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DESTRUCTION OF BIOLOGICAL WEAPONS BY GAMMA RADIATION

İbrahim TÜKENMEZ[1], Özge Birden KIRCI[2]

1-Prof. Dr., Gazi University, Faculty of Engineering, Department of Chemical Engineering, Ankara, Turkey, 06570

2- M.Sc. Student, Gazi University, Graduate School Of Natural And Applied Sciences, Teknikokullar, Ankara, Turkey, 06500

Corresponding author: ibrahim.tukenmez@gazi.edu.tr

BİYOLOJİK SİLAHLARIN GAMA RADYASYONUYLA İMHASI

Abstract:

The mechanistic aspects of the radiation induced inactivation process of microorganisms were analyzed; a reaction mechanism was proposed, and accordingly a dose-dependent inactivation kinetics was developed for the inactivation and sterilization of microbial weapons. The kinetic model was validated using experimental survival values of *Bacillus anthracis 34F2 sterne*. Dried samples of *Bacillus anthracis 34F2 sterne* spores mixed with non-fat milk were irradiated at 0-31 kGy gamma radiation doses at 25 ^{o}C and then eluted with buffered peptone water, and plated on tryptic soy agar by the pour technique in petri dishes. The plates were then incubated at 37 ^{o}C for 18-24 h and microorganism colonies were counted. By using the experimental survival data in the developed kinetic model, the inactivation parameters including initial cell number in a microorganism colony (n_o: 3.17 cells/CFU), radiation sensitivity (G: 0.45 kGy^{-1}), threshold dose (D_e: 2.59 kGy), decimal reduction dose (D_{10}: 5.18 kGy), sterility assurance dose (D_{SAL}: 31.05 kGy), and minimum sterilization dose (D_{MRD}: 62.10 kGy) were evaluated. These parameters were correlated with each other and the proposed mechanism and their values were compared with literature. By using the numerical values of kinetic parameters in the model equation, the dose dependent inactivation kinetics of *Bacillus anthracis 34F2 sterne* was simulated and compared with experimental data. Correlation coefficient (r: 0.9709) of the inactivation line indicated that the kinetic equations and corresponding mechanism were acceptable ($p<0.01$) for describing the dose dependent inactivation processes of *Bacillus anthracis 34F2 sterne*. It is concluded that developed model and model parameters can be used for the process efficacy control and dose setting for radiation sterilization of biological weapons.

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Özet:

Mikroorganizmaların radyasyona bağlı inaktivasyon prosesinin mekanistik özellikleri analiz edildi; bir reaksiyon mekanizması önerildi ve bu mekanizmaya uygun olarak mikrobiyal silahların etkisiz hale getirilmesi ve sterilizasyonu için doza bağlı bir inaktivasyon kinetiği çıkarıldı. Kinetik model, *Bacillus anthracis 34F2 sterne'*in canlı kalan deneysel konsantrasyon değerleri kullanılarak doğrulandı. Yağsız süt ile karıştırıldıktan sonra kurutulmuş *Bacillus anthracis 34F2 sterne* örnekleri, 25 °C'de 0-31 kGy gama radyasyon dozlarında ışınlandı ve daha sonra tamponlanmış peptonlu suyla yıkanarak alındı ve petri kaplarındaki triptik soy agar üzerine dökme yöntemiyle kaplanarak ekildi. Petri kapları daha sonra 37 °C'de 18-24 saat inkübe edildi ve mikroorganizma kolonileri sayıldı. Geliştirilen kinetik modelde deneysel canlı kalan koloni verileri kullanılarak, bir mikroorganizma kolonisinde başlangıçtaki hücre sayısı (n_o: 3.17 hücre / CFU), radyasyon hassasiyeti (G: 0.45 kGy^{-1}), eşik dozu (D_e : 2.59 kGy), ondalık azalma dozu (D_{10}: 5.18 kGy), sterilite güvence dozu (D_{SAL}: 31.05 kGy) ve minimum sterilizasyon dozu (D_{MRD}: 62.10 kGy) hesaplandı. Bu parametreler birbirleriyle ve önerilen reaksiyon mekanizmasıyla ilişkilendirildi, değerleri başka çalışmalarda rapor edilmiş değerlerle karşılaştırıldı. Model eşitliğinde kinetik parametrelerinin sayısal değerleri kullanılarak *Bacillus anthracis 34F2 sterne'*in doza bağımlı inaktivasyon kinetiği simüle edildi ve deneysel verilerle karşılaştırıldı. İnaktivasyon doğrusunun korelasyon katsayısı (r: 0.9709), kinetik denklemlerin ve karşılık gelen mekanizmanın *Bacillus anthracis 34F2 sterne'*in doza bağlı inaktivasyon proseslerini tarif etmek için uygun olduğunu ($P < 0.001$) gösterdi. Önerilen model ve model parametrelerinin biyolojik silahların radyasyonla sterilizasyonu için proses etkinliği kontrolü ve doz ayarlamada kullanılabileceği sonucuna varıldı.

