumerate the viability of microorganism in presence of an antibiotic. Various methods, such as use of agar plate to measure viability and determination of antibiotic zone using agar cavity (Ryan et al., 1987; Smith et al., 1986) and broth disk method (Wilkins et al., 1973; Romano et al., 1993) are commonly used for inhibition assay. These methods are time consuming (taking days), error prone (Woolfrey et al., 1979), and tedious. Other assays such as determination of endogenous ATP levels or immunoassay can correlate cellular viability (Harber and Asscher, 1977). These assays are highly sensitive, accurate and fast, but involve expensive instruments and reagents. Other methodologies such as quantification based on flow cytometry (Jepras et al., 1997) and PCR techniques (Bowman et al., 2001) quantify viable cell numbers accurately. For example, flow cytometry was used to study the effects of different antibiotics such as ampicillin, gentamicin and cefotaxime etc. on bacterial morphology (Gant et al., 1993). However, the key parameter evaluated through these methods are the MIC50 values, which determines the minimum inhibitory concentration necessary to kill 50% of the cells in a specific amount of time (typically 24 h). MIC50 is evaluated through streaking cells on agar plates containing different concentrations of the antibiotic. The methodology requires more than a day to enumerate MIC50 and yields considerable variability.

The above methods used for inhibition assays can also

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be used for exhibition assay however with similar disadvantages (Smith et al., 1986). Vitamin B12 is one of the growths promoting compound essential for growth of specific microorganism. Some species of *Escherichia coli* requires vitamin B12 for their growth. Many soil bacteria also have been shown to be dependent on vitamin B12. Similar to inhibition assays, experiment can be carried out using agar plates to determine the minimum activation concentration (MAC) necessary for growth. The problems associated with determining cell count using agar plates such as variability and time consuming remain with the methodology.

Recently, we have used Methylene Blue Dye Reduction Test (MBRT) in evaluating cell viability in a very short time (Nandy et al., 2007). The methodology employed the enzymatic reduction of methylene blue by a metabolically active organism turning the Methylene Blue colorless. The rate of decoloration by the metabolically active cells can be correlated to the number of viable cells. For this purpose, the slope of the MB decoloration rate was calibrated with respect to colony forming units (CFU) obtained through plating. This method was successfully employed to characterize the viability of *E. coli* and *B. subtilis* (Bapat et al., 2006). Further, the methodology was used to characterize the cannibalistic tendency of *B. subtilis* under nutritional limiting conditions (Nandy et al., 2007; Nandy and Venkatesh, 2008). These studies revealed that MBRT can be successfully employed to quantify viable cell count in a very short time (less than 4 min).

Methylene Blue (MB) dye has been employed to check for the overall microbial load and quality control of milk and other liquid foods (Impert et al., 2002). Because of its size and positive charge, it does not enter into the cells appreciably. It gets reduced to 'leuko' or colorless form of MB at the cell surface via reductase enzymes present in the cell membrane. This colorless form of methylene blue (MBH) is uncharged, lipophilic, and enters cells by diffusion across the plasma membrane where it is re-oxidized and thus sequestered within the cells (May et al., 2003).If oxygen is available, reduced MB can be oxidized by the mitochondrial electron transport system. This will result in the reappearance of the blue color. Up to now, the exact mechanism of dye reduction is not known, but some reports available suggest that MB is reduced by transmembrane reductases (Bongard et al., 1995; Merker et al., 1997). This mechanism is applied to evaluate the microbial load in a liquid medium. The shorter time required for the disappearance of the blue color is indicative of a higher microbial load. It is assumed that greater the number of microorganisms, more the oxygen demand and lesser the oxygen concentration in the medium resulting in the faster disappearance of the color. This fact has been used as a broad indicative test of a microbial load representing microbial quality of milk.

In the current study, MBRT was adopted to develop a protocol for the antibiotic and exhibition assays. The developed protocol was extended to demonstrate the effect of kanamycin on the viability of *E. coli K12*. Further, we extend the protocol to study the effect of vitamin B12 on the growth of *E. coli 113-3D*. The main advantage of the assay is that only 2.5 h was necessary to determine the Minimum Activating Concentration in the exhibition assay and minimum inhibition concentration (MIC) in the inhibition assay. Further, the values of MAC and MIC were correlated to growth and death kinetics of the organism, respectively.

MATERIALS AND METHODS

Microorganisms

E. coli K12 (MTCC 1302) and *E. coli 113-3D* were used throughout this study. The strains were obtained from MTCC, IMTECH Chandigarh, India. *E. coli K12* was used for inhibition assay and *E. coli* 113-3D was used for the exhibition assay. The working cultures of these organisms were maintained on Luria agar (LA) slants at 4°C. The culture was revived once every month. A loopful of the culture from the slant was sub cultured into 100 ml of sterile Luria broth (LB) and grown for 6 h at 37°C at 240 rpm. The cells were centrifuged and introduced into test tubes containing minimal media with different concentration of the antibiotic, kanamycin or vitamin B12.

Chemicals

LB (Hi-media, Mumbai, India) was used throughout the experiments to grow the organism. LA (Hi-media, Mumbai, India) was used for culture maintenance on slant and for viable count using spread plate method. Methylene blue dye was obtained from E. Merck, India. Kanamycin and vitamin B12 (Hi-media, Mumbai, India) were used for all the experiments in antibiotic and exhibition assays respectively.

