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Death kinetics of *Bacillus subtilis* spores during sterilization of aseptic packaging material by hydrogen peroxide

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Abstract
Aseptic packaging is a method of packaging in which pre-sterilized liquid food is packed and sealed in pre-sterilized container under aseptic conditions to produce shelf-stable products that do not need refrigeration. Sterilization of the packing material is a critical step before aseptic filling of liquid foods. Hydrogen peroxide was used for sterilization of packaging material. The death kinetics of *Bacillus subtilis* ATCC 6633 was studied during sterilization of packaging material. The initial count of spore taken for experimental purpose was 10^8 cfu / ml. In this study, inoculated packaging film ($2x2cm^2$) was used for sterilization at hydrogen peroxide concentration of 10-30 % v/v, temperature of 50-80 ºC and contact time of 30-90 second. The death kinetic parameters 'k', 'D' and Q_{10} were evaluated. It was found that reaction rate constant (k value) increases with rise in temperature while decimal reductio22n time (D value) decreases with rise in temperature at a given contact time and concentration of hydrogen peroxide. The temperature of hydrogen peroxide solutions had a pronounced effect on the rate of spore destruction. The estimated Q_{10} value was found be in the range of 1.00 to 2.1678828. The result could be used for designing sterilization process of food packaging material in aseptic packaging.

Keywords: Death kinetics, *Bacillus subtilis*, during sterilization, packaging material

Introduction
Aseptic packaging is used for filling a commercially sterile product into a sterile container under aseptic conditions to keep the product without refrigeration. This packaging technique is very useful for temperate countries where refrigeration is not prevalent as a means to preserve liquid foods (Ansari *et al*. 2003) [1]. The most important example of an aseptically processed and packed food is Ultra-High-Temperature (UHT) milk which undergoes ultrahigh-temperature-short-time sterilization with subsequent delivery and rapid sealing into presterilized containers. Spore forming *bacilli* present on equipment surface and packaging material cause spoilage of aseptically packed food products and hence decontamination of the packing material is very essential in order to prevent spoilage. Testing of packaging film sterilization is essential before commercial production of aseptic packaging system and it is carried out by using various chemical and physical methods. A combination of physical and chemical methods is, in general, more efficient, being the method most commonly used by industry (Cardoso *et al*. 2011) [5]. The most common sterilants used for aseptic packing applications worldwide are hydrogen peroxide, gamma irradiation, hot air/steam, UV light, halogens or combinations of these methods (Ansari *et al*. 2003) [1] as well as a combination of peroxy acetic acid and hydrogen peroxide. Among these sterilants, H_2O_2 is the most popular worldwide for sterilization of packaging material as it does not leave toxic residues in the food (Smith and Brown, 1980) [10]. It is reported that the traces of residual of H_2O_2 left in food is less than 0.25 ppm does not have any harmful effect when consumed (Smith and Brown, 1980; Hedrick, 1973) [10, 8]. Concentrated solutions of H_2O_2 at high temperature (60-70 ºC) are generally used to increase sporicidal activity (Ansari and Rai, 2017) [2]. For a 10% solution of H_2O_2 at 60 °C, the Q_{10} value was about 1.6. Increases in concentration from 10% to 15% and from 15% to 20% each gave an increase of about 50% in the rate constant (Swartling and Lindgren, 1968) [12]. In general, bacterial spores are more resistant to H_2O_2 than are vegetative cells, and spores are generally the major targets in peroxide sterilization.