Key Words: Anthrax, Bacillus anthracis 34F2 sterne, bilogical weapon, radiation sterilization, inactivation kinetics, decimal reduction dose, sterility assurance level, minimum sterization dose

Anahtar Kelimeler: Şarbon, Bacillus anthracis 34F2 sterne, biyolojik silah, radyasyon sterilizasyonu, inaktivasyon kinetiği, ondalık azaltma dozu, sterilite güvence seviyesi, minimum sterilizasyon dozu

1. Introduction

A biological weapon (also called biological agent, bio-agent, biological threat agent, biological warfare agent, or bioweapon) is microorganism such as a bacterium, virus, protozoan, parasite, or fungus that can be used purposefully as a weapon in bioterrorism or biological warfare. Biological warfare also known as germ warfare is the use of microorganisms such as bacteria, viruses, and fungi or their toxins with the intent to kill or incapacitate humans, animals or plants as an act of war (Wheelis, Rózsa and Dando, 2006). Biological weapons are easily produced, stored, transported, distributed, difficult to detect and have significant mortality and morbidity making them appeal to terrorists. A biological weapon consists of 4 major parts: biological agent, protective armor, carrier system and spreading mechanism. Propagation is linear or point welded (David and Sauzanne, 2003). Linear spreading technique is the most effective technique. A truck or aerial vehicle moves perpendicular to the wind and emits the biological agent. The biological agents that hang in the air collapse at dawn, evening or night and they spread in wide area. The spot-induced propagation ways may be a detonation of a bomb, the introduction of a biological agent into

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aeration or drinking water, or an agent in envelopes. No matter how the way is spread, victims are contaminated in three ways: skin, gastrointestinal system, and the lung. (David and Sauzanne, 2003; William, Deanna and Jay, 2002).

Anthrax (Anthrax germ) is a spore forming aerobic bacterium. *Basicillus Anthracis* can withstand disinfectants, changes in temperature and humidity as a result of spore forming properties. For this reason, anthrax spores can stay alive for many years. Anthrax bacillus as a biological weapon is used as a durable form of sports and it is ensured that target masses receive spores through respiration. It can be detected in blood, cerebrospinal fluid (CSF), vesicular fluid and infected tissue. Contamination occurs from infected herbivorous animals, ungulate mammals and their products. There is no transmission from human to human. The anthrax bacillus used in bioterrorism is sensitive to all antibiotics except cephalosporin. In recent years, ciprofloxacin has been recommended since some countries are known to produce resistant strains. Anthrax will remain active in the soil for at least forty years and is resistant to environmental conditions. It can takes years for the effects of biological warfare agents to disappear. For this reason, the microorganisms which are used as the biological weapon in the environment, should be killed and sterilized.

Destruction of biological weapons is possible with the chemical and physical extermination methods used to kill microorganisms. At the same time, they destroy the environment and other living and non-living things in the environment (process damage) and creates pollution residue (Gazso and Ponta, 2005)**.** Therefore, in this study, radiation sterilization of dry antrax microbe spores were studied by applying ionizing radiation for an effective and non-destructive elimination of anthrax microbe. Besides, the mechanistic and kinetic aspects of the radiation induced inactivation process of microoganisms were studied. Then, the process kinetics was developed and kinetic parameters were used for process efficacy control, dose setting and the design of the proper irradiation process for inactivation and sterilization of anthrax and similar microorgansms.