Bacterial growths and killing assay

Media development

Minimal media was used for the antibiotic and exhibition assays respectively. Minimal media was prepared by dissolving (56.4 gm in 1 L) 5 X minimal salts (Hi-media, Mumbai, India). $MgSO_4$ (0.5 g/2 ml), $Cacl_2$ (0.3 g/2 ml) and 0.4% Glucose were also used in the minimal media. Kanamycin and Vitamin B12 (Hi-media, Mumbai, India) in different concentrations were used for the antibiotic and exhibition assay respectively.

Protocol development

Inhibition assay

In our previous study, we had reported a protocol using Methylene blue Reduction Test (MBRT) to evaluate CFU (Bapat et al., 2006) of an aerobic microorganism. The protocol was extended to evaluate the MIC in an inhibition assay. The cultures were firstly grown on LB seed media. One loop of the culture (10% v/v) was inoculated from the seed media into a LB media of 100 ml LB in a 500 ml Erlenmeyer flask. After 9 h of incubation at 37°C with 240 rpm, the broth was separated using 10 centrifuge tubes and aseptically centrifuged at 10000 rpm (Remi C-24 centrifuge, India) for 10 min. The supernatant was discarded in these tubes and different kana-

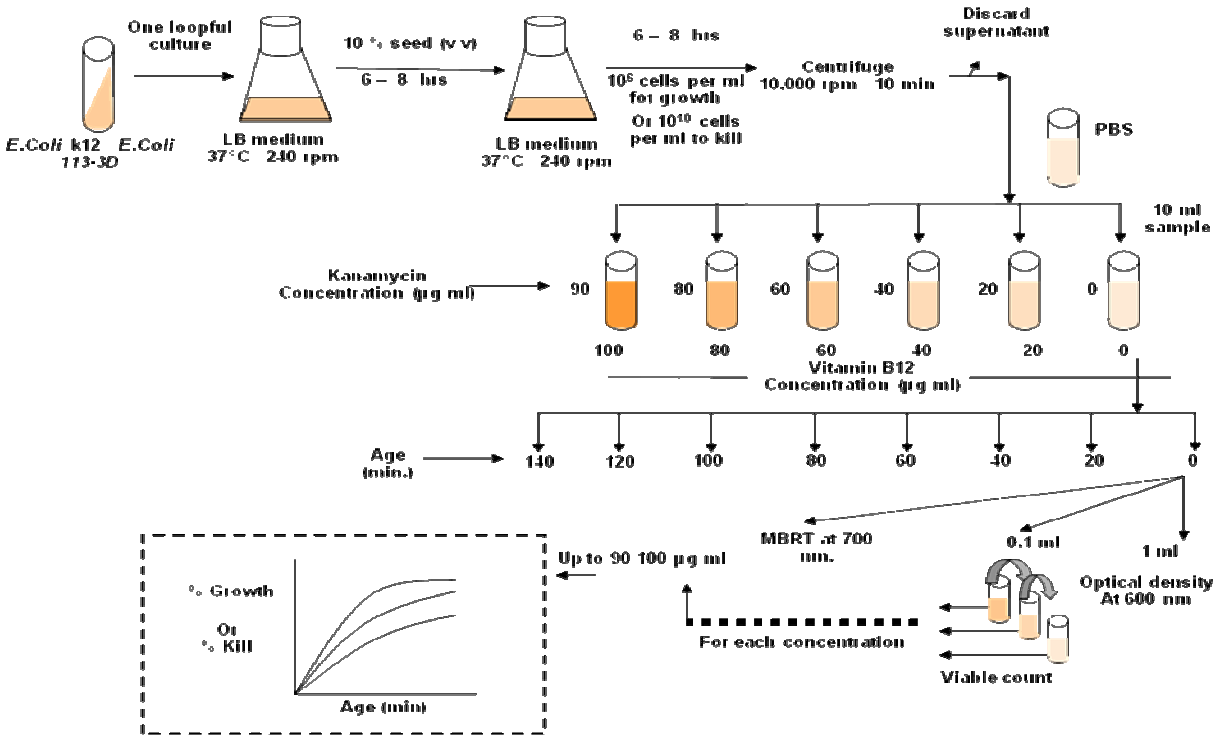

Figure 1. Schematic representation of the Antibiotic and Exhibition Assays. Fresh slant was inoculated with *E. coli* stock culture. After incubating overnight (9 h), the whole broth was separated in 10 centrifuge tubes having 10 ml sample each and the tubes were aseptically centrifuged at 10000 rpm for 10 min. These cells were resuspended in 10 ml sterile minimal media containing **(a)** Kanamycin or **(b)** Vitamin B12 and further used for estimation of colony forming units (Cfu /ml) and MBRT slope. The kanamycin or the vitamin B12 concentrations of each tube were varied. For each concentration one test tube was used as a control and other test tubes were incubated at 37°C with a constant time intervals of 20 min. for one hour.

mycin was added along with the minimal media. The concentrations of kanamycin used were 0, 20, 40, 60, 80 and 90 µg/ml. Each was further divided into 8 test tubes, which were used for 8 time points for rate determination. For each antibiotic concentration the test tubes were incubated at 37°C and a single test tube was used for evaluating CFU every 20 min. Each test tube containing 10 ml sample was used for the MBRT (6 ml), OD determination (1 ml) and viable count through plating (0.1 ml). The above experimental protocol was repeated thrice and the average is reported here. Figure 1 shows the detailed protocol for the method to evaluate the inhibition assay.