The first successful aseptic filling system for cartooning the aseptic Tetra Pak of 1961 used a combination of H_2O_2 and heat for the sterilization of the surface of container material (Burton, 1988) [4]. Hydrogen peroxide kills a wide variety of organisms (Wang and Toledo, 1986) [14] and viruii (Mentel and Schmidt, 1973) [9]. Both linear (Swartling and Lindgren, 1968; Toledo *et al*. 1973) [12, 13] and non-linear (Cerf and Hermier, 1972) [6] relationships between log number of survivors and time of exposure to different concentrations of hydrogen peroxide have been reported. The degree of sterilization required for packaging materials is associated with the potential number of microorganisms that exist on the contact surface of the packaged food and the degree of sterilization used in packages of low acid products varies from 4 to 6 cycles of logarithm reductions (Cardoso *et al*. 2011 [5]. Literatures are not available for death kinetics of *B. Subtilis* ATCC 6633 by hydrogen peroxide. The main objective of this study is to explore the death kinetics of *B. Subtilis* ATCC 6633 by hydrogen peroxide sterilization of food packaging material. The result could be used for designing sterilization process of food packaging material in aseptic packaging.

Materials and Methods

Preparation of test organism

Bacteriological effectiveness of the process was tested with *Bacillus subtilis* as target organism as it is the most resistant to hydrogen peroxide (Smith and Brown, 1980 and Ansari and Datta, 2003) [10, 1]. First of all 50 ml nutrient broth media solution in four 100 ml flask was sterilized in autoclave for 15 min at 121 °C. Pure culture of *B. subtilis* (ATCC 6633) obtained from National Collection Centre, Dairy Bacteriology Division, NDRI, Karnal, India was inoculated in each flask and kept into incubator at 37 °C for seven days. After sufficient growth of strain, flasks were taken out from incubator and then nutrient agar surface was inoculated with those cultures and kept into incubator for growth at 37 °C for 24 hours. The spore crop was grown on the surface of nutrient agar for 4 days at 37 °C. Sterile distilled water was added to each Petri dish simultaneously scraping gently the agar surface with sterile rod. The scraped strain was then added into sterile distilled water. The liquid containing cells from all Petri dishes was pooled, centrifuged and washed thrice with sterile distilled water. The cell obtained from centrifugation were suspended in sterile water and heated at 80 °C for 10 minutes to kill the vegetative cells (Ansari and Rai, 2017) [2] and then spore suspensions were stored at 4 °C approximately for 2 months. Standard plate count was done to enumerate the concentration of spores. Initial concentration was 10^{10} cfu/ml and it was diluted with sterile distilled water to make its concentration up to 10^{8} cfu/ ml. This concentration was used for experimental study and finally the results were calculated for a reference initial count for the ease of interpretation.

Preparation of H_2O_2 solution of different concentrations

A commercially stabilized 30% (v/v) concentrated solution of food grade hydrogen peroxide (Merck, Calcutta) was diluted with sterilized distilled water to get required concentrations of hydrogen peroxide solution and stored at 4 °C. Hydrogen peroxide of 10% v/v, 20% v/v and 30% v/v concentrations were used to sterilize the food packaging material.

Procedure of sterilization of food packaging material

The packaging material was tested for sterility by using hydrogen peroxide solution at different levels of concentration, temperature and treatment time of packaging material. For estimation of the number of bacteria in inoculated packaging material before and after sterilizing, standard plate count method was used. First of all the nutrient agar media (Hi Media, Mumbai, India) was prepared by taking nutrient agar and distilled water in recommended proportions and sterilized the flask at 121 ^{0}C for 15 minutes. The other necessary glass and plastic wares like Petri dishes, test tubes, plastic tips etc. along with the nutrient agar media were sterilized at 121 ^{0}C for 15 minutes in the autoclave. After that all sterilized materials except the bacterial cell solution were taken to the laminar flow chamber and UV light was kept ON for 20 to 30 minutes. Then 2×2 cm^2 of the plastic packaging film was taken. First the film was thoroughly dipped and shaken into spore suspension. Inoculated sample were given different treatment of hydrogen peroxide at various combination of contact time and solution temperature. Film was exposed at hydrogen peroxide concentration of 10-30% at temperature of 50-80 °C and at contact time of 30-90 second. The H_2O_2 solution temperature was maintained using water heater monitored by digital temperature controller.