2. Materials and Methods

The Sterne 34F2 vaccine strain (obtained from the International Biological Standards Laboratory, Weybridge and currently used in the EVMKAE Anthrax Vaccine Production Laboratory) was produced in Casein Digest according to the World Health Organization's international spore vaccine production method (WHO, 1967). Using FTS, by collecting growing microorganisms on the surface the germ suspension was obtained. In order to eliminate the vegetative forms of *anthrax bacillus*, the sample was kept in a 60 ºC water bath for 20 min. Then the spores were separated from the media by centrifugation at 3000 rpm for 20 min. After washing twice with FTS, 10-fold dilutions of the sediment were made and sport counts were made by casting method (Halkman and Ayhan, 1999). 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} dilutions were made from 10% v/w suspension of non-fat milk powder in distilled water and was divided into 3 ml in 15 ml neutral bottles. In the EDWARDS Mini-Fast MOD 3400 freeze dryer, lyophilization was performed from -40 ºC to -20 ºC for 24 hours, from -20 ºC to +30 ºC for 10 hours (Lefevre, 1977). Dried samples of *Bacillus anthracis 34 F2* mixed with non-fat milk powder were irradiated in triplicate at 0, 4,81; 10,33; 15,76; 21,93 and 30,85 kGy doses of gama radiation with 360 Gy/h dose rate at 25ºC, 1 atm. The irradiation process carried out in experimental

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laboratory irradiator (Ob-Servo Sanguis Co-60 Research Radiator model) in Radiation and Acceleration Technologies Department of Turkey Atomic Energy Authority (TAEK).

Following irradiation, *Bacillus anthracis 34 F2* spores were eluted with buffered peptone water and diluted ten-fold and aliquots of dilutions were plated in triplicate on tryptic soy agar in 100x25 mm petri dishes by the pour plating technique. The plates were then incubated at 37 ^{o}C for 12 h and surviving microorganism colonies were counted. For each dose, irradiated samples and controls (0 kGy) were maintained under the same conditions without irradiation. Counting results were performed by taking the average of three samples studied as parallel. Counts (CFU) corresponding to applied doses were converted to the concentration of bacteria by dividing the weight of the sporte suspensions (CFU/g; Colony Forming Unit / g). Then, the average bacterial concentration (CFU/g), that is averaged of parallel three samples, was used in the model equation to find the numerical values of kinetic parameters and to investigate the success of fit between kinetic model simulation and experimental data.

3. Results (equation numbers should be in same level), please corrected all)

With direct effect of radiation, damage (*) occurs in the healthy cell (A) as a result of ionization, and endogenous repair enzymes (E) in microorganism are stimulated simultaneously in microorganisms. Thus, a damaged live intermediate microorganism (*AE) is formed. In this step, free radicals (R·) are formed as a result of radiation effects in both healthy cells and water, protein, lipid, carbohydrate and other molecules (RH) associated with cells. Thus, the radiation induced reaction in this initial reaction was expressed with reaction yield (k_1, 1/kGy) as follows.

$$A + RH \xrightarrow{k_1} {}^{*}AE + R\cdot \qquad (1)$$

In the indirect effect of radiation, the reactions of free radicals with healthy cells cause damage to the healthy cell and the simultaneous stimulation of endogenous repair enzymes (E) in the cell:

$$A + R\cdot \xrightarrow{k2} {}^{*}AE + R'H \qquad (2)$$

in the Equation (2), k_2 is rate constant in the propagation reaction, (R'H) is a stable radiolysis product, which is formed by (R·)'s. If radiation damage is successfully repaired with the radiation-induced repair endogenous enzymes (E) in the damaged cell (*AE), the cell becomes healthy cell again. Hence (*AE) is an intermediate state. As seen in the reaction in Equation (3), if the cell successfully repairs the radiation damage, it is transformed into healthy cell (A) and remains viable. If not, the cell dies as shown in the reaction in Equation(4).