Exhibition assay

A similar protocol was also used for the exhibition assay. The steps were identical till the centrifugation of 10 centrifuge tubes. Further, aseptically minimal media were transferred along with various concentrations of vitamin B12 into the tubes. The concentrations of vitamin B12 used were 0, 20, 40, 60, 80 and 100 µg/ml. Each tube was further divided into 8 test tubes for rate determination. Similar to inhibition assay, the samples were used for MBRT, OD determination and viable cell count through agar plating. Figure 1 also shows the detailed protocol for the exhibition assay.

Standard calibration curve

The relation between OD, CFU and MBRT of antibiotic and exhibition assay using *E. coli K12*, *B. subtilis 168trpC2* and *E. coli 113-3D*, respectively were obtained as mentioned earlier. For each assay controls (minimal media) were used without antibiotic or vitamin B12. Each sample were incubated for time span of 20 min to maximum of 1 hr at 37°C and then analyzed those samples with OD, MBRT and plated in triplicate and incubated overnight at 37°C.

RESULTS

Experiments were conducted using equal number of cells in a minimal media with various concentrations of antibiotic. Further, the dynamics of cell viability was evaluated using agar plating and MBRT at various time points. Figure 2 shows the rate of decoloration of Methylene Blue after the cells were exposed to different antibiotic concentrations for 40 min. It can be noted from the figure that the rate of decoloration was dependent on the amount of antibiotic present. The rate of decoloration decreased with increase in the antibiotic concentration indicating that the viable cell count decreased with increase in the antibiotic concentration, since only the presence of viable cells oxidizes Methylene Blue and discolors it. Thus, the rate of decoloration can be used to quantify the effect of antibiotic. The initial slope of decoloration decreased from 0.0142 -3.6 x 10^{-5} for anti-

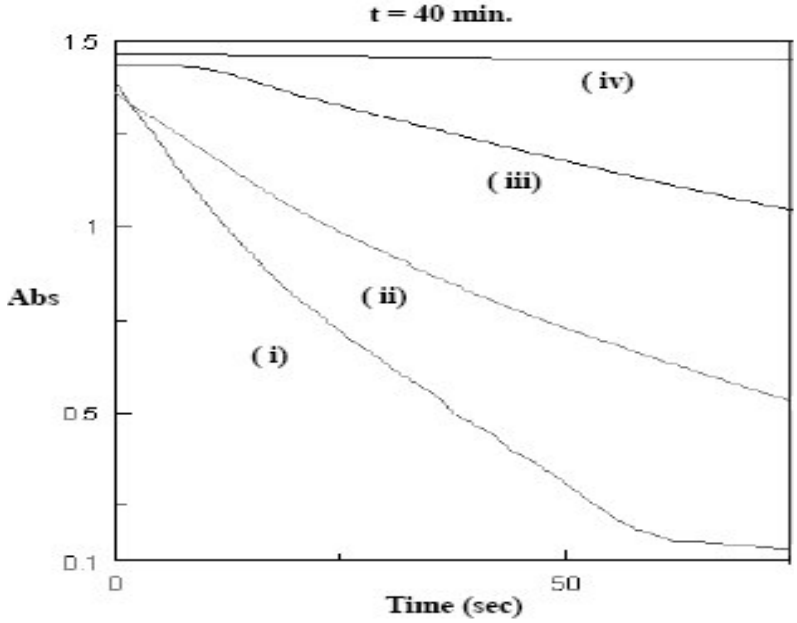

Figure 2. Variation of MBRT slopes at various antibiotic concentration in PBS with *E. coli K12* at the end of 40 min. The profile shows the decrease in absorbance due to decoloration of methylene blue by viable cells. The decrease in the MBRT slope with increase in antibiotic concentration relates to the decrease in the viable count. Different profiles indicate different antibiotic concentrations; (i) 0 µg/ml; (ii) 30 µg/ml; (iii) 60 µg/ml; and (iv) 90 µg/ml in the figure 2 represent the increase in MBRT slope with time.

biotic concentration varying from 0 - 90 µg/ml.

Plots were obtained based on the CFU measurement using agar plates for various antibiotic concentrations at different time points. Figures 3a and b show the Log values of CFU/CFU_0 [defined as *x*], where CFU_0 was the CFU obtained at the initial condition (that is t = 0). It can be noted that the dynamics of death of *E. coli* can be captured as a linear profile in Log terms. For a first order kinetics, the dynamics can be represented by,

$$\frac{dx}{dt} = -K_d \times x \quad \text{..... (1)}$$

On integrating the above equation, 'x' can be expressed as,

$$\ln x = -K_d \times t \quad \text{..... (2)}$$

Thus, the linearity in the profile of Log (CFU/CFU_0) demonstrated a first order kinetics and the slope of the line yielded the value of the rate constant 'K_d'. It can be noted that the slope of the linear profile increased with increase in the antibiotic concentration which indicated that the rate constant for the death of *E. coli* increased with the antibiotic concentration. Since the agar plates were incubated for 24 h for each part, the time required to determine the 'K_d' value was more than 24 h.