Determination of Survivors

After exposing the film for required combination of treatment time, concentration of hydrogen peroxide solution and temperature, it was immediately taken out and placed aseptically into 10 ml sterile dilution blank and properly shaken for few seconds to ensure separation of surviving spores from film and uniform distribution of surviving spores. This spore suspension was used to get the final bacterial count after the treatment. The standard plate count method was used up to the required dilutions to determine the number of spore survivors. After plating, the Petri dishes were kept in the incubator for 24 hours at 37 ^{0}C. The initial bacterial load on the packaging film was also calculated using the film without any treatment following the same method. Then colony count was done using colony counter for all the dilutions and the average value was taken as a result of the treatment.

Death kinetics parameters of microorganisms

In order to evaluate the microbiological effectiveness of sterilization process, knowledge about death kinetics of microorganisms is essential. The death kinetic parameters like k-value (death rate constant), D-value (decimal reduction time: time required to reduce the number of microorganisms to one tenth of their original population subjected to sterilization process) and Q_{10} (number of times sporicidal rate of microorganism changes with a 10 °C change in temperature) were calculated using following equations (Toledo, 1991):

$$\log\left(\frac{N_o}{N}\right) = \frac{kt}{2.303} \quad (1)$$

Where,
k = Death rate constant, s^{-1}
t = Sterilization time, s
N_o = Initial count, cfu/cm^2
N = Final count, cfu/cm^2

$$D = \frac{2.303}{k} \quad (2)$$

Where,
k = Death rate constant, s^{-1}

$$Q_{10} = \frac{k_{\theta+10}}{k_{\theta}} \quad (3)$$

Where,
k_0= Death rate constant at reference temperature, s^{-1}

Results and Discussion

Death kinetics of spores

The estimated values of death kinetic parameters of spores are presented in Table 1, Table 2 and Table 3. From these Tables, it is clear that for same concentration of hydrogen peroxide, k-value decreases while D-value and Q_{10} value increases with increase in contact time and temperature of solution. Similar effects of hydrogen peroxide for destruction of *B. subtilis* has been reported by (Swartling and Lindgren, 1968) [12]. Toledo (1975) [15] has also reported that the antimicrobial power of hydrogen peroxide increases with increase in temperature. The decrease in spores is due to destruction of spore protein (Stevenson and Shafer, 1983) [11] and degradation of outer spore layers including spore coats and cortex (Shin *et al*. 1994) [17] when exposed to hydrogen peroxide. Spore coats are disrupted by oxidizing sporicidal agents such as hydrogen peroxide which may cause extraction of spore coat material, facilitating the penetration of hydrogen peroxide into the cortex and protoplast (Bayliss and Waites, 1976) [3]. Setlow and Setlow (1993) [16] believe that hydrogen peroxide, or possibly the free hydroxyl radicals resulting from its degradation, gained access to the core of spores of certain *B. subtilis* mutants and killed these spores at least in part by DNA damage. The effect of temperature on survival count at a given concentration are given in Fig. 1, Fig. 2 and Fig. 3 respectively. The microbial count was found to be zero at all temperature, concentration and treatment time of 90 second. So higher treatment time has more influence on cell destruction. The complete destruction of spores could be due to destruction of spores protein (Stevenson and Shafer, 1983) [11]. The estimated Q_{10} value was similar to reported values. Q_{10} value is the influence of a 10 °C temperature rise for the sporicidal rate of hydrogen peroxide and has been reported ranging from 1.6 (Swartling and Lindgren, 1968) [12] to 2.5 (Cerf and Metro, 1977) [7]. The sterilization system tested showed satisfactory performance in the sterilization of food packaging film being capable of reaching up to up to 8 log cycle reductions of spores.