$$^{*}AE \xrightarrow{k3} A \qquad (3)$$

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$$*AE \xrightarrow{k4} A_d \qquad (4)$$

Here k_3 and k_4 are rate constants. A_d is dead cells. In Equation (1), applied on the kinetic approach developed by Tükenmez et al., (1997) the rate equations according to the above reaction equations can be written as follows:

$$\frac{dC_A}{dt} = - k_1 C_A C_{RH} \frac{d}{dt} \left(\frac{\partial E}{\partial W}\right)_{T,V} - k_2 C_A C_{R\cdot} + k_3 C_{*AE} \qquad (5)$$

$$\frac{dC_{*AE}}{dt} = k_1 C_A C_{RH} \frac{d}{dt} \left(\frac{\partial E}{\partial W}\right)_{T,V} + k_2 C_A C_{R\cdot} - (k_3+k_4) C_{*AE} \qquad (6)$$

$$\frac{dC_{R\cdot}}{dt} = k_1 C_A C_{RH} \frac{d}{dt} \left(\frac{\partial E}{\partial W}\right)_{T,V} - k_2 C_A C_{R\cdot} \qquad (7)$$

Here, C_A and C_{*AE} are colony concentrations of A, and *AE, respectively (CFU/g; colony forming unit/gram); C_{RH} and $C_{R\cdot}$ concentrations of components RH and ($R\cdot$) respectively (mol/g). t is irradiation time (hours), and E in the paranthesis is the average radiation energy (kJ) absorbed by the mass (W, kg) in the irradiated unit volume element. Radiation dose (D, kGray, kGy = kJ / kg), at constant temperature (T) and constant volume (V):

$$D = \frac{d}{d} \left(\frac{\partial E}{\partial W}\right)_{T,V}$$

For the dose rate (dD / dt, kGy/hour) that expresses the amount of radiation energy absorbed by the unit mass per unit time:

$$\frac{d}{d} \left(\frac{\partial E}{\partial W}\right)_{T,V} = \frac{dD}{d} \qquad (8)$$

can be written. This statement is put in place in Equaation (5), (6) and (7). When the steady state is applied for intermediate products *AE and $R\cdot$, the concentration change rates given in Equation (6) and Equation (7) will be $dC_{*AE}/dt = 0$, $dCR\cdot/dt=0$.

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$$k_1 C_A C_{RH} \frac{dD}{dt} - k_2 C_A C_{R\cdot} = 0; \quad k_2 C_A C_{R\cdot} = k_1 C_A C_{RH} \frac{dD}{dt} \qquad (8.1)$$

after substituting the Equation (8.1) in Equation (6) and applying steady state gives

$$2\, k_1 C_A C_{RH} \frac{dD}{dt} - (k_3 + k_4) C_{*AE} = 0$$

Where, C_{*AE} is obtained in terms of measured quantities as Equation (9)

$$C_{*AE} = \frac{2k1}{k3+k4} C_A C_{RH} \frac{dD}{dt} \qquad (9)$$

After substitution Equation (9) in Equation (5) and when both sides of the equation are divided by dD/dt, a dose dependent inactivation kinetics was obtained as following:

$$\frac{dC_A}{dD} = -2\, k_1 \left(1 - \frac{k3}{k3+k4}\right) C_A C_{RH} \qquad (10)$$

As irradiation is a non-destructive process (Diehl J.F.,1990), since the concentration of macro-components such as water, proteins, lipids and carbohydrates in association with healthy microorganisms (RH) is much greater than that of microorganisms, ($C_{RH} >> C_A$), C_{RH} will be very low compared to change in exchange C_A. In this case, equation (10) can be shown as zero-order $(C_{RH})^0$ with respect to C_{RH} in this equation considering that change in (dC/dD) is not affected. In Equation (10), velocity coefficients can be grouped as follows:

$$G = \left(\frac{2k1}{1+k3/k4}\right) \qquad (11)$$

In Equation (10), substituting $(C_{RH})^0 = 1$) and G coefficient in Equation (11) and by using C without A sub-index in C_A for living microorganism colony concentration, the dose-dependent inactivation kinetics that can be used for inactivation and sterilization of microorganisms:

$$\frac{dC}{dD} = -GC \qquad (12)$$

Here G is defined as dose dependent inactivation yield or sensitivity in unit (1/kGy). The coefficient G indicates the reduction fraction in live microorganisms per absorbed unit

