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Comparison of sterilization reaction rate for *Bacillus subtilis* and *Escherichia coli* using UV-visible spectroscopy

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ABSTRACT

Sterilization is a process that eliminates or kills all microbes, including transmissible agents present on a surface, contained in a fluid, medication or biological culture media (UCLA Department Epidemiology). Dry heat sterilization is one of the important methods of eliminating microbes, as it coagulates the proteins in an organism, causing oxidative free radical damage thus leading to cell death. Heat is lethal to micro organisms, but each species has its own particular heat tolerance. This study is based on effect of sterilization, at varied time periods as well as constant temperature and pressure conditions, on sporulating and non-sporulating bacteria. Bacillus subtilis and Escherichia coli cultures were inoculated in nutrient media and incubated. After incubation, the concentration of bacterial cells in the media, before and after sterilization at 80°C in hot air oven, was found out. Bacterial cultures were subjected to sterilization and OD values of both the cultures were taken at an interval of 10 minutes, using UV Spectrophotometer. The reaction constant (K) and sterilization reaction rates were calculated from the obtained data, for both cultures and an increased sterilization rate was observed for E.coli. A reaction equation was also designed for sterilization reaction for both cultures. This study would help us understand the dynamics of sterilization and methods of varying specified conditions in order to increase the rate of sterilization of pathogenic, sporulating bacteria.

Keywords: Dry heat sterilization, bacterial cultures, reaction constant, sterilization reaction rate, reaction equation

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INTRODUCTION

To determine proper conditions for thermal processing of media which would ensure product sterility and quality, it is necessary to know both thermal inactivation of microorganisms that are present in the medium and the parameters which determine their heat resistance. Therefore, in the case of thermal destruction of microorganisms, such factors as the physiological state of cells and chemical and physical characteristics of the medium in which spores are suspended, should be taken into account. Due to high heat resistance of bacterial spores, their inactivation is very difficult when their nutrient values are to be retained [1].

Certain microbes like *Bacillus* species are seen to be more tolerant to heat, given to their spore forming property. Endospores can survive without nutrients. They are resistant to ultraviolet radiation, desiccation, high temperature, extreme freezing and chemical disinfectants. Endospore formation is usually triggered by a lack of nutrients, and usually occurs in Gram-positive bacteria. Whereas, Gram-negative bacteria do not undergo sporulation and are therefore, more heat labile compared to spore forming bacteria. It has been known for years that, bacterial spores are more resistant to biocides than non-sporulating bacteria [2]. The bacterial spore is a more complex structure than a vegetative cell and consists of a spore-coat surrounding the cortex which is itself external to the spore core. The cortex controls the water content of the core, an important aspect in considering the resistance of spores to moist heat, dry heat and radiations.

Microbial inactivation is a kinetic process wherein the viability of organisms exposed to a biocidal agent varies as a function of time. Inactivation kinetics depends on the type of organism, type and concentration of biocide, environmental conditions such as temperature and pH. Death rates make it possible to compare the heat resistance of different species at the same temperature or the heat resistance of one species at different temperatures. It also enables us to describe in quantitative terms the effect of environmental factors such as concentration or pH, upon heat sterilization [3].

An opinion prevails that thermal inactivation of spores can be described by a single first order reaction [4][5]. In many references [6-8] as well as in former studies [9][10] it has been shown that the kinetics of thermal spores inactivation is closely connected with the physiological state of cells and in special cases only it can be described by the first order reaction [9]. Thermal inactivation of microbes follows a first order reaction due to death of the bacteria by protein inactivation.

Inactivation of this particular protein in bacterial cells forms the limiting step for sterilization reaction, thus the concentration of protein decreases with increased time of sterilization, at constant temperature. Therefore, sterilization reaction equation follows the first order:

$$-r_A = -dC_A/dt = KC_A$$

It is known that in spore population there are individuals who differ significantly in heat resistance. This follows from the fact that particular spores can be in different physiological states, i.e. activated, dormant and the so-called ‘‘super dormant’’ state. It is generally accepted that activated spores are more sensitive to disadvantageous environmental conditions than the dormant forms. It is also known that the state of spore dormancy can be interrupted by activation (a reversible process) which leads to the state enabling germination, i.e. growing in the form of colonies on proper substrates. On the other hand, spores destruction (inactivation) is a complete loss of their ability to live. When only activated spores are in the medium, thermal treatment will cause their destruction and the spore viability curve will be linear. In other cases, spores destruction in the semi-logarithmic system is curvilinear. Particular types of viability curves and equations that describe them can be found in literature [4][7][8].