Table 1: Kinetic parameters of spores of test organisms to inactivation in 10 % v/v hydrogen peroxide solution

Time, s	Temperature, °C	k-value, s^{-1}	D-value, s	Q_{10}
30	80	0.310523	7.416511	1.238141
	70	0.250798	9.182687	1.701153
	60	0.147428	15.62115	2.167828
	50	0.068007	33.86398	-
60	80	0.230259	10.0018	1.488274
	70	0.154715	14.88542	1.91257
	60	0.080894	28.4694	1.620182
	50	0.049929	46.12562	-
90	80	0.205128	11.22715	1
	70	0.205128	11.22715	1
	60	0.205128	11.22715	1
	50	0.205128	11.22715	-

Table 2: Kinetic parameters of spores of test organisms to inactivation in 20 % v/v hydrogen peroxide solution

Time, s	Temperature, °C	k-value, s^{-1}	D-value, s	Q_{10}
30	80	0.383764	6.001081	1.171617
	70	0.327551	7.030969	1.098184
	60	0.298266	7.721299	1.803357
	50	0.165395	13.92426	-
60	80	0.238772	9.645174	1.15938
	70	0.205948	11.18242	1.142066
	60	0.18033	12.77106	1.489419
	50	0.121074	19.02145	-
90	80	0.205128	11.22715	1
	70	0.205128	11.22715	1
	60	0.205128	11.22715	1
	50	0.205128	11.22715	-

Table 3: Kinetic parameters of spores of test organisms to inactivation in 30 % v/v hydrogen peroxide solution

Time, s	Temperature, °C	k-value, s^{-1}	D-value, s	Q_{10}
30	80	0.470106	4.898891	1.029266
	70	0.456739	5.042263	1.263445
	60	0.361503	6.370621	1.261769
	50	0.286505	8.03825	-
60	80	0.248736	9.258804	1.080248
	70	0.230259	10.0018	1.026577
	60	0.224297	10.26762	1.192037
	50	0.188163	12.23939	-
90	80	0.204674	11.25203	1
	70	0.204674	11.25203	1
	60	0.204674	11.25203	1
	50	0.204674	11.25203	-

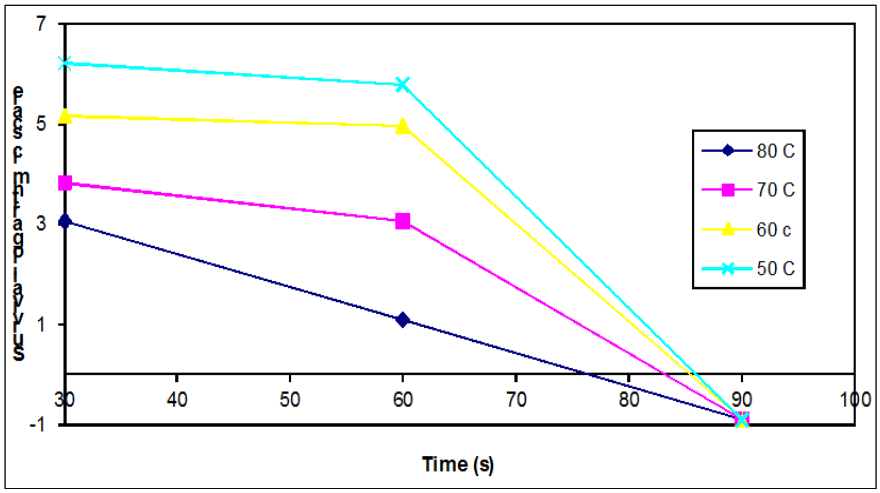

Fig 1: Survival counts for *B. subtilis* spores in 10 % hydrogen peroxide solution at various temperatures