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dose. Equation (12) is divided into variables, and integrated with the initial conditions at D = D_e (D_e is threshold dose) C = C_0,

$$\int_{Co}^{C} \frac{dC}{C} = -G\int_{De}^{D} dt$$

$$ln(C/C_0) = GD_e - GD \quad (13)$$

Threshold dose (De) is the dose at which inactivation begins to be observed. In Equation (13)

when D = 0, C = n_oC_0 in ordinate;

$$ln(n_o) = GD_e \quad (14)$$

$$\log(n_o) = \frac{G}{2{,}303}D_e \quad (14.1)$$

$$n_o = \exp(GD_e) \quad (14.2)$$

gives cell number (n_o) in a colony at the beginning. When Equation (14) is used in Equation (13) and rearranged it;

$$C = n_oC_0exp(-GD) \quad (15)$$

gives the concentration (C) of viable cells at an irradiated dose (D). After this equation is rearranged and subtracted from 1, in obtained equation $1-C/Co=1-n_oexp(-GD)$ the term on the right can be written as $(1-exp(-GD))^{no}$ by employing binomial theorem, and it gives the dose variable form of classical target theory expression (Lea, 1955) as

$$C/Co = 1 - (1-exp(-GD))^{no} \quad (16)$$

Equation (13) can be converted to logarithmic form as follows;

$$log\frac{C}{Co} = \frac{G}{2.303}x\ De - \frac{G}{2.303}x\ D \quad (17)$$

$$log\frac{Co}{C} = \frac{G}{2.303}x\ D - \frac{G}{2.303}x\ D_e \quad (17.1)$$

With Equation (17.1), a linear regression process using experimental $\log(C_o/C)$ data against the irradiation doses (D) gave a straight line with a slope (b = 0.1932) and ordinate intercept (a = -0.5011) and a correlation coefficient (r=0.9709) in Figure 1. as

$$log\frac{Co}{C} = 0.1932x\ D - 0.5011, \quad (r = 0.9709) \quad (18)$$

Inactivation yield (G) in Equation (17.1) from the slope of the regression line in Equation (18):

G=2.303 x b=2.303 x 0.1932 = 0.4449 kGy^{-1},

Threshold dose (D_e) from ordinate intercept:

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D_e= (2.303 x a)/G= 2.303 x 0.5011)/0.4449 = 2.5935 kGy were calculated (Table 1.)

The numerical values of the dose dependent inactivation kinetics parameters evaluated as: (n_o) was calculated from Equation (14.2). In Equation (17) Decimal reduction dose (D_{10}) was evaluated by using (C/Co):1/10 at D=D_{10} (D>D_e);

$$D_{10}=2.303/G \qquad (19)$$

Which is reported as D_{10} in linear region of inactivation graph Figure (2). Using (C/Co):1/10 at D>0

$$D_{10}= D_e + \frac{2.303}{G} \qquad (19.1)$$

by which, the D_{10} is calculated by taking D_e into account. Likewise, sterility assurence level

(D_{SAL}) and minimum radappertization (sterilization) dose (D_{MRD}) values were calculated by using (C/Co):$1/10^6$ and $1/10^{12}$ at D>De (De=0) D = D_{SAL} and D_{MRD} respectively, and D>0 by (D_{SAL}) and minimum radappertization (sterilization) dose (D_{MRD}) values were calculated by using (C/Co):$1/10^6$ and $1/10^{12}$ at D>De (De=0) D = D_{SAL} and D_{MRD} respectively, and D>0 by adding De are given in Table 1.