EXPERIMENTAL SECTION

The methodology adopted can be broadly classified into pre and post-sterilization operations. Pre-sterilization operation included preparation of nutrient broth solution. 100 ml of nutrient broth solution was prepared by mixing 1.3 grams of nutrient broth in 100 ml of distilled water. 6 ml of nutrient broth solution was added in 13 test tubes labelled as TEST, $E_\circ$, $B_\circ$, E1, E2, E3, E4, E5, B1, B2, B3, B4 and B5 and all the test tubes were plugged with cotton. All the test tubes, along with nutrient broth solution were kept for sterilization (121°C, 30 minutes) to remove any unwanted microbes which could hinder the experimental results.

Fig.1:Sterilized conical flasks containing broth,under LAF

Fig2:Sterilized test tubes with nutrient broth,under LAF

Sterilized test tubes $E_\circ$, E1, E2, E3, E4 and E5 were then inoculated with 0.1 ml of *E.coli* culture each, while test tubes $B_\circ$, B1, B2, B3, B4 and B5 were inoculated with 0.1 ml of *B.subtilis* culture each. These test tubes were then placed in an incubator for 24 hours, to allow the two microbial populations to grow in number, in each test tube.

Meanwhile, standard nutrient broth curve was derived using UV-Vis spectroscopy. Test tube ‘TEST’ was used to take the spectroscopic readings of the nutrient broth at different concentrations of the broth. The optical density of nutrient broth solution was taken at 480 nm after standardizing the spectrophotometer using 1ml of distilled water. Volume 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of nutrient broth (1.0, 0.8, 0.6, 0.4, 0.2 ml of distilled water respectively)

were used and optical density values were recorded, using spectrophotometer. A standard OD_{480} Vs concentration (µg/ml) graph was plotted using the obtained data. Optical density values of nutrient broth inoculated with *E.coli* ($E_\circ$) and *B.subtilis* ($B_\circ$) culture were also recorded at 480 nm, before sterilization. Concentration of microbial cells before sterilization was deduced from the standard nutrient broth graph.

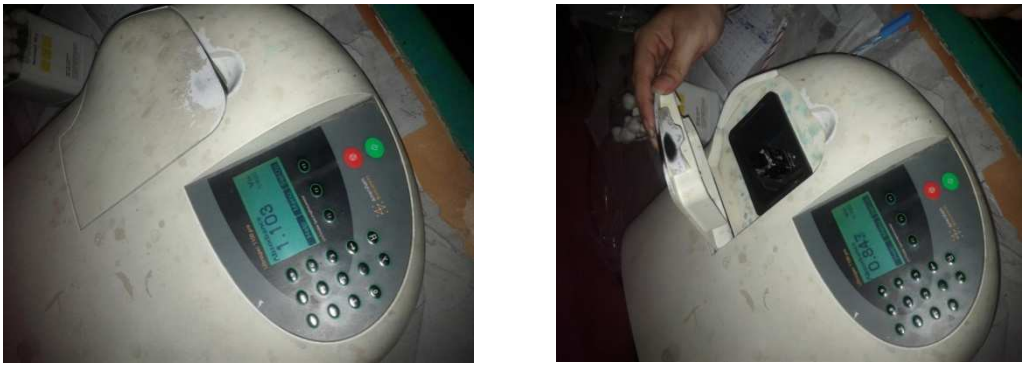

Fig3:The optical density values of inoculated nutrient broth using spectrophotometer

After 24 hours of incubation, the test tubes E1-E5 and B1-B5 were placed in a pre-sterilzed test tube stand.The test tube stand, with test tubes, was then placed in a hot air oven at 80°C for sterilization. Test tube E1 and B1 were taken out of the oven after 10 minutes and the OD values for both the cultures were taken at 480 nm. After another 10 minutes, test tubes E2 and B2 were taken out and the respective OD values were taken at 480 nm. The same procedure was followed for test tubes E3-B3, E4-B4 and E5-B5 at an interval of 10 minutes. All the OD_{480} values were tabulated and plotted on the standard nutrient broth graph. Using projection of point method, concentration of microbial cells in all the test tubes was calculated. These concentration values were then compared with initial concentration of microbial cells (before sterilization) and subsequently OD_{480} Vs time and concentration Vs time graphs were plotted. The rate of sterilization and sterilization reaction constant was then calculated for both microbial cultures, using first order kinetic equation:

$$-r_A = -dC_A/dt = KC_A$$

The sterilization reaction rates for *E.coli* and *B.subtilis* were compared and the results were analysed.

RESULTS AND DISCUSSION

Heat sterilization reaction conditions depend on a variety of factors like moisture content of the microorganism cells before and after the sterilisation, flow rate of gaseous atmosphere, physical and chemical composition of the surrounding medium of the cells, and total pressure of the system. [11].