Plots were also obtained based on the slope of decoloration of Methylene Blue for various antibiotic concentrations at different time points. Figures 3c and d show the Log of normalized values of decoloration slopes (defined as *y*) for various antibiotic concentrations. The slopes were normalized with the initial slope obtained at t = 0. Since the slope of decoloration decreased with decrease in the number of viable cells, the value of y also decreased. It can be observed in Figures 3c and d that the Log (*y*) also demonstrated a linear profile as obtained for the values of CFU. This indicated that the first order kinetics can be captured through the measurement of the decoloration rate of Methylene Blue. Since the slope of decoloration was directly proportional to the CFU, '*y*' could also be related to '*x*' as, *y = mx*, where *m* was a proportional constant. Thus, by replacing '*x*' in terms of '*y*' in equation 1, we obtain

$$\frac{d\frac{y}{m}}{dt} = -K_d \times x \quad \text{..... (3)}$$

On integrating the above equation, 'y' can be expressed as

$$\ln y = -K_d \times t \quad \text{..... (4)}$$

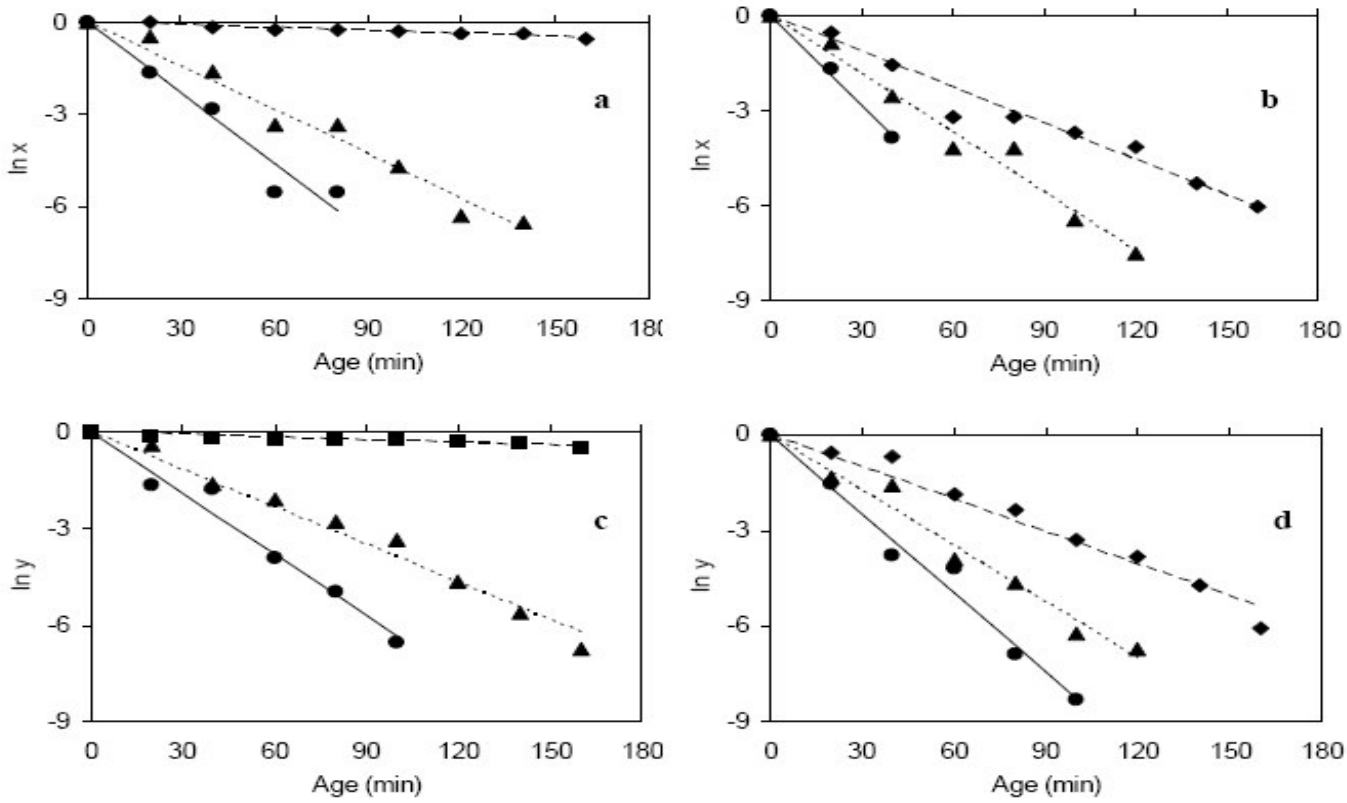

Figure 3. Variation in viability of *E. coli* cells in PBS in presence of different concentration of kanamycin, where x = (CFU/CFU$_0$) and y = (MBRT slope/MBRT slope$_0$); **(a)** Plot of CFU obtained using agar plating method for 80 µg.ml^{-1} (●), 40 µg.ml^{-1} (▲) and 0 µg.ml^{-1} (♦); **(b)** Plot of CFU obtained using agar plating method for 90 µg.ml^{-1} (●), 60 µg.ml^{-1} (▲) and 20 µg.ml^{-1} (♦); **(c)** Plot of MBRT slope for 80 µg.ml^{-1} (●), 40 µg.ml^{-1} (▲) and 0 µg.ml^{-1} (♦); **(d)** Plot of MBRT slope for 90 µg.ml^{-1} (●), 60 µg.ml^{-1} (▲) and 20 µg.ml^{-1} (♦). Note that the CFU and MBRT slope were normalized using the initial CFU and MBRT slope obtained at the beginning of the experiment (that is, at t=0). The slope of the linear profile yielded the kinetic constant for death.