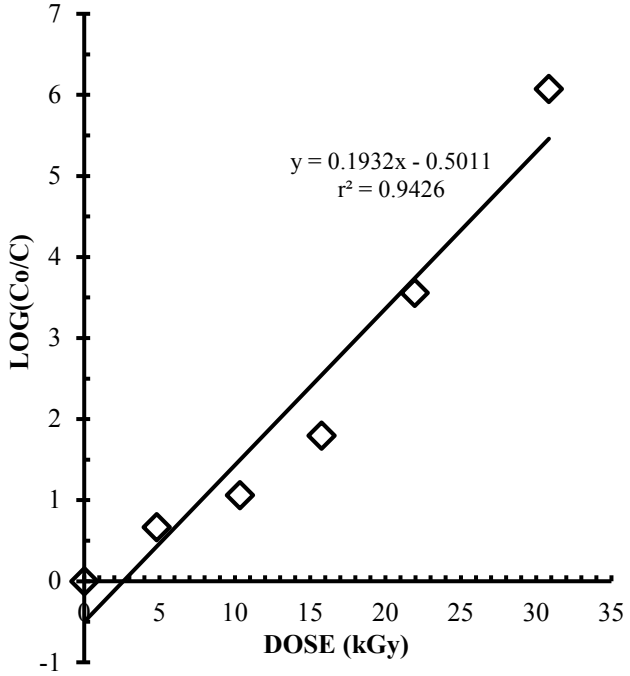

Figure 1. Evaluation of the parameter values (yield from slope, and threshold dose from abscissa intercept) and comparison of the dose dependent inactivation kinetics model (solid line) of with experimental survival data of *Bacillus anthracis* $34F_2$

By using numerical values of G and (n_o) in Equation (16);

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$$C/Co=1-(1-\exp(-0.4449D))^{3.1697} \quad (20)$$

Equation (20) are derived; increasing the amount of irradiation dose D values gave the surviving microorganism fraction (C/C_o) in semi-logarithmic graph as seen in Figure 2. gave the simulation of the dose dependent inactivation kinetics of *Bacillus anthracis* $34F_2$.

As shown in Figure 1 and Figure 2, there is a good agreement between the experimental data shown with symbols and the kinetic model simulation lines. As seen in Table 1, correlation coefficients (*r: 0.9709*) of the inactivation lines in Eq.(17) indicated that the kinetic equations in Equations (5)-(16) and the corresponding mechanism in Equations (1) - (4) were convenient*($p<0.01$)* for describing the dose dependent inactivation and sterillization kinetics of the microorganisms such as *anthrax bacteria* to be used as biological weapons.

Table 1. Calculated values of kinetic parameters of radiation induced inactivation of *Bacillus anthracis* $34F_2$

PARAMETER	**VALUE**
N	6
a	-0.5011
b	0.1932
r	0.9709
C_0 (CFU/g)	3.61E+10
G(kGy^{-1})	0.4449
D_e (kGy)	2.5935
n_o (Number of Cell/CFU)	3.1697
D_{10} (kGy), ($D>D_e$)	5.1755
D_{10} (kGy), ($D>0$)	7.7690
D_{SAL} (kGy) ($D>D_e$)	31.053
D_{SAL}(kGy), ($D>0$)	33.6521
D_{MRD}(kGy), ($D>D_e$)	62.100
D_{MRD}(kGy), ($D>0$)	64.6996
Significance	($0.01<P$)

When substituting G value from Equation (19) in Equation (14.1), a useful relationship among n_o, G, D_e, and D_{10} can be proved from the developed kinetic model as following:

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$$\log(n_o) = \frac{G}{2.303} D_e = \frac{De}{D10} \quad (21)$$

Which states that radiation resistances (D_{10}) of microorganisms increase with threshold dose (D_e), decreases with sensitivity (G) and cell number per colony (n_o).