The kinetics of media (inoculated with microbe species) sterilization describes the rate of destruction of micro organisms (by dry heat sterilization method) using a first order reaction rate model [12]. Death follows a logarithmic pattern thereby implying a monomolecular reaction [13].

An explanation for this phenomenon is that the first order or logarithmic death rate is due to an expression of a monomolecular reaction of protein penetration or damage essential to reproduction of microbes. It is must also be realized that microbial death is a result of the failure of the micro- organism to reproduce even when placed in a favorable environment and optimal recovery medium [14].

As the population of the microorganisms decreases with time, the rate is defined by the following equation:

$$\mathbf{-r_A = k.C_A = -dC_A/dt}$$

Where,
A = reactant: protein involved in sterilization reaction
C_A=concentration of A.
k = reaction rate constant.

After exposure to dry heat in the hot air oven, the following data was obtained using UV Vis-spectrometer:

Table 1: OD values of standard nutrient media and nutrient media inoculated with microbial cultures (before sterilization)

SNo.	Volume of Nutrient media (ml)	Volume of water (ml)	Concentration (µg/ml)	OD at 480 nm
1.	0.0 (Blank)	1.0	0.0	0.000
2.	0.2	0.8	20.0	0.229
3.	0.4	0.6	40.0	0.259
4.	0.6	0.4	60.0	0.392
5.	0.8	0.2	80.0	0.556
6.	1.0	0.0	100.0	0.690
7.	1.0 ml of nutrient media inoculated with E.coli culture (before sterilization,E_o)	0.0	207 (calculated from graph)	1.424
8.	1.0 ml of nutrient media inoculated 2.0 with B.subtilis culture (before sterilization,B_o)	0.0	254 (calculated from graph)	1.796

Values, as seen in the above table were plotted in graph representing concentration Vs OD value. From the standard nutrient media graph, we obtain values of concentration of the bacterial culture after equal interval of time .The following tables and graphs show the data related to the OD value and corresponding concentration of each of bacteria:

Table 2: OD values of nutrient media inoculated with B.subtilis culture at evey 10 minutes of sterilization at 80°C

S.No.	Sample	Time (minutes)	OD at 480 nm
1.	B1	10	1.759
2.	B2	20	1.560
3.	B3	30	1.495
4.	B4	40	1.475
5.	B5	50	1.366

Table 3: OD values of nutrient media inoculated with E.coli culture at evey 10 minutes of sterilization at 80°C

S.No.	Sample	Time (minutes)	OD at 480 nm
1.	E1	10	1.231
2.	E2	20	1.207
3.	E3	30	1.170
4.	E4	40	1.072
5.	E5	50	1.026