Thus, the value of the rate constant for death (K_d) could also be estimated by measuring the slope of the linear profile obtained by plotting Log (y) versus time, as shown in Figures 3c and d for various concentrations of the antibiotics. Thus, the value of the rate constant 'K_d' was also estimated using the Log (y) plot versus time.

The value of the first order rate constants (K_d) for death of *E. coli* at various concentrations of antibiotics obtained using the slopes of the linear profile of Log (CFU) and the Log (MBRT) slopes were compared for different values of antibiotic concentration (Figure 4a) and found to yield similar values. Thus, it is clear that the slopes of decoloration of MBRT can be used to evaluate the rate constant for the death of an organism but in less than 2 h. The value of the rate constant for *E. coli* ranged from 0.004 to 0.01 h^{-1} for samples with no antibiotic to 90 µg/ml of antibiotic, respectively.

The kinetic constant for death (K_d) can be used to determine the extent of death for different exposure times for a given antibiotic concentration. If an organism with an initial cell count of N_0 is exposed for a time, t, in PBS, then Extent of death (E_D) is defined as;

$$E_D = \frac{\left(N^* - N\right)}{N^*} = 1 - \frac{N}{N^*} \quad \text{..... (5)}$$

Where, N is the final cell count at the end of time't' in presence of the antibiotic, while N^* is the final cell count at the end of the same exposure time in the absence of the antibiotic. Based on the kinetic constant (K_d), the extent of death can be given as,

$$E_D = 1 - \frac{N_0 \,.\, e^{-K_d t}}{N_0 \,.\, e^{-K_d^* t}} = 1 - e^{\left(K_d - K_d^*\right)t} \quad \text{..... (6)}$$

The above equation can be used to determine the extent of death (E_D) for a specific exposure time using the kinetic constant for death (K_d) at different antibiotic concentration. It should be noted that K_d is a function of antibiotic concentration and was determined using slopes of MB decoloration.

Figues 4b shows the values of E_D obtained for different exposure times using the kinetic rate constant at various

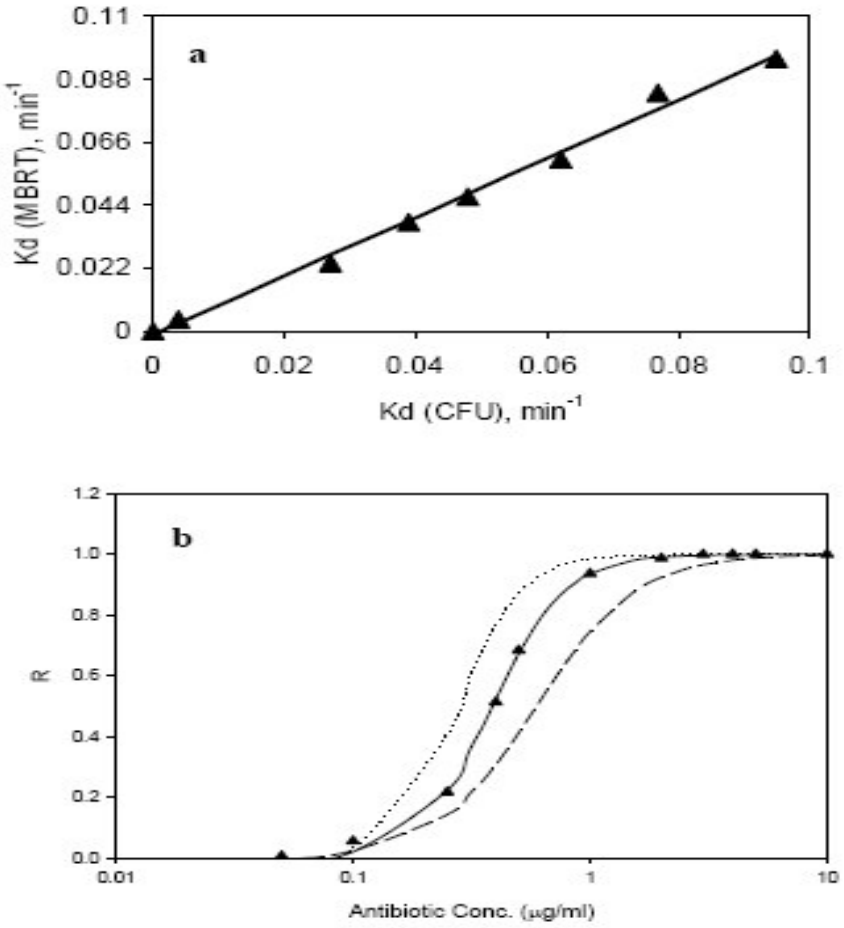

Figure 4 (a) Comparison of the kinetic rate constant (K_d) for death of *E. coli* obtained through the agar plate method and MBRT. It can be noted that the constants lie on the line with a slope of 45°, the standard deviation for the rate constant obtained through plate method was higher by 1.3 fold as compared to that for MBRT. **(b)** The dose-response curves for different exposure times. The plot shows the Extent of Death (E_D) on the y-axis and the kanamycin concentration on the x-axis. The exposure times were 6h (dashed line), 12 h (solid line) and 24 h (dotted line). The symbol (▲) represents the calculated E_D values using experimental values of the rate constant. The lines represent the fit obtained using Hills equation.