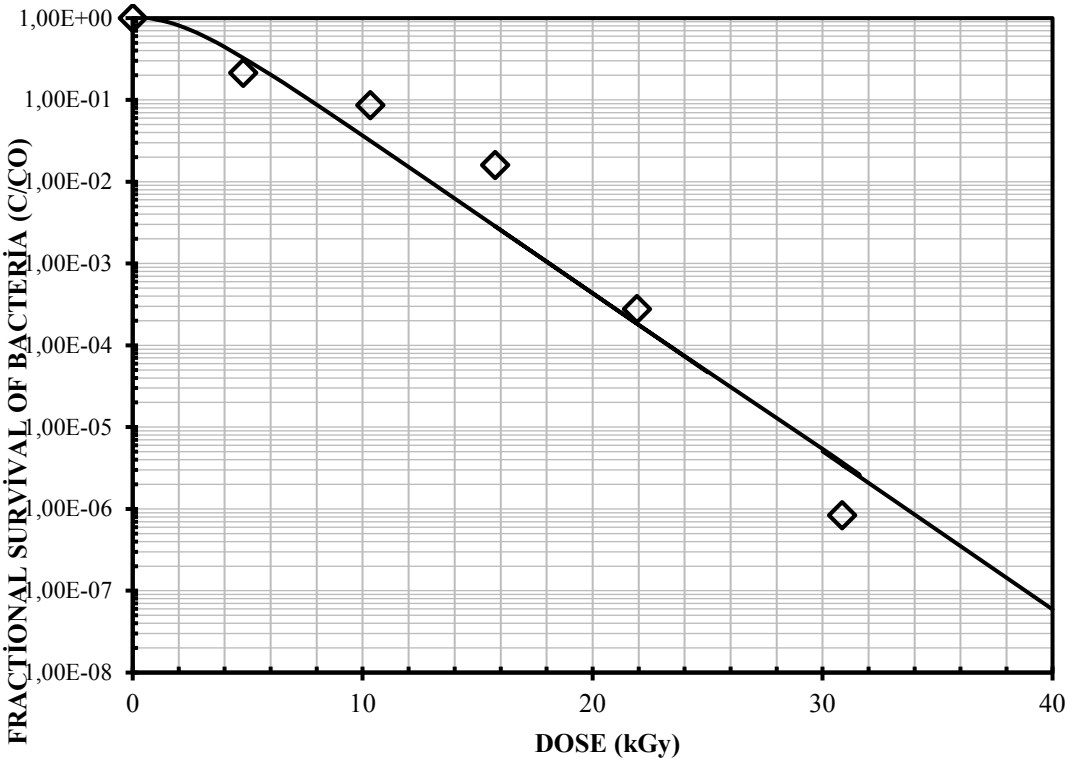

Figure 2. Dose-dependent inactivation kinetics with gamma radiation of *Bacillus anthracis 34F2* (Symbols show experimental data, line refers to fitted one)

When examined Equation (11) together with Equation (19), the following equation is obtained;

$$G = \frac{2.303}{D10} = \left(\frac{2k1}{1+k3/k4}\right) \quad (22)$$

Where, unlike G, D_{10} decreases with k_1 and k_4, and increases with k_3 as implied in proposed mechanism given by Equation (1) - (4).

Gazsoi and Gyulai (2004) reported that the dose range of D_{10} values for viruses: a) for ribovirols: 3.5-4.7 kGy, b) for Doxyvirals: 3.6-4.7 kGy; D_{10} values for bacteria, a) for Gram-negative bacteria: 0.02-2.49 kGy, b) For Gram-positive bacteria: 0.14-0.68 kGy; D_{10} values for spore-forming bacteria, a) for spore-forming aerobic bacteria: 0.84-5.5 kGy, b) for anaerobic spore forming bacteria: 1.75-13.28 kGy. In the same study, D_{10} was reported as 2.8-5.50 kGy for the anthrax *Bacillus anthracis strain* among the aerobic spore forming bacteria. In our study,

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the D_{10} is obtained as 5.18 kGy value (Table 1) that we found for the *Bacillus anthracis sterne 34F2* strain of anthrax is in this range and it was found to be consistent with D_{10} values found by different evaluation methods.

4. Conclusion

Consequently, this study showed that the proposed reaction mechanism and the kinetic equations based on that mechanism is described well the radiation inactivation kinetics for anthrax microbe and other similar resistant microorganisms to be used as biological weapons. It is concluded that developed model and model parameters may be used for the process efficacy control and dose setting for radiation sterilization of biological weapons.

5. Acknowledgments

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