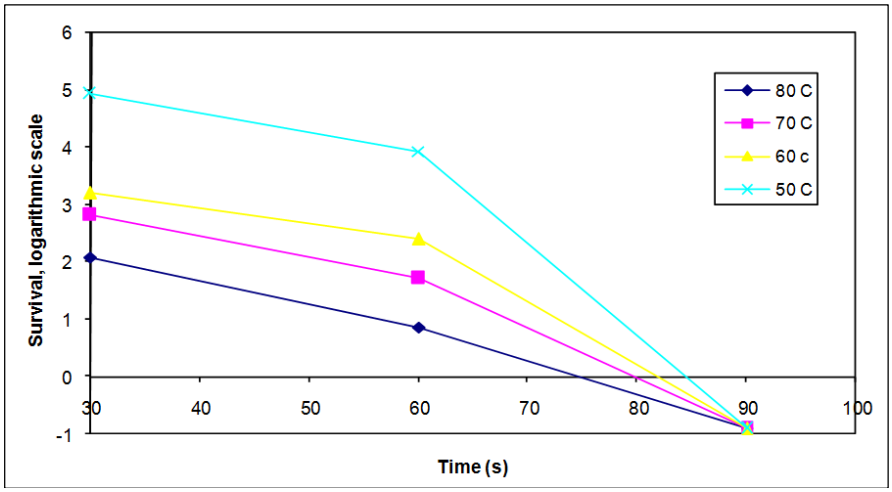

Fig 2: Survival counts for *B. subtilis* spores in 20 % hydrogen peroxide solution at various temperatures

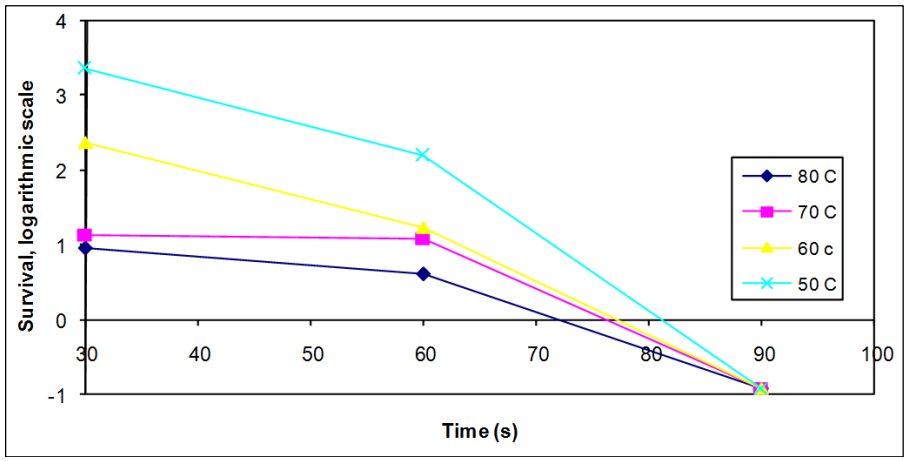

Fig 3: Survival counts for *B. subtilis* spores at 30 % hydron peroxide solution at various temperatures

Conclusion

Hydrogen peroxide is an effective sterilant with potential applications in the decontamination of food packaging materials. A flexible aseptic food packaging material (2 cm x 2 cm) was treated with hydrogen peroxide at different operating conditions (concentration: 10-30% v/v, temperature of solution: 50-80 °C and contact time: 30-90 s) to inactivate Bacillus subtilis spores. The death kinetic parameters ('k', 'D' and Q_{10}) during sterilization of packaging film by hydrogen peroxide were evaluated. The destruction of spores increased with increase in concentration, contact time and temperature of solution of hydrogen peroxide. The reaction rate constant (k value) increases with rise in temperature while decimal reduction time (D value) decreased with rise in temperature at a given contact time and concentration of hydrogen peroxide. The estimated Q_{10} value was found be in the range of 1.00 to 2.1678828. The sterilization system tested showed satisfactory performance in the sterilization of food packaging film being capable of reaching up to up to 8 log cycle reductions of spores. Such results could be used to design systems for the sterilization of plastic food packaging material used for the aseptic filling of liquid foods.

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