antibiotic concentrations. The value of E_D at different antibiotic concentrations demonstrated a typical dose response curves and was fitted using Hills equation, given below;

$$E_D = \frac{A^n}{K_A^n + A^n} \quad \text{..... (7)}$$

Where; '*A*' rep resents the antibiotic concentration, while the parameter *n* and K_A represent the Hill coefficient and half saturation constant, respectively. The dose response curves for E_D versus the antibiotic concentration were dependent on the time of exposure. The curve shifted to the left at higher exposure time indicating higher extent of death with higher exposure times. This was also captured by the lower values of K_A at higher exposure times. The value of K_A were 0.6, 0.39 and 0.28 (µg/ml) for t = 6, 12 and 24 h, respectively. Since the value of half saturation constant K_A indicated the concentration of antibiotic required for 50% enhanced kill due to the exposure of the antibiotic, K_A is therefore equal to MIC_{50} for the given exposure time. Note that MIC_{50} is dependent on the time of exposure and the rate constant for death (K_D). The value of '*n*' indicated the steepness of the response. A value of one represented a typical Michaelis-Menten response, while $n>1$ represented an ultra sensitive response and $n<1$ indicated a sub sensitive response. The values of '*n*' were 2, 2.8 and 3.3 for exposure time of 6, 12 and 24 hrs respectively indicating an ultra sensitive

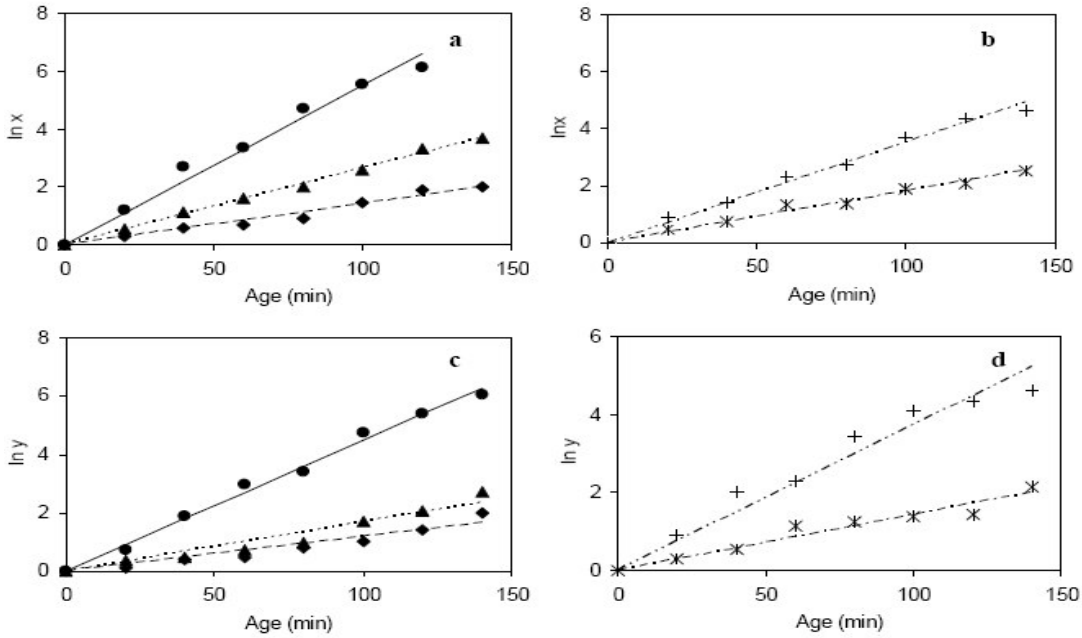

Figure 5. Variation in viability of *E. coli 113 3D* cells in minimal medium in presence of different concentration of vitaminB12 where x = (CFU/CFU$_0$) and y = (MBRT slope/MBRT slope$_0$); **(a)** Plot of CFU obtained using agar plating method for 100 µg/ml (●), 60 µg/ml (▲) and 20 µg/ml (♦); **(b)** Plot of CFU obtained using agar plating method for 80 µg/ml (+), and 40 µg/ml (*); **(c)** Plot of MBRT slope for 100 µg/ml (●), 60 µg/ml (▲) and 20 µg/ml (♦); **(d)** Plot of MBRT slope for 80 µg/ml (+), and 40 µg/ml (*). Note that the CFU and MBRT slope were normalized using the initial CFU and MBRT slope obtained at the beginning of the experiment (i.e., at t=0). The slope of the linear profile yielded the growth rate of various vitamin B12 concentrations.

response.