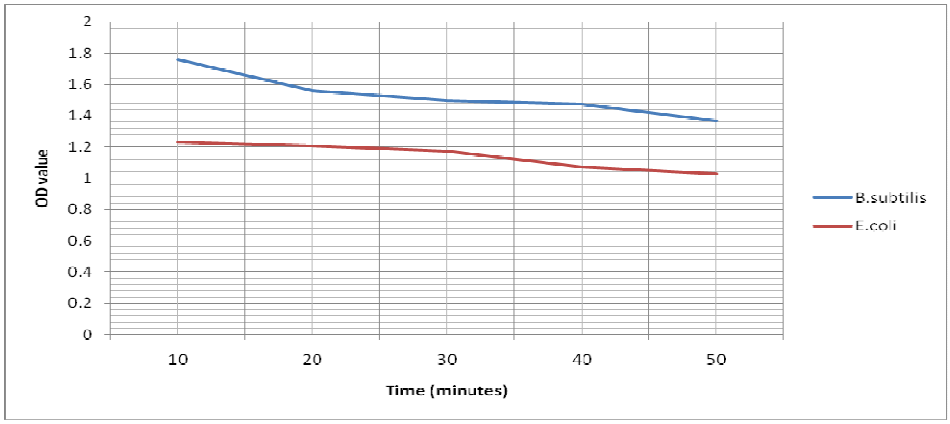

Fig. 4: OD_{480} Vs time graph of *B.subtilis* and *E.coli*

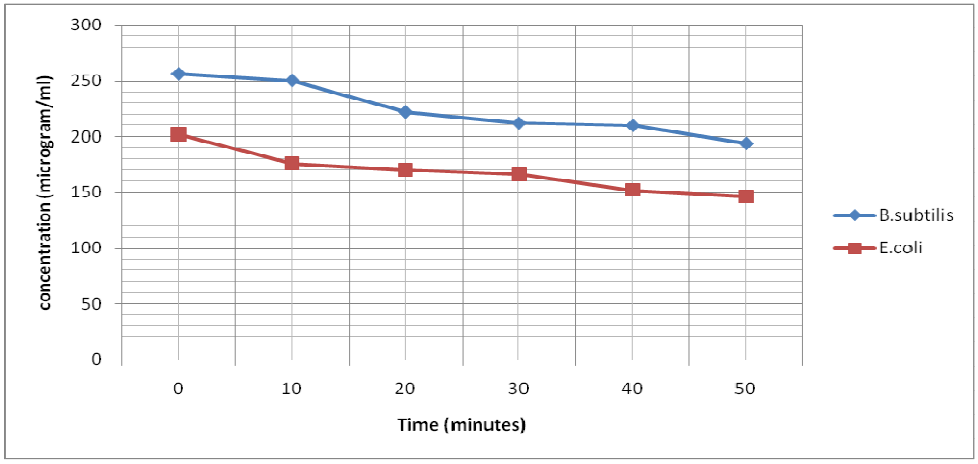

Fig. 5: Concentration Vs time graph for *B.subtilis* and *E.coli*

From the data obtained, we analyse that the sterilization for *E.coli* is more than that of *Bacillus subtilis.* From the logarithmic first order rate reaction we calculate and analyse the difference in rate constant and subsequently the difference in rate of sterilization reaction of the two bacterial cultures at 80^0C.

$$-dC_A/dt = k.C_A$$

Using separation of variables and integrating with initial condition, the following expression is obtained:

$$C_A(t) = C_{A0}.e^{kt}$$

Taking natural logarithm of the above expression,

$$\ln C_A(t) / C_{A0} = -kt$$

Using this formula we calculate the reaction rate constant 'k', and then determine the rate of the reaction 'r'.

Table 4: Calculation of rate constant and rate of reaction for *Bacillus subtilis*

SNo.	Concentration (micro-gram/ml)	Time (minutes)	Rate constant (k)	Rate of reaction
1.	256	0	-	-
2.	250	10	0.0024	0.600
3.	222	20	0.0071	1.576
4.	212	30	0.0062	1.314
5.	210	40	0.0049	1.039
6.	194	50	0.0055	1.067
7.	Average rate of reaction			1.119

Table 5: Calculation of rate constant and rate of reaction for *Escherichia coli*

SNo.	Concentration (micro-gram/ml)	Time (minutes)	Rate constant (k)	Rate of reaction
1.	202	0	-	-
2.	176	10	0.0137	2.411
3.	170	20	0.0086	1.462
4.	166	30	0.0065	1.079
5.	152	40	0.0071	1.079
6.	146	50	0.0065	0.949
7.	Average rate of the reaction :			1.390

Thus, we observe that the rate of reaction for *E.coli* (1.390) > rate of reaction for *B.subtilis* (1.119). The action of heat on bacterial cells and spores during sterilisation can be understood from the recent studies done on sterilisation reaction, [15][16] which explain that the water activity of A_w of the cells themselves and that of the environment in which the spores are heated affects heat resistance. Other works [17-21] have demonstrated that spores are highly permeable and that a free exchange of water occurs between the spore and its environment. The water activity of spores may be expected, therefore, to change in relation to the water activity of the suspending fluid or with the

relative humidity of the environment. The important factors to be considered when measuring the dry heat resistance of spores are: the initial moisture content of the spore, the rate of spore desiccation during heating, the water retention capacity of the material in or on which spores are located, and the relative humidity of the system at the test temperature. Also, heating dry spores, they show lower resistance to heat because of the lost water vapour in the spores and cells, and the environment [22].

CONCLUSION

From the above experimental analysis and the obtained data, we conclude that the sterilization reaction rate constant is more for *E.coli* culture than for *B.subtilis* culture, indicating a faster rate of sterilization reaction of *E.coli* culture. The faster rate for the sterilization reaction indicates a faster death rate of the bacterial culture, as implied by the fast decrease in concentration or the number of viable cells in the test tubes from the initial concentration of bacterial cells of *E.coli* before sterilization (C_{A0}). The explanation for this phenomenon is that *B.subtilis* a sporulating, Gram positive bacteria, is more heat resistant than *E.coli* culture, which is a non-sporulating Gram –negative bacteria, and more heat labile. Aerobic mesophilic bacterial spore-formers, such as *Bacillus subtilis* and *Bacillus coagulans*, are the most resistant among several species of spore-forming bacteria to dry heat sterilization. In comparison to the heat resistant bacteria, the non-sporulating Gram negative bacteria are more efficiently killed at 80^0C when sterilised for the same time interval. It is believed that the higher flow rates of dry gas causes greater dehydration of the spores and that spore moisture loss is one of the major factors in determining the dry-heat thermal destruction rate of bacterial spores. Thus, we infer that the sterilisation of sporulating bacteria requires more time or exposure to heat at higher temperatures for death of all viable cells present in the culture, given their heat resistant characteristic.

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