Thus, the response curve gets steeper as the exposure time was increased. Equation 7 can also be used to determine MIC_{90} by determining the concentration of antibiotic required for E_D = 0.9 indicating 90% death. Therefore,

$$MIC_{90} = 9^{\frac{1}{n}} . MIC_{50} \quad \text{.....(8)}$$

In case of the effect of kanamycin on the death of *E. coli* MIC_{90} was determined to be 1.8, 0.86 and 0.55 (µg/ml) for exposure times of 6, 12 and 24 h, respectively. This implied that 1.8 µg/ml was required to kill 90% of the cells in 6 h; while only 0.55 µg/ml was necessary to kill the same amount in a day. Equation 8 indicated that an antibiotic with higher value of n is preferred when two antibiotics with similar MIC_{50} are compared. Thus, MIC_{90} captured both the effect of sensitivity (value of *n*) and MIC_{50} and is therefore a better parameter to quantify the dose-response curves of an inhibitor.

Experiments were also conducted to determine the dependency of growth of *E. coli* on vitamin B12. In this case, CFU measurement and MBRT were used to evaluate the dependency of vitamin B12 on the growth rate of *E. coli*. Figures 5a and 5b show the log of CU/CFU$_0$ (that is ln (x)) versus time. In this case, with time CFU increased due to growth and thus ln(x) also increased with time. It can be noted that in the absence of vitamin B12 no growth was observed as the strain used in the experiments genetically required vitamin B12 for growth. Even in this case, a linear profile of the Log plot was observed. The growth rate can be mathematically represented as,

$$\frac{dx}{dt} = \mu \times x \quad \text{..... (9)}$$

On integrating we obtain,

$$\ln x = \mu \times t \quad \text{..... (10)}$$

Thus, the above equation can be used to determine the value of 'μ' at different concentrations of vitamin B12 by plotting ln(x) versus t (Figures 5a and b). The same samples that were used to determine CFU using agar plates were also used to determine the viability using the slope of MBRT. Figures 5c and 5d show the Log of slope of decoloration versus time. As seen in this case of the inhibition assay, the log of MBRT slope also demonstrated a linear profile. Thus, the slope of decoloration can also be used to determine the growth rate 'μ' to a li-

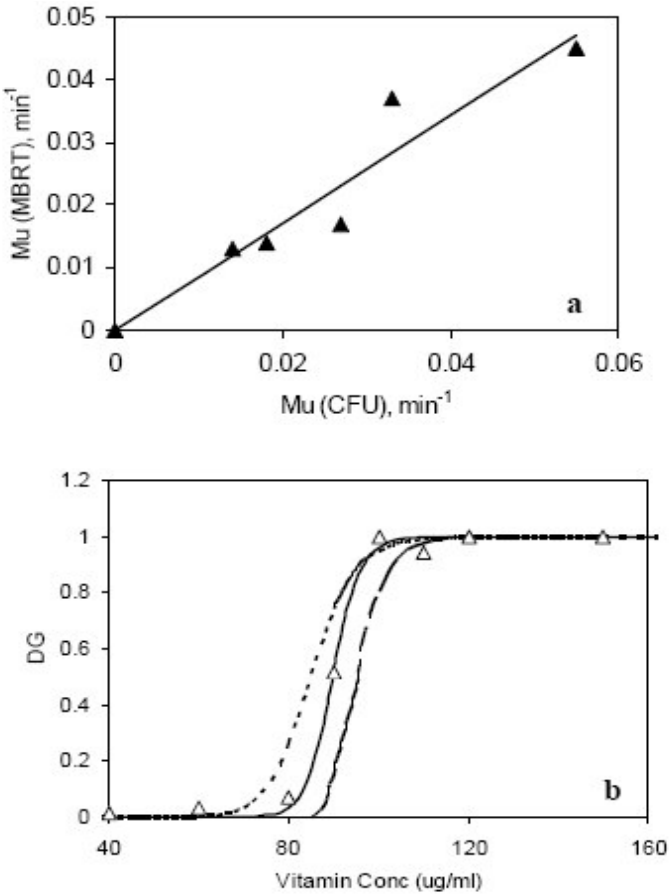

Figure 6 (a) Comparison of the values for Specific growth rate (μ) for *E. coli* 113-3D obtained through the agar plate method and MBRT. It can be noted that the values for 'μ' i.e. on the line with a slope of 45^0 indicating a good match. The standard deviation for the rate constant obtained through plate method was higher by 1.25 fold as compared to that for MBRT. **(b)** The dose-response curves for different exposure times. The plot shows the Extent of Growth (E_G) on the y-axis and the vitamin B12 concentration on the x-axis. The exposure times were 1h (dashed line), 2 h (solid line) and 4 h (dotted line). The symbol (Δ) represents the calculated E_G values using experimental values of the specific growth rate. The lines represent the fit obtained using Hills equation.

miting activator. Figure 6a shows the growth rate 'μ' evaluated through CFU measurement and also through the slope of decoloration. It can be seen that both the methods yield similar values of growth rate since the points lie around the 45° line.

The growth rate can be used to define the extent ofgrowth for different times at various concentrations of Vitamin B12. If an organism is grown in a media containing a specific activator concentration for a specific time, then the Extent of Growth (E_G) can be defined as,

$$E_G = \frac{N}{N^*} \quad \text{..... (11)}$$

Where, N is the number of cells at the end of time 't' at a given concentration of the vitamin B12 (activator) and N^* is the maximum number of cells formed at the end of time 't' for saturation concentration of the activator. Thus,

$$E_G = \frac{N_0 \cdot e^{\mu t}}{N_0 \cdot e^{\mu_{\max} t}} = e^{(\mu - \mu_{\max})t} \quad \text{..... (12)}$$

The above equation can be used to determine the extent of growth (E_G) for a specific vitamin B12 concentration at the end of time 't'. E_G will be dependent on the specific growth rate (μ) obtained through the slope of MBRT. Figure 6b shows the values of E_G for *E. coli* obtained at

different exposure times using the growth rate at different vitamin B12 concentrations. E_G also shows a typical dose-response curve as shown for extent of death (E_D) and can be fitted by Hills equation (similar to equation 7),

$$E_G = \frac{V^n}{K_G^n + V^n} \quad \text{..... (13)}$$

Where, V represents vitamin B12 concentrations while the parameters *n* and K_G represent the Hills coefficient and half saturation constant, respectively.

As expected, the dose-response curves for E_G was dependent on the growth time. Increase in the growth time yields high cell counts resulting in the shift of the dose response curves to the right. Thus, K_G, the half Saturation constant were determined to be 84.8, 89.7 and 91 (µg/ml) for growth times of 1, 2 and 4 h respectively.

The value of K_G can be defined to be Minimum Activating Concentration (MAC) essential for growth. Thus, K_G can be equivalently defined as MAC_{50}. The value of '*n*' was determined to be 17.6, 29.6 and 88 for growth times of 1, 2 and 4 h, respectively. Thus, the dose-response curves indicated that growth of *E. coli* demonstrated a switch like dependency on vitamin B12 concentration. Similar to MIC_{90}, a parameter MAC_{90} can be defined for relating vitamin B12 required for 90% of the maximum growth.

$$MAC_{90} = 9^{\frac{1}{n}} . MAC_{50} \quad \text{..... (14)}$$

The MAC_{90} of vitamin B12 necessary for the growth of *E. coli* was 96.6, 96.1 and 93.3 (µg/ml) for 1, 2 and 4 h of growth time respectively. MAC_{90} captured the effects of both the hill coefficient ('*n*') and MAC_{50}. It can also be noted that MAC_{90} was maximum for growth time of 2 h, being less for 4 hrs due to higher ultra sensitivity at higher growth times. Thus, a value of 96.6 (µg/ml) of MAC_{90} for vitamin B12 can be used as a global value for *E. coli* that represents the amount of activator required for growth.

DISCUSSION

Antibiotic and exhibition assays were performed to evaluate the Minimum Inhibitory Concentration (MIC) and Minimum Activating Concentration (MAC), respectively. Typically agar plates or antibiotic zone methods are used to determine the value of MIC. These methods have a high variability and consume lot of time. Here the MBRT was effectively used to determine the values of MIC or MAC. The method presented here not only enumerated the amount of inhibitory or activation concentration but also the sensitivity (in terms of Hill Coefficient) of the effect of antibiotic or vitamin B12 on an organism.

The dynamics of death (in case of inhibitor) and growth (in case of an activator) were quantified in terms of rate constants using MBRT. The values of the rate constants were comparable to that obtained from CFU enumerated using agar plate method. However, the variability in the estimation was lower in the case of the MBRT method as compared to that obtained from plating. Also, the time required for evaluating the rate constants was less for the MBRT as compared to that using CFU (> 24 h). Further, the rate constants were used to determine the Extent of Death (E_D) or Extent of Growth (E_G) as the case may be. These definitions help in characterizing the dose-response curves for death and growth using an antibiotic or a growth factor, respectively.

The dose-response curves were fitted using Hills equation which characterized the dose-response curves through a sensitivity parameter (Hills Coefficient) and the MAC_{50}/MIC_{50} value (the half saturation constants). The Hills Coefficients characterized the steepness of the dose-response curve, while the half saturation constant determined the amount of activator/inhibitor required for 50% response. The Hills Coefficient for both the effect of antibiotic and vitamin B12 on death and growth of *E. coli*, respectively demonstrated a sensitivity response with $\eta_H > 1$. The effect of vitamin B12 on growth was move switch like as compared to that of antibiotic on the death of *E. coli*.

Similarly, the MIC_{50} for antibiotic (~ 0.3 µg/ml) was order of magnitude lower than that for MAC_{50} for vitamin B12 (~ 96.6 µg/ml). It was also observed that MIC_{90} or MAC_{90} that is the amount of activator/inhibitor required for 90% response captured both the effect of steepness and the half saturation constant and can be a better measure of the amount of activator/inhibitor required for effective response. The methodology presented here is general and can be easily adapted to other systems. The use of MBRT to evaluate viability can decrease the experimentation time with a higher accuracy of measurement. The dose-response curves for extent of death / growth can be used to establish the MIC90 or MAC90 for an inhibitor or an activator, respectively. The use of kinetic constants to determine the MIC/MAC values establishes a rational basis for determination of these constants instead of just being an empirical definition